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RESEARCH ARTICLE

Ability of *Seidenfadenia mitrata* (Rchb.f.) Garay, pollinia stored at low temperature on fertilization, pod formation and *in-vitro* hybrid seed germination

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Abstract

Seidenfadenia mitrata (Rchb.f.) Garay, is one of the beautiful Thai orchids that is rare and has become endangered. Stored pollinia of *Seidenfadenia mitrata* at 10ºC for 17, 42, 44, 49, 307, 314 and 339 days were used to pollinate the flowers of *Vanda liouvillei*, *Aerides multiflora*, *Aerides rosea*, *Rhynchostylis retusa*, *Rhynchostylis gigantea*, *Vanda lilacina*, and *Ascocentrum miniatum*, respectively to produce intergeneric hybrids. After hand-pollination, the fertilizing ability of seven crosses of intergeneric hybrids remained high with respect to pod formation (50–100%). Hybrid pods of three crosses using stored pollinia of *Seidenfadenia mitrata* for 17, 24 and 314 days involving *Vanda liouvillei*, *Aerides multiflora* and *Vanda lilacina*, respectively, produced seeds and germinated into protocorms after sowing on modified Vacin and Went agar medium supplemented with 150 mlL^{-1} coconut water, 10 gL^{-1} sucrose, 7 gL^{-1} agar and pH 5.2 for 113, 22 and 36 days, respectively. The survival of three intergeneric hybrid plantlets was 100 % for pollinia stored for 180 days and they were successfully grown in greenhouse conditions for 360 days. Storage of *Seidenfadenia mitrata* pollinia at low temperatures is successful on keeping the desirable male parents for hybridization and conservation.

Keywords: Orchid, pollination, protocorm, conservation, hybridization.

Introduction

Seidenfadenia mitrata (Rchb.f.) Garay, an epiphyte orchid, is native to Thailand and Myanmar. It is one of the rare beautiful Thai orchids and has recently become endangered due to deforestation, climate change, global warming and wild orchid trade. Thus, the population of *S. mitrata* is decreasing, affecting the breeding and leading to poor genetic diversity. The leaves are cylindrical with a longitudinal groove and hang down like strings (Figs. 1 A, B). The flowering period is in March to May, depending on climatic conditions. The colour of small flowers is light blue-purple and white (Figs. 1B and 2A). Pollinia are very small (Fig. 1C) and size of mature pods is about 3.9 cm in length x 0.5 in width (Fig. 1D). Pollen bears high importance for plant conservation, successful and efficient breeding which are depending on the quality, longevity, and pollen storage (Parton et al. 2002; Song and Tachibana 2007). Pollen storage facilitates the hand-pollination of two plants grown at different times, and locations, with variation in the durability of the flowers and non-synchronization (Zambon et al. 2018) and keep the desirable male parents for hybridization at the required time (Masum Akond et al. 2012; Wang et al. 2015). The viability of orchid pollen storage at 4 to 6°C has been described in *Dendrobium, Vanda, Cymbidium*, *Arachnis* (Shijun 1984) and *Phalaenopsis* hybrid at 4°C (Yuan et al. 2018). Currently, no report is available on the storage of *S.mitrata* pollinia at low temperatures for conservation and hybridization. Therefore, an investigation was carried out on the storage of *S. mitrata* pollinia at 10°C for utilizing

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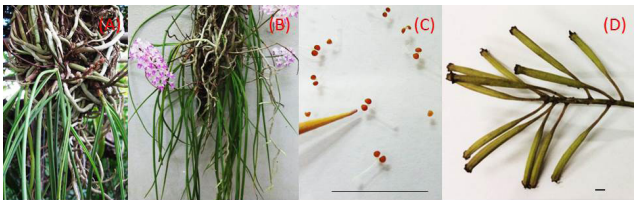

Fig. 1. *Seidenfadenia mitrata,* (A) leaves, (B) leaves and flowers, (C) pollinia and (D) mature pods (scale = 1 cm)

in hand-pollination (hybridization) with different species of Thai orchids flowering at different times. The fertilizing ability, pod formation, hybrid seed germination *in-vitro*, and survival of hybrid plantlets in the greenhouse to produce intergeneric hybrids was also studied.

Materials and methods

Plant materials

Seidenfadenia mitrata was used as a male parent and seven species of Thai orchids used as female parents, namely, *Rhynchostylis gigantea* (Lindl.) Ridl. (Fig. 2B), *Rhynchostylis retusa* (Lindl.) Blume (Fig. 2C), *Aerides multiflora* Roxb. (Fig. 2D), *Aerides rosea* Lodd. ex Lindl. & Paxton (Fig. 2E), *Ascocentrum miniatum* (Lindl.) Schltr. (Fig. 2F), *Vanda lilacina* Teijsm. & Binn. (Fig. 2G) and *Vanda liouvillei* Finet (Fig. 2H) were maintained in the greenhouses at the Rajamangala University of Technology Isan, Surin Campus, Surin province, Thailand in November 2017 to September 2019.

Pollinia collection, storage and hand-pollination

S. mitrata pollinia were collected from open flowers, transferred to 2 mL cryotubes, and stored at 10°C in the refrigerator for 17, 42, 44, 49, 307, 314 and 339 days (d) for hand-pollination with seven species of Thai orchids (Table 1). The viability of *S. mitrata* stored pollinia was assessed by staining with 0.6% 2,3,5-triphenyltetrazolium chloride (TTC). The stored pollinia were removed from 10°C and then put

Fig. 2. Flowers of Thai orchids for pollination, (A) *Seidenfadenia mitrata*, (B) *Rhynchostylis retusa*, (C) *Rhynchostylis gigantea*, (D) *Aerides multiflora*, (E) *Aerides rosea*, (F) *Aerides miniatum*, (G) *Vanda lilacina* and (H) *Vanda liouvillei* (scale = 1 cm)

at room temperature for 1 h before hand-pollination with orchid flowers at different times. When completely open flowers of female parents occurred, anthers were removed from flowers to prevent self-pollination and then the stored pollinia were transferred on the stigma of *V. liouvillei*, *A. multiflora*, *A. rosea*, *R. retusa*, *R. gigantea*, *V. lilacina*, and *A. miniatum*, respectively, to produce intergeneric hybrids (Table 2). Pollinated orchid plants were maintained in the greenhouse.

Pod formation and in-vitro hybrid seed germination

After 30 d of hand-pollination with stored pollinia of *S. mitrata*, the fertilizing ability was determined by pod formation. Mature pods were collected from female plants when pod color changed from green to yellow, then age and size of pods were recorded. Hybrid pods were cleaned and soaked in 95% ethyl alcohol for 1 min inside a laminar air-flow cabinet and then flamed with a lamp until the flame stopped. Hybrid seeds were removed and sown on modified Vacin and Went (1949, VW) agar medium supplemented with 150 mL^{-1} coconut water, 10 gL^{-1} sucrose, 7 gL^{-1} agar, and pH at 5.2. They were cultured at 25 ± 2°C under the illumination of about 37 $mmol.m^{-2}.s^{-1}$ provided by fluorescent tubes (Philips, Thailand) for 10 h/d. Hybrid seeds germinated into protocorms was recorded. The presence of embryos in hybrid seeds was examined under a light microscope at 40X magnification. Protocorms developed into shoots and roots after 150 d of culture on modified agar medium described above. The plantlets were removed from bottles and then washed with water to remove the culture medium. They were transplanted into plastic pots carried out in the greenhouse for 180 d, and plantlet survival was recorded.

Results and discussion

Ability of seidenfadenia mitrata (Rchb.f.) Garay pollinia stored at low temperature on hybridization and pod formation

After hand-pollination, pod formation was observed to confirm successful fertilization. The ability of pod formation depends on the pollen's storage temperature and storage time (Mesnoua et al. 2018). Pollen age and pollen moisture content affect pollen viability (Masum-Akond et al. 2012). Survival of *S. mitrata* pollinia was stored at 10 °C for 17 to 339 d was 100% using TTC test. The fertilizing ability remained high and pod formation about 50 to 100%, among which seven crossesof intergeneric hybrids (Table 2). *S. mitrata* pollinia storage for 49 and 307 d were used to pollinate with flowers of *R. retusa* and *R. gigantean* showed 100% pod formation, followed *A. multiflora* (95.2%) and *A. rosea* (66.7 %) pollinated with stored pollinia of *S. mitrata* for 42 and 44 d, respectively. Storage time of pollinia at 10°C pollinated with flowers did not affect pod formation, but it depended on species of orchids. In addition, the success of

Table 1. Flowering time, age and size of mature pods of Thai orchids in the greenhouse

Species	Flowering time	Age of Mature pod (d)	Size of mature pod (length x width; cm)
V. lilacina	January-February	173.3 ± 11.8	3.2 ± 0.2x1.0 ± 0.1
A. miniatum	March-April	145 ± 5.0	1.8 ± 0.1x 0.6 ± 0.1
S. mitrata	March-May	154 ± 1.0	3.9 ± 0.2x0.5 ± 0.1
V. liouvillei	April-May	211.3 ± 2.1	6.2 ± 0.3x1.5 ± 0.2
A. multiflora	April-May	128.7 ± 4.0	1.7 ± 0.3x0.8 ± 0.1
A. rosea	May-June	122.7 ± 3.8	1.5 ± 0.2x0.6 ± 0.2
R. retusa	June-July	196.7 ± 15.3	2.1 ± 0.2x0.8 ± 0.1
R. gigantea	December-January	212.3 ± 2.1	3.9 ± 0.1x1.6 ± 0.2

Values represent means ± SD.

Table 2. Storage period of *Seidenfadenia mitrata* pollinia at 10°C on pod formation after hand-pollination with open flowers of seven species

Female	Male	Storage period of pollinia (d)	No. of flowers pollinated	No. of pod formation	Pod formation(%)
V. liouvillei	*S. mitrata*	17	2	1	50.0 ± 50
A. multiflora	*S. mitrata*	42	21	20	95.2 ± 5.0
A. rosea	*S. mitrata*	44	12	8	66.7 ± 14.2
R. retusa	*S. mitrata*	49	5	5	100.0 ± 0.0
R. gigantea	*S. mitrata*	307	4	4	100.0 ± 0.0
V. lilacina	*S. mitrata*	314	14	8	57.1 ± 13.7
A. miniatum	*S. mitrata*	339	10	6	60.0 ± 16.3

Values represent means ± SD

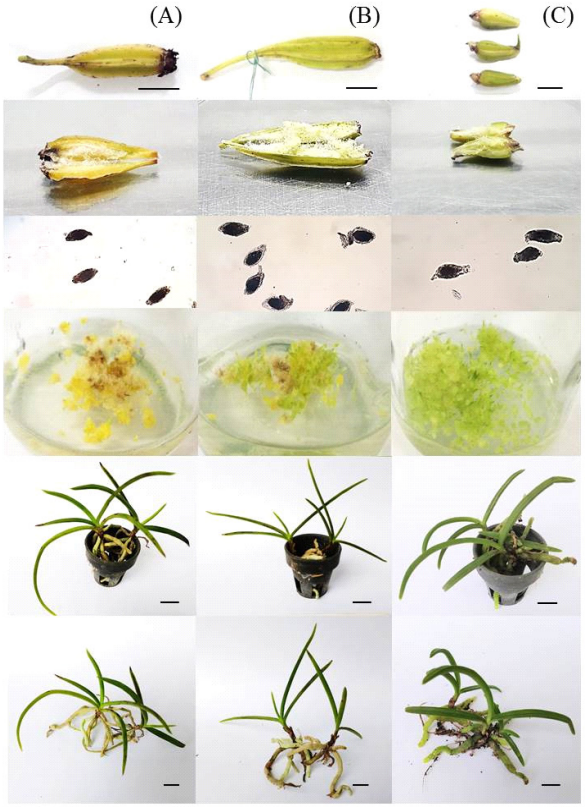

Fig. 3. Mature pods, seeds, seed germination on modified VW agar medium and plantlets of (A) *Vanda liouvillei* x *Seidenfadenia mitrata*, (B) *Vanda lilacina* x *Seidenfadenia mitrata* and (C) *Aerides multiflora* x *Seidenfadenia mitrata* after culture in the greenhouse for 360 d (scale = 1 cm)

pollination depends on flower shape, inflorescence size and flowering time (Proctor 1998). The stored pollen is longer, the pollen ability to grow will be reduced (Soares et al. 2013); however, the longest time to store *S. mitrata* pollinia for 339 d pollinated with *A. miniatum* flowers showed higher pod formation (60%) than pollinated with *Vanda* spp. Flowers. The lower percentage of pod formation of *V. liouvillei* and *V. lilacina* in a cross with stored pollinia of *S. mitrata* for 17 and 314 d was about 50 and 57%, respectively, might perhaps be due to intergeneric incompatibilities, selective abortion (Shiau et al. 2002), the difference in the flower age of female parents, and unhealthy state of the off-season flowers (Kishor et al. 2006) and elongation of pollen tube among incompatible crosses (Devadas et al. 2016).

The time duration of development of hybrid pods age and size of mature pods depend on the species involved in the cross, the health of the female plants and the environmental factors. Mature pods of seven crosses of intergeneric hybrids were harvested in about 124-197 d. The characteristics of hybrid pods are similar to those of the female parents (Fig. 3). In addition, the higher of pollen viability increased the size of formed fruit (Padureanu and Patras 2018). The shortest of *V. liouvillei* x *S. mitrata* pod age may be due to intergeneric incompatibilities, health of female plant and the environment. Seed formation and embryos were observed on six crosses of intergeneric hybridization (Figs. 3A–3C). Pods from *R. retusa* flowers pollinated with stored pollinia of *S. mitrata* for 49 d did not produce seeds indicating that pollen failed and do

Table 3. Age and size of mature hybrid pods using stored pollinia of *Seidenfadenia mitrata* pollinated with flowers of orchids and hybrid seed germination on modified Vacin and Went (1949) agar medium

Female	Male	Storage period of pollinia (d)	Age of mature pod (d)	Size of mature pod (length x width; cm)	Seed germination (d)
V. liouvillei	*S. mitrata*	17	80±0.0	2.0 ± 0.0x0.8 ± 0.0	113
A. multiflora	*S. mitrata*	42	172 ± 17.2	1.7 ± 0.0x0.9 ± 0.1	22
A. rosea	*S. mitrata*	44	124 ± 2.0	1.5 ± 0.1x0.7 ± 0.1	-
R. retusa	*S. mitrata*	49	124 ± 0.0	1.8 ± 0.1x0.8 ± 0.1	-
R. gigantea	*S. mitrata*	307	172.5 ± 48.5	2.6 ± 0.2x1.0 ± 0.1	-
V. lilacina	*S. mitrata*	314	197.2 ± 13.9	2.7 ± 0.5x0.9 ± 0.1	36
A. miniatum	*S. mitrata*	339	163 ± 69	1.6 ± 0.1x0.5 ± 0.0	-

Values represent means ± SD

not necessarily to form seeds, even though pollen grains germinated *in-vitro* (Niimi and Shiokawa 1992). Stanley (1962) observed that pollen grains failed to penetrate the flower's stigma or styler region and hence did not form zygotes.

In vitro hybrid seed germination

Pod formation may not be successful after hybridization in several cases, whereas the development and germination of seeds is important. Seed germination is not only depends on successful of intergeneric hybrids but also on the type of medium, undefined organic supplements, plant growth regulator, age of the pods, the growth and development of the embryo. Hybrid seeds of three intergeneric hybrids involving *V. liouvillei*, *A. multiflora* and *V. lilacina*, out of six crosses made using stored pollinia of *S. mitrata* stored for 17, 24 and 314 d germinated into protocorms after sowing on modified VW (1949) agar medium for 113, 22, and 36 d, respectively (Table 3, Figs. 3A-3C). Protocorms were developed into plantlets for 150 d after culture on modified Vacin and Went (1949) agar medium supplemented with 100 g L^{-1} banana, 150 ml L^{-1} coconut water, 20 g L^{-1} sucrose and 2 g L^{-1} activated charcoal which activated charcoal was added for adsorption of all toxic compounds released by explants (Kim et al. 2019). It induced a high number of buds and roots in seedlings of the orchid hybrid (Eliane et al. 2012). Survival of three intergeneric hybrids plantlets including *V. liouvillei* x *S. mitrata* (Fig. 3A), *V. lilacina* x *S. mitrata* (Fig. 3B), and *A. multiflora* x *S. mitrata* (Fig. 3C) were transplanted into the greenhouse conditions was about 100% after 180 days and showed successful acclimatization and grew even after 360 d. The developed plants showed intermediate morphological characteristics/features between the parents and expected to be qualitatively inherited obviously due to introgression of genes from the species used (Devadas et al. 2019; Tasanai et al. 2021).

Authors' contribution

Conceptualization of research (NJ, KT); Designing of the experiments (NJ); Contribution of experimental materials (NJ, AC); Execution of field/lab experiments and data collection (NJ, AC, VP); Analysis of data and interpretation (NJ, AC, KT); Preparation of manuscript (NJ, AC, VP, KT).

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