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Intergeneric hybridization of *Aerides, Rhynchostylis* and *Vanda* genera with stored pollinia of Thai orchids

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Abstract

Preservation of orchid pollinia is required for the intergeneric hybridization of species with different flowering times. Pollinia were collected from the flowers of three genera and fifteen species of male orchid plants. The pollinia were then refrigerated at 10 °C for a duration ranging from 4 d to 274 d, depending on the blooming period of *Aerides*, *Rhynchostylis*, and *Vanda* genera and eleven species of female parent plants. A total of thirty-six crosses of intergeneric hybridization were studied. Pod formation, the size and age of the hybrid pods, and *in vitro* seed germination were used to assess the capacity of the pollinia for fertilization. Results showed that pod formation (8.33% to 100%) was observed in twenty-seven crosses (75%), with the success of sixteen crosses of intergeneric hybridization (44%) having seeds with embryos that germinated into protocorms on modified VW (1949) medium or MS (1962) medium including, *R. coelestis* x *A. falcata* (48 d), *V. liouvillei* x *A. houlletiana* (4 d), *V. liouvillei* x *A. multiflora* (8 d), *R. coelestis* x *A. multiflora* (11 d), *V. liouvillei* x *R. coelestis* (18 d), *A. rosea* x *R. coelestis* (25 d), *V. lilacina* x *R. gigantea* "Alba" (47 d), *V. lilacina* x *R. gigantea* "Cartoon" (17 d), *V. lilacina* x *R. gigantea* "Red color" (23 d), *A. multiflora* x *V. curvifolia* (44 d), *A. odoratum* x *V. curvifolia* (44 d), *R. coelestis* x *V. curvifolia* (71 d), *A. rosea* x *V. curvifolia* (72 d), *A. rosea* x *V. garayi* (95 d), *R. coelestis* x *V. garayi* (95 d), and *V. garayi* x *V. lilacina* (53 d). All protocorms developed into plantlets after culturing on modified VW agar medium supplemented with 100 g L^{-1} banana, 150 mL L^{-1} coconut water, 20 g L^{-1} sucrose, 2 g L^{-1} activated charcoal, and 7 g L^{-1} agar. The intergeneric hybrid plantlets successfully survived in the greenhouse. Male pollinia can be stored at 10°C and utilized for the creation of orchid hybrids to progress orchid breeding and the preservation of Thai orchid species. The methods are uncomplicated and direct, needing no chemicals, tools, or complex procedures.

Key words: Preservation, pollination, breeding, hybrid, pod, seed, protocorm,

Introduction

Thailand is known for its diversity of tropical orchids, with approximately 1,300 species and 180–190 genera of orchids found throughout the country (Thammasiri, 2016). Thai orchids are renowned for their vibrant colors, unique inflorescence, and ability to bloom all year round, distinguishing them from orchids found in other countries. Currently, Thailand export both orchid plants and cut-flowers to foreign countries, leading to the commercialization and improvement in the cultivation of orchids. One method used to enhance orchids is through hand-pollination, where the pollinia of a male plant is transferred to the flower of a female plant, resulting in the creation of a new orchid hybrid. These new hybrids often exhibit different characteristics compared to their original counterparts, such as vibrant colors, unique inflorescence, year-round blooming, and ease of cultivation. Breeding orchids not only provides commercial benefits but also helps to prevent the risk of traditional orchid species becoming extinct. By creating new hybrids, there is a greater chance of preserving and promoting the cultivation of these beautiful flowers. Pollen plays a crucial role in hybridization and the conservation of plant genetic resources (Sacks and Clair, 1996). The quality, longevity, and storage of pollen are important factors that contribute to these processes (Song and Tachibana, 2007). The viability and age of pollen are important factors in achieving successful pollination (Dafni and Firmage, 2000). Pollen storage allows for the hand-pollination of plants that are grown and have different blooming times and locations, and it also helps to deal with variations in flower durability and unsynchronized flowering times (Zambon *et al.*, 2018). Research has shown that storing orchid pollinia from *Dendrobium*, *Vanda*, *Cymbidium*, and *Arachnis* at a temperature of 4°C to 6°C can maintain its viability (Shijun, 1984). Similarly, pollinia from *Phalaenopsis* hybrids can be stored at 4°C (Yuan *et al.*, 2018). Pollinia from *Seidenfadenia mitrata* can also be preserved at 10°C and used for hybridization with *Vanda liouvillei*, *Aerides multiflora*, and *V. lilacina* (Jitsopakul *et al.*, 2023). The successful hybridization of orchids also requires the formation of pods with viable seeds and the ability to germinate seeds. Orchid seeds are tiny and do not have endosperm, which contributes to the development of embryo stopping at the globular stage (Arditii, 1967). However, orchid seeds can still be germinated *in vitro* through the provision of nutrient rich media that can supply carbon and other minerals which are required for seeds to germinate and grow into protocorms *in vitro* (Zhao *et al.*, 2024).

The Vacin and Went (Knudson, 1922; Vacin and Went, 1949) medium and Murashige and Skoog (MS) medium (Murashige and Skoog, 1962; Kishor and Sharma, 2008) are commonly used culture media for tissue culture of hybrid orchids derived from different species or genera (Sivanaswari *et al.*, 2011).

At present, there is limited research into the storage of Thai orchid pollinia at low temperatures for the purpose of pollination, which have different flowering times. This study aimed to investigate the intergeneric hybridization of Thai orchids, including *Aerides*, *Rhynchostylis*, and *Vanda* species, using pollinia of Thai orchids that were stored at 10°C for 4 d to 274 d. The fertilizing ability, pod formation, and *in vitro* hybrid seed germination were studied to develop new orchid hybrids.

Materials and methods

Plant materials: Thai orchid plants were maintained in the greenhouses at Rajamangala University of Technology Isan, Surin Campus, Surin province, Thailand. Plants used as female parents included three genera (*Aerides*, *Rhynchostylis*, and *Vanda*) and eleven species (*A. houlletiana*, *A. multiflora*, *A. odoratum*, *A. rosea*, *R. coelestis*, *R. retusa*, *V. bensonii, V. curvifolia* (formerly known as *Ascocentrum curvifolium* (Lindl.) Schltr.), *V. garayi* (formerly known as *Ascocentrum miniatum* (Lindl.) Schltr.), *V. lilacina*, and *V. liouvillei* (Fig. 1).

Plants used as male parents included three genera (*Aerides*, *Rhynchostylis*, and *Vanda*) and fifteen species (*A. falcata*, *A. houlletiana*, *A. multiflora*, *A. odoratum*, *A. rosea*, *R. coelestis*, *R. gigantea*, *R. gigantea* “Alba”, *R. gigantea* “Cartoon”, *R. gigantea* “Red color”, *R. retusa*, *V. curvifolia*, *V. garayi*, *V. lilacina*, and *V. liouvillei*). Studies were conducted between November 2017 to September 2019.

Pollinia collection and storage condition: Pollinia were collected from the blooming flowers of fifteen species of male orchid plants with toothpicks and placed in 2 mL cryotubes. Pollinia were kept in the refrigerator at 10°C and hand-pollinated with blooming flowers from eleven different female parent species. The pollinia storage period for each orchid species was determined by the flowering times of the female plants used for hand pollination (Table 1). Female plants were not pollinated with fresh pollinia from male plants during the experiment because their flowering times differed.

The fertilizing ability of stored pollinia in intergeneric hybridization: The intergeneric hybridization abilities of fifteen stored Thai orchid pollinia were assessed by hand-pollinating blooming flowers of eleven different species of female plants (Table 2). To prevent self-pollination, the pollinia were removed from fully blooming flowers of female plants before transferring pollinia from male plants to the stigma of the female parents to produce intergeneric hybrids (Jitsopakul *et al.*, 2022; 2023). Pollinia, which had been stored at 10°C for durations ranging from 4 d to 274 d, were taken out of the refrigerator and allowed to reach room temperature for 1 h before being used for hand-pollination of blooming flowers on a single female plant. The flowers of female plants were pollinated and then maintained in the greenhouse. The fertilizing ability of the stored pollinia was determined by observing pod formation after 30 d of hand-pollination. Mature hybrid pods were collected from the female plants when the pod color changed from green to yellow, and the age and size of the mature pods were recorded.

Table 1. Flowering time of Thai orchids for the collection of pollinia and hand-pollination for intergeneric hybridization in the greenhouse at Rajamangala University of Technology Isan Surin campus, Surin province, Thailand from November 2017 to November 2019

Genus	Species	Flowering time
Aeride	*Aerides falcata* Lindl. & Paxton	May-June
	Aerides houlletiana Rchb.f.	April-May
	Aerides multiflora Roxb.	April-May
	Aerides odoratum Lour.	April-May
	Aerides rosea Lodd. ex Lindl. & Paxton	May-June
Rhynchostylis	*Rhynchostylis coelestis* Rchb.f.	May-June
	Rhynchostylis gigantea (Lindl.) Ridl.	December-January
	Rhynchostylis gigantea “Cartoon”	December-January
	Rhynchostylis gigantea “Red color”	December-January
	Rhynchostylis gigantea “Alba”	December-January
	Rhynchostylis retusa (Lindl.) Blume	June-July
Vanda	*Vanda bensonii* Batem	March-April
	Vanda curvifolia (Lindl.) L.M.Gardiner (Formerly known as *Ascocentrum curvifolium* (Lindl.) Schltr.)	March-April
	Vanda garayi (Christenson) L.M.Gardiner (Formerly known as *Ascocentrum miniatum* (Lindl.) Schltr.)	February-April
	Vanda lilacina Teijsm. & Binn.	January- February
	Vanda liouvillei Finet	April-May

Hybrid seed germination *in vitro* culture and seedling growth: Seeds were sown on two media types: a) modified VW agar medium (1949) supplemented with 150 mL L^{-1} coconut water, 10 g L^{-1} sucrose, 7 g L^{-1} agar, and adjusted pH to 5.2, and b) MS agar medium (1962) supplemented with 30 g L^{-1} sucrose, 7 g L^{-1} agar, and adjusted pH to 5.7 to compare the germination of hybrids. The seeds were cultured at 25±2°C under fluorescent tubes (Philips, Thailand) providing an illumination of about 37 mmol m^2 s^{-1} for 10 h d^{-1}. The day of seed germination into protocorms was recorded, which are green, pointed, shoot-like structures that appear (Pereira *et al.*, 2015). When the protocorms developed into shoots, they were transferred to modified VW agar medium supplemented with 100 g L^{-1} banana, 150 mL L^{-1} coconut water, 20 g L^{-1} sucrose, 7 g L^{-1} agar, and adjusted pH to 5.2 for the development of shoots and roots. The hybrid plantlets, which were 3 cm long, were carefully removed from the bottles and then washed with clean water to remove the culture media before being transplanted into plastic baskets and placed in the greenhouse for further growth. The hybrid plantlets, which were 3 cm long, were carefully removed from the bottles and then washed with clean water to remove the culture media before being transplanted into plastic baskets and placed in the greenhouse for further growth.

Results and discussion

Intergeneric hybridization of *Aerides*, *Rhynchostylis*, and *Vanda* genera with stored pollinia on pod formation: Thai orchid pollinia of male parents from three genera and fifteen species were stored at 10°C for different durations (4 d to 274 d) based on the flowering time of the female parents (Table 1). These pollinia were then used to hand-pollinate flowers of the female parents, including three genera and eleven species. This resulted in a total of thirty-six crosses of intergeneric hybrids (Table 2). The ability of storing pollinia at 10°C in facilitating hybridization was assessed by observing pod formation. The results showed that twenty-seven crosses (75%) out of the thirty-six crosses of

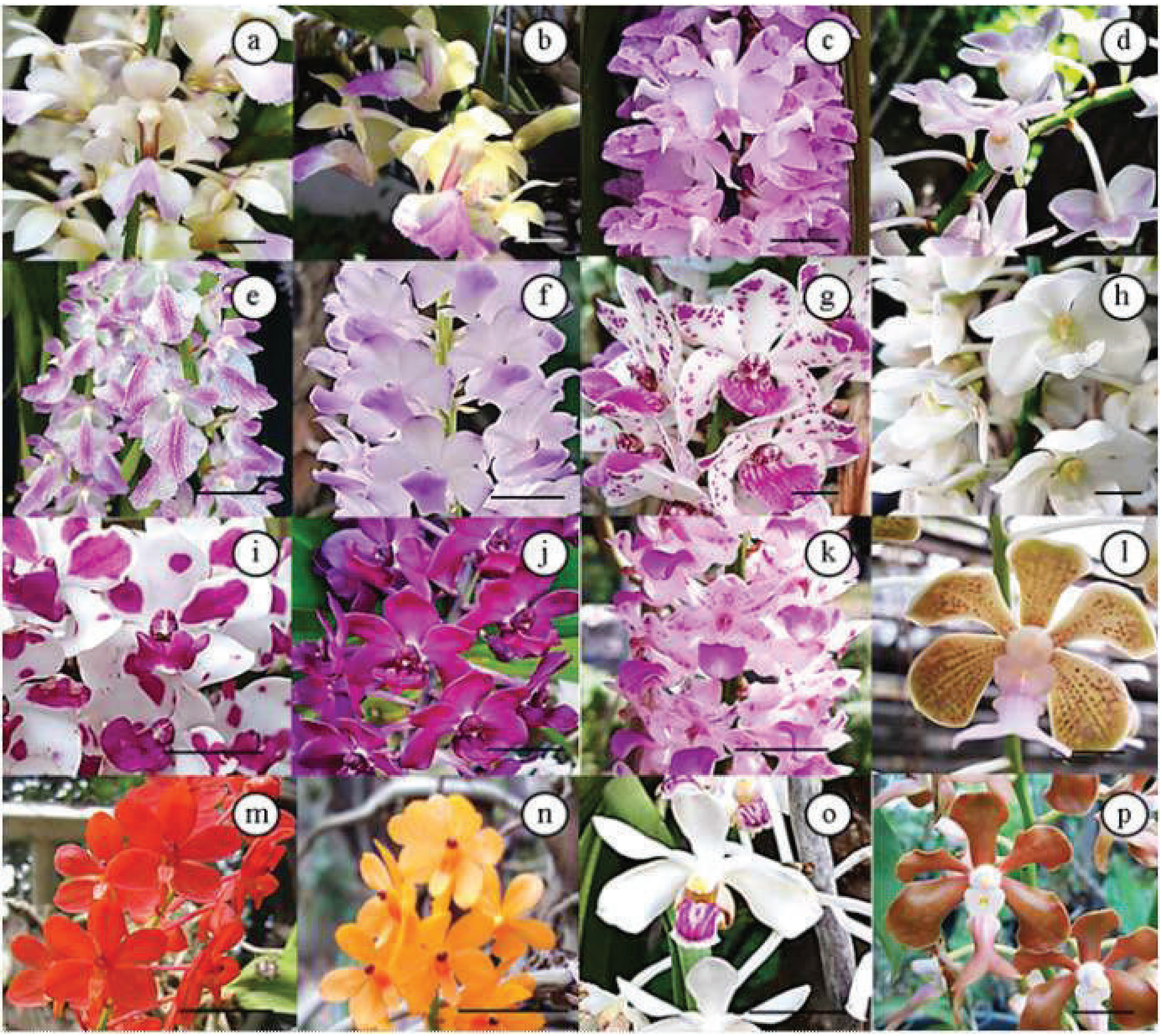

Fig. 1. Flowers of orchids for intergeneric hybridization. (a) *A. falcata*, (b) *A. houlletiana*, (c) *A. multiflora*, (d) *A. odoratum*, (e) *A. rosea*, (f) *R. coelestis*, (g) *R. gigantea*, (h) *R. gigantea* "Alba", (i) *R. gigantea* "Cartoon", (j) *R. gigantea* "Red color", (k) *R. retusa*, (l) *V. bensonii*, (m) *V. curvifolia*, (n) *V. garayi*, (o) *V. lilacina*, and (p) *V. liouvillei* (scale = 1 cm).

intergeneric hybridization with pollinia stored for 4 d to 245 d resulted in pod formation, with success rates ranging from 8.33% to 100% (Table 2).

Intergeneric hybridization of the *Vanda* genus, pod formation was observed with *V. lilacina* pollinated with stored pollinia from *R. gigantea* varieties, achieving up to 100% success in specific combinations, especially *R. gigantea* "Cartoon" and *R. gigantea* "Alba" (Table 2). *V. garayi* and *V. curvifolia* are receptive to their crosses, with *V. garayi* x *V. lilacina* and *V. garayi* x *R. retusa* both showing 100% success. However, *V. bensonii* x *R. coelestis* achieved 50% pod formation, while *V. liouvillei* reached 100% with *A. houlletiana* and *R. coelestis* but lower with *A. multiflora.* The strong compatibility with *A. houlletiana* indicated effective pollination even after 4 d of storage. In contrast, the lower rates with *A. multiflora* (33.33%) and *R. coelestis* (50%) suggested that the longevity of the stored pollinia and viability of pollinia can affect pollination success (Dafni and Firmage, 2000).

Additionally, the intergeneric hybridization experiments with *Rhynchostylis* genus, specifically *R. coelestis* and *R. retusa*, yielded higher pod formation rates. *R. coelestis* was successfully pollinated with various species using different durations of pollinia storage and achieved a 100% pod formation rate when pollinated with *V. curvifolia* (Table 2). In contrast, *R. retusa* showed pod formation rates of 60% with *V. garayi* and 90% with *V. curvifolia*. However, attempts to pollinate both *R. coelestis* and *R. retusa* using stored pollinia from different *Aerides* species did not result in any pod formation.

Lastly, in the study of intergeneric hybridization of *Aerides* genus (Table 2), *A. houlletiana*, *A. odoratum*, and *A. rosea* demonstrated high rates of pod formation when pollinated with various *Vanda* species using stored pollinia, achieving up to 100% pod development in crosses. In contrast, *A. multiflora* had lower pod formation rates when pollinated with *Vanda* and *Rhynchostylis* species. The success rate of the *A. rosea* x *R. coelestis* cross was 72.73%.

The failure of hand-pollination in orchids can be attributed to

Table 2. Pod formation of *Aerides, Rhynchostylis*, and *Vanda* genera after hand-pollination with different storage periods of orchid pollinia at 10°C

No.	Flower (Female parent)	Pollinia (Male parent)	Storage periods of pollinia (d)	No. of flowers pollinated	Pod formation (%)
1	*R. coelestis*	*A. falcata*	48	15	33.33
2	*R. retusa*	*A. falcata*	52	5	0
3	*V. liouvillei*	*A. houlletiana*	4	1	100
4	*R. coelestis*	*A. houlletiana*	48	12	8.33
5	*V. liouvillei*	*A. multiflora*	8	3	33.33
6	*R. coelestis*	*A. multiflora*	11	31	54.84
7	*R. retusa*	*A. multiflora*	49	15	0
8	*V. garayi*	*A. multiflora*	76	5	0
9	*R. retusa*	*A. odoratu*m	45	5	0
10	*R. retusa*	*A. rosea*	33	5	0
11	*V. garayi*	*A. rosea*	274	5	0
12	*V. liouvillei*	*R. coelestis*	18	1	100
13	*A. rosea*	*R. coelestis*	25	22	72.73
14	*V. bensonii*	*R. coelestis*	36	2	50
15	*V. lilacina*	*R. gigantea*	21	6	33.33
16	*A. multiflora*	*R. gigantea*	89	21	38.10
17	*V. lilacina*	*R. gigantea* "Alba"	47	3	100
18	*V. curvifolia*	*R.* gigantea "Alba"	114	9	0
19	*V. lilacina*	*R. gigantea* "Cartoon"	17	1	100
20	*V. lilacina*	*R. gigantea* "Red color"	23	5	60
21	*V. garayi*	*R. retusa*	245	5	100
22	*A. multiflora*	*V. curvifolia*	44	28	85.71
23	*A. odoratum*	*V. curvifolia*	44	2	100
24	*A. houlletiana*	*V. curvifolia*	44	2	100
25	*A. odoratum*	*V. garayi*	67	2	0
26	*R. coelestis*	*V. curvifolia*	71	5	100
27	*A. rosea*	*V. curvifolia*	72	4	100
28	*R. retusa*	*V. curvifolia*	100	10	90
29	*A. multiflora*	*V. garayi*	67	28	75
30	*A. odoratum*	*V. garayi*	67	2	0
31	*A. rosea*	*V. garayi*	95	4	50
32	*R. coelestis*	*V. garayi*	95	30	80
33	*R. retusa*	*V. garayi*	123	10	60
34	*A. houlletiana*	*V. liouvillei*	40	3	100
35	*A. odoratum*	*V. liouvillei*	40	3	66.67
36	*V. garayi*	*V. lilacina*	53	10	100

various factors. One possible cause is female sterility (Stort, 1987), which can result from disrupted ovule development (Stort, 1984). Another factor could be pollen sterility, leading to low fertility. Abnormal meiosis in orchid hybrids may also contribute to reduced fertility. Additionally, male sterility or low fertility could be linked to issues with anther tissue functionality (Hu *et al.*, 2018). Several factors may contribute to lower pod formation in intergeneric hybridization of Thai orchids, including intergeneric incompatibilities and selective abortion (Shiau *et al.*, 2002), pollinia size (Sivanaswari *et al.*, 2011), differences in the flower age of the female parents, the unhealthy state of off-season flowers (Kishor *et al.*, 2006) and elongation of pollen tubes in incompatible crosses (Devadas *et al.*, 2016). Pod formation after hand-pollination may be due to the viability of the pollinia after storage for more than 50 d. Pollen longevity of some orchids under felid greenhouse condition about 4-51 d. In the experiment, the viability of the pollinia was not measured before pollination, but the success of the pollination was measured by the formation of pods. However, pollination using fresh pollinia was not performed in the experiment, and the viability of the pollinia was not measured before hand-pollination. The success of the pollination was measured by the formation of pods. The failure to set pods may not be due to the incompatibility of the orchid species or the environment, but rather to the viability of the pollinia after storage at 10°C for more than 50 d (Table 2). Pod formation following hand-pollination can be used as a measure of success in orchid breeding (Proctor, 1998). The viability of stored pollen is influenced by various factors, including genotype and environmental conditions such as moisture content (Custodio *et al.*, 2020), light and temperature (Dafni and Firmage, 2000; Soares *et al.*, 2013). Pollen viability, along with flower durability and synchronization of blooming, plays a significant role in breeding (Zambon *et al.*, 2018). Therefore, it is crucial to store pollen collected from male parents for hand-pollination. However, the optimal storage temperature for pollen may differ among species and varieties (Loupassaki *et al.*, 1997; Du *et al.*, 2018).

In this study, it was observed that the highest pod formation occurred when using pollinia stored at 10°C from the genus *Vanda* as male parents for intergeneric hybridization of orchids. The longest period for storage of Thai orchids for an intergeneric hybridization was *V. garayi* pollinia for 95 d. The pollinia of *V. garayi* is the smallest when compared to pollinia of fifteen species of male orchids. The pollinia of Thai orchids have been found to tolerate storage at 10°C. It is believed that sucrose or other chemicals present in the cytoplasm and elastoviscin in pollinia provide protection against freezing injury. Sucrose helps in storing pollen at low temperatures by maintaining membrane integrity and facilitating intracellular glass formation, which prevents the formation of ice crystals (Speranza *et al.*, 1997; Firon *et al.*, 2012).

However, the fertility of the stored pollen decreases over time, indicating gradual physical and chemical changes (Natalia *et al.*, 2016). Mature pods of thirty-two crosses of intergeneric hybrids were harvested when the hybrid pods changed from green to yellow color at 33 d to 220.8 d after hand-pollination (Table 3, Fig. 2). Factors affecting the age and size of hybrid mature pods including the species of crossing, the health of the female plants, and environmental factors (Jitsopakul *et al.*, 2022; 2023) in the greenhouse.

Hybrid seed germination *in vitro* culture and seedling growth: The success of hybridization of orchids is determined not only by pod formation but also by the germination of hybrid seeds and the development of seedlings (Wilfret and Kamemoto, 1969). Several factors influence seed germination, including the successful formation of intergeneric hybrids, pod age, culture media, organic supplements, plant growth regulators, and embryo growth and development (Hossain *et al.*, 2013; Jitsopakul *et al.*, 2023). In this study, hybrid seeds from twenty-seven crosses of intergeneric hybrids were removed from pods and cultured on two types of media: modified VW agar medium and MS agar medium. The results showed that sixteen crosses (44%) %) out of the thirty-six of intergeneric hybrids produced embryos and germinated on the media culture included *R. coelestis* x *A. falcata* (48 d), *V. liouvillei* x *A. houlletiana* (4 d), *V. liouvillei* x *A. multiflora* (8 d), *R. coelestis* x *A. multiflora* (11 d), *V. liouvillei* x *R. coelestis* (18 d), *A. rosea* x *R. coelestis* (25 d), *V. lilacina* x *R. gigantea* "Alba" (47 d), *V. lilacina* x *R. gigantea* "Cartoon" (17 d), *V. lilacina* x *R. gigantea* "Red color" (23 d), *A. multiflora* x *V. curvifolia* (44 d), *A. odoratum* x *V. curvifolia* (44 d), *R. coelestis* x *V. curvifolia* (71 d), *A. rosea x V. curvifolia* (72 d), *A. rosea* x *V. garayi* (95 d), *R. coelestis* x *V. garayi* (95 d), and *V. garayi* x *V. lilacina* (53 d) (Table 3, Fig. 2).

Table 3. Age and size of pods and *in vitro* hybrid seed germination of intergeneric hybrid after hand-pollination with different storage periods of pollinia at 10°C

No.	Flowers (Female parent)	Pollinia (Male parent)	Storage periods of pollinia (d)	Age of mature pod (d)	Length of pod (cm)	Width of pod (cm)	Day of seed germination (d)	Media for seed germination
1	*R. coelestis*	*A. falcata*	48	100	1.96	0.94	25	VW
2	*V. liouvillei*	*A. houlletiana*	4	159	3.00	1.20	36	MS
3	*R. coelestis*	*A. houlletiana*	48	158	1.70	0.80	-	MS
4	*V. liouvillei*	*A. multiflora*	8	154	3.67	1.43	37	MS
5	*R. coelestis*	*A. multiflora*	11	113.7	1.54	0.75	35.7	MS, VW
6	*V. liouvillei*	*R. coelestis*	18	145	5.00	1.80	36	MS
7	*A. rosea*	*R. coelestis*	25	109.7	1.40	0.64	81	MS, VW
8	*V. bensonii*	*R. coelestis*	36	33	3.40	1.20	-	MS
9	*V. lilacina*	*R. gigantea*	21	213	2.20	0.60	-	VW
10	*A. multiflora*	*R. gigantea*	89	187	1.75	0.83	-	MS, VW
11	*V. lilacina*	*R. gigantea* "Alba"	47	192.3	3.07	0.97	27	MS
12	*V. lilacina*	*R. gigantea* "Cartoon"	17	184	4.0	1.1	29	MS, VW
13	*V. lilacina*	*R. gigantea* "Red color"	23	153	2.17		61.5	VW
14	*V. garayi*	*R. retusa*	245	97	1.52	0.48	-	MS, VW
15	*A. multiflora*	*V. curvifolia*	44	170.8	1.61	0.73	47	MS, VW
16	*A. odoratum*	*V. curvifolia*	44	122	1.75	0.95	81	VW
17	*A. houlletiana*	*V. curvifolia*	44	201	1.65	1.05	Without seed	-
18	*R. coelestis*	*V. curvifolia*	71	172.7	2.11	0.93	68	VW
19	*A. rosea*	*V. curvifolia*	72	119.5	1.55	0.76	151	VW
20	*R. retusa*	*V. curvifolia*	100	100	2.14	0.69	Without seed	-
21	*A. multiflora*	*V. garayi*	67	124.5	1.40	0.75	-	MS, VW
22	*A. rosea*	*V. garayi*	95	119.5	1.63	0.80	298	MS
23	*R. coelestis*	*V. garayi*	95	220.8	1.82	0.96	83.3	VW
24	*R. retusa*	*V. garayi*	123	87	1.53	0.63	Without seed	-
25	*A. houlletiana*	*V. liouvillei*	40	186	1.63	0.90	Without seed	-
26	*A. odoratum*	*V. liouvillei*	40	107	1.60	0.70	-	VW
27	*V. garayi*	*V. lilacina*	53	184	1.50	0.45	36	VW

Fig. 2. Mature pods and seeds of intergeneric hybrids using stored pollinia at 10 °C for hand-pollination. (a) *R. coelestis* x *A. falcata* (48 d), (b) *V. liouvillei* x *A. houlletiana* (4 d), (c) *V. liouvillei* x *A. multiflora* (8 d), (d) *R. coelestis* x *A. multiflora* (11 d), (e) *V. liouvillei* x *R. coelestis* (18 d), (f) *A. rosea* x *R. coelestis* (25 d), (g) *V. lilacina* x *R. gigantea* "Alba" (47 d), (h) *V. lilacina* x *R. gigantea* "Cartoon" (17 d), (i) *V. lilacina* x *R. gigantea* "Red color" (23 d), (j) *A. multiflora* x *V. curvifolia* (44 d), (k) *A. odoratum* x *V. curvifolia* (44 d), (l) *R. coelestis* x *V. curvifolia* (71 d), (m) *A. rosea* x *V. curvifolia* (72 d), (n) *A. rosea* x *V. garayi* (95 d), (o) *R. coelestis* x *V. garayi* (95 d), and (p) *V. garayi* x *V. lilacina* (53 d). (scale = 1 cm).

However, seven crosses (25.93%) out of twenty-seven intergeneric hybrids did not germinate on either medium culture or four crosses (14.82%) did not produce seeds. The lack of seed production was also observed by Stanley (1962), who noted that pollen grains failed to penetrate the flower's stigma or styler region, resulting in the failure to form zygotes. Additionally, Niimi and Shiokawa (1992) found that although pollen grains germinated *in vitro*, they did not lead to the formation of seeds. The time taken for hybrid seeds to germinate into protocorms varied depending on the intergeneric hybrids, ranging from 25 d to 298 d. It was observed that hybrid seeds germinated better on modified VW medium compared to MS medium. When comparing the modified VW medium to the MS medium, the VW medium demonstrated better performance in germination rates. A total of ten hybrids successfully germinated on the VW medium, whereas only eight hybrids germinated on the MS medium. Both media effectively promoted germination in four hybrids, but for twelve hybrids, only one medium was able to induce germination. This suggests that using VW medium can enhance the germination success rate for hybrid seeds. VW medium, which is rich in phosphates, has been shown to promote higher seed germination rates in hybrid seeds. The presence of coconut water in VW medium also has a positive effect on seed germination and plantlet development (Kishor and Sharma, 2008; Zahara *et al.*, 2017; Kunakhonnuruk *et al.*, 2018; Kang *et al.*, 2020). However, it is important to note that activated charcoal and banana should not be added to the VW medium for sowing hybrid seeds, as they inhibit seed germination but are beneficial for development of seedlings (Prizao *et al.*, 2012). Activated charcoal can be added to the modified VW agar medium (1949) to absorb toxic compounds released by hybrid plantlets (Kim *et al.*, 2019).

Once protocorms developed into shoots and roots, they were transferred to a modified VW agar medium supplemented with 100 g L^{-1} banana, 150 mL L^{-1} coconut water, 20 g L^{-1} sucrose, 2 g L^{-1} activated charcoal, 7 g L^{-1} agar, and adjusted pH at 5.2 (Jitsopakul *et al.*, 2022; 2023). After 90 d, shoots and roots started to develop into plantlets (Fig. 3). These plantlets, which were a combination of morphological traits from the parent plants (Devadas *et al.*, 2019; Tasanai *et al.*, 2021) were then transferred to plastic baskets and grown in the greenhouse. The growth of intergeneric hybrid plantlets is influenced by the environment and climate within the greenhouse. In this study, the plants successfully survived and exhibited morphological characteristics like those of their parent plants after three years in the greenhouse.

Thai orchid pollinia were stored at 10°C for 4 d to 95 d, which proved to be an effective short-term preservation method for intergeneric hybridisation. This method enables successful crosses between female blooming flowers at various stages, resulting in a development of new orchid varieties. This approach resulted in sixteen successful intergeneric hybridisation crosses out of thirty-six attempts. This study provides insight into the storage and use of Thai orchid pollinia, which can help advance orchid breeding and conservation initiatives. The methods described in this study are straightforward and do not require any chemicals, tools, or complex protocols.

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Fig. 3. Hybrid pods of *R. coelestis* x *V. garayi* (95 d) after 120 d of hand-pollination (a), germination of hybrid seeds on modified VW agar medium (b), shoot and root development on modified VW agar medium supplemented with 100 g L^{-1} banana, 150 mL L^{-1} coconut water, 20 g L^{-1} sucrose, 7 g L^{-1} agar, and adjusted pH at 5.2 for 90 d (c), growth of hybrid plantlets after culture in the greenhouse for 2 years (d) and 4 years (e) (scale = 1 cm).

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