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Asymbiotic germination of *Habenaria rhodocheila* Hance on different culture media and impact of plant growth regulators

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Abstract

Habenaria rhodocheila Hance is a very popular terrestrial orchid due to the diversity of its floral color. Since its seed germination is low, modification of the culture media by adding hormones could enhance the germination rate. This study focused on the asymbiotic seed germination of *H. rhodocheila*'s orange and pink forms. Four media (Vacin and Went (VW), ½ VW, Murashige and Skoog (MS), and ½ MS), and three hormones (6-benzylaminopurine (BAP), gibberellic acid (GA), and thidiazuron (TDZ) at 1, 3, 5 mg/L concentrations were investigated for seed germination and protocorm formation for 16 weeks. The results revealed that seeds of both orange and pink forms germinated within four weeks after inoculation and the pink form seeds had a higher seed germination percentage than the orange form seeds in all tested media. Both orange and pink forms had the highest seed germination percentages on ½VW with 15.78±3.82% and 27.92±3.79%, respectively. Protocorm development of the pink form reached stage 5 in all tested media, but for the orange form it was observed only in VW and 1/2 VW. The addition of 1 mg/L BAP in ½VW media enhanced seed germination and facilitated the advanced prototocorm stage when compared with the control, while GA and TDZ showed an inhibitory effect on germination and protocorm formation. The results indicate that asymbiotic seed germination on ½ VW when added 1 mg/L BAP is an effective condition for this orchid species. This result may be applied for commercial production of this species and other terrestrial orchids.

Keywords: Conservation, Cytokinins, Terrestrial orchid, Seed germination, Seedling development

1. Introduction

The genus *Habenaria,* belonging to subfamily Orchidoideae, Orchidaceae [1], is comprised of more than 800 terrestrial orchid species worldwide. It is well represented in Thailand with 46 species, which are mainly found in the northern and northeastern regions of the nation [2]. *Habenaria rhodocheila* Hance is a species found in Southeast Asia with showy flowers and a wide range of color lips such as orange, pink, red, and yellow, which has been used for ornamental purposes as well as an herbal medicine [3]. Recently, *H. rhodrocheila* has become possibly at risk of extinction due to the decrease in their natural habitats and over-collection [4], but it has not yet been evaluated for the International Union for Conservation of Nature (IUCN) Red List. An effective method for propagation is necessary for this orchid's conservation program as well as for commercial purposes.

Although terrestrial orchid species produce large numbers of dust-like seeds, propagation is often considered difficult because their seeds lack an endosperm and in nature they need mycorrhizal fungi in a symbiotic relationship for seed germination, growth, and development [5]. It is well established that terrestrial orchids can germinate asymbiotically (without mycorrhiza) under laboratory and glasshouse conditions. Additionally, it has been shown that asymbiotic seed germination is an effective propagation approach for a wide range of orchid species [6,7]. However, In vitro seed propagation depends on many factors, for example, capsule maturity [8,9], nutrient composition [10,11] culture method [12,13], photoperiod [10,14], and plant growth regulators [15,16]. In the genus *Habenaria*, the asymbiotic germination protocol for *H. macroceratitis* was reported by Stewart and Kane (2006) [10]. At week 16, their seed germination and protocorm growth was the best on Vacin and Went

(VW), Knudson C (KC), and Malmgren Modified (MM) media, in the range 95.3 - 98.6%. Only the MM medium supported protocorm growth to stage 4 (emergence of first leaf) after 16 weeks of incubation, but VW and KC induced stage 2 protocorm development. The addition of cytokinin plant growth regulators, zeatin, and kinetin at 1 µM increased the seed germination [10]. On the contrary, *H. edgeworthii* Hook. f. ex. Collett cultured on MS medium with 1.0 M α-naphthalene acetic acid (NAA) added, an auxin hormone, exhibited the highest seed germination [17].

Little information exists about asymbiotic seed germination of *H. rhodocheila.* Piyattrakul and Apavatjrut (2004) [8] reported the age of the seed capsule affected the quality of the seeds and germination rate in *H. rhodocheila* (no data about flower color form), and it showed that the seed capsule at seven weeks old gave the highest germination rate, but only 2.46% on modified VW media the chorismate mutase (CMU1) at 20 weeks after sowing. In this paper, the effect of different basal media and various plant growth hormones on seed germination and protocorm formation of *H. rhodocheila* was investigated. A comparative study on asymbiotic seed germination between orange and pink forms has been reported for the first time. The results from this research study will assist future orchid conservation and exploitation of the *Habenaria* species and other terrestrial orchid species.

2. Materials and methods

2.1 Collection of seeds and surface disinfection

Mature indehiscent capsules (7-8 weeks old) from hand cross-pollination of *H. rhodocheila* orange form were collected with a permit from Phitsanulok Province and *H. rhodocheila* pink form was collected from Sakon Nakhon Province, Thailand in 2019 (Figure 1). The capsules were dried with silica gel until dehiscence and then stored in paper envelopes. The viability of seeds was tested using a tetrazolium test at a concentration of 1% TTC (2, 3, 5-triphenyl tetrazolium chloride, Sigma, St-Louis, USA) [12] within seven days. Brown seeds from dehisced capsules were kept at 4 °C in sterile Eppendorf tubes until needed. Seeds were surface sterilized with 0.6% (v/v) sodium hypochlorite solution with 0.1% Tween-20 for 10 min. Then, seeds were washed with sterile deionized water for five min and sterilized again with 3% H_2O_2 for 10 min. After this, seeds were washed again three times in sterile deionized water for five min each time. The dried seeds were later inoculated on Petri plates containing 20 mL of solidified medium.

2.2 Effect of different media on seed germination

The ability for germination and formation of protocorm were assessed by spreading seeds on four different types of culture media: (1) Murashige and Skoog (MS) [18], (2) half-strength MS (½MS), (3) VW [19], and (4) half-strength VW (½VW). All media used in the experiment were further modified by adding 2% sucrose, 0.8% agar, and 15% coconut water. The pH adjustment of MS and ½MS medium was 5.8, while VW and ½VW medium was 5.0 before sterilization and autoclaving at 121 °C for 20 min. Each treatment consisted of four replicates of approximately 100 viable seeds each. All the plates were kept at 25 ± 2 °C in the dark for one month, followed by a 16 h photoperiod for three months.

2.3 Effect of 6-benzylaminopurine (BAP), gibberellic acid (GA) and thidiazuron (TDZ) on seed germination and protocorm formation

Following the first experiment, ½VW medium supplemented with BAP (1, 3, and 5 mg/L), GA (1, 3, and 5 mg/L), and TDZ (1, 3, and 5 mg/L) was used in this experiment. The media modification and culture conditions were the same as in the prior experiment.

2.4 Data collection

For the experiments, germination of seeds and protocorms were observed every two weeks for four months under a stereomicroscope. According to Stewart and Kane (2006) [10], the germination and developmental stages were graded on a 1-5 scale of progressive growth. Seed germination and protocorm percentages for each developmental stage were determined from the total viable seeds. The germination rate index (GRI) at stage 1 and the developmental rate index (DRI) at stages 2-5 were calculated according to Papenfus *et al*. [20].

2.5 Data analysis

The experimental design was completely random. Before data processing, the arcsine square root transformation was used to normalize variability. Using the SPSS V16.0 statistical software (SPSS Inc.,

Chicago, USA), an analysis of variance (ANOVA) was performed, and the means were compared using Duncan's multiple range test (DMRT) ($p< 0.05$).

Figure 1 Flowers of *H. rhodocheila*. (A): orange form, (B): pink form.

3. Results

3.1. Effect of different media on seed germination

The tetrazolium test showed that the seeds of *H. rhodocheila* orange and pink forms, had 56.71% and 71.96% viability, respectively (Table 1). Overviews of the seed germination and protocorm formation of the orange and pink forms are presented in Figure 2 and Figure 3, respectively. Seeds of both orange and pink forms geminated to stage 1 (embryo swollen) within four weeks. Similar protocorm development in both forms was observed, where stage 2 protocorms have embryo enlargement with ruptured test at six weeks. Stage 3 protocorms with the appearance of protomeristem and more rhizoids were recorded at eight weeks. Stage 4 was determined with first leaf emergence, followed by stage 5 with second leaf emergence. Protocorms of the orange form reached stage 5 at 16 weeks on both VW and ½VW, while those of the pink form were at 14 weeks on all tested media (Figures 2 and 3).

The results of the seed germination and development of *H. rhodocheila* on various media for 16 weeks after sowing are shown in Table 2. For the orange form, the GRI was highest on ½VW (12.95 ±1.41% per week), VW (10.12±1.39% per week), MS (8.58±2.16% per week), and ½ MS (6.67±1.98% per week). Seeds of the orange form reached stage 1 at four weeks after sowing in all tested media and developed to stage 5 only on VW and ½VW media, whereas they were arrested at stage 2 on MS and at stage 4 on ½MS. The ½VW media gave the highest DRI of protocorms at stages 2-5 among the tested media. At 16 weeks after sowing, the greatest frequency of germinated seeds was observed with the ½ VW medium (15.78±3.82 %) compared with VW (9.78 ±2.44%), MS (9.73 ±5.31%), and ½ MS (8.37±4.24%) (Figure 4).

For the pink form, the GRI was highest on the ½ VW medium (23.63±2.19% per week), MS (20.48±4.13% per week), VW (12.68±1.44% per week), and 1/2VW (12.29±2.06% per week). Protocorm development was supported to stage 5 on all tested media, whereas the highest DRI of stages 3-5 was found on the ½VW medium. Similar to the result of the orange form, the total seed germination percentage of the pink form at 16 weeks after sowing was highest on the ½VW (27.92±3.79%) compared with MS (20.94 ±7.70%), ½MS (15.68± 9.05%),

and VW (13.39± 4.38%). In addition, the pink form showed comparatively greater seed germination percentages than the orange form in all tested media (Figure 4).

Table 1 Seed viability with tetrazolium test of *H. rhodocheila*.

Form	Seed viability (%)
Orange form	56.71±1.23
Pink form	71.96±3.91

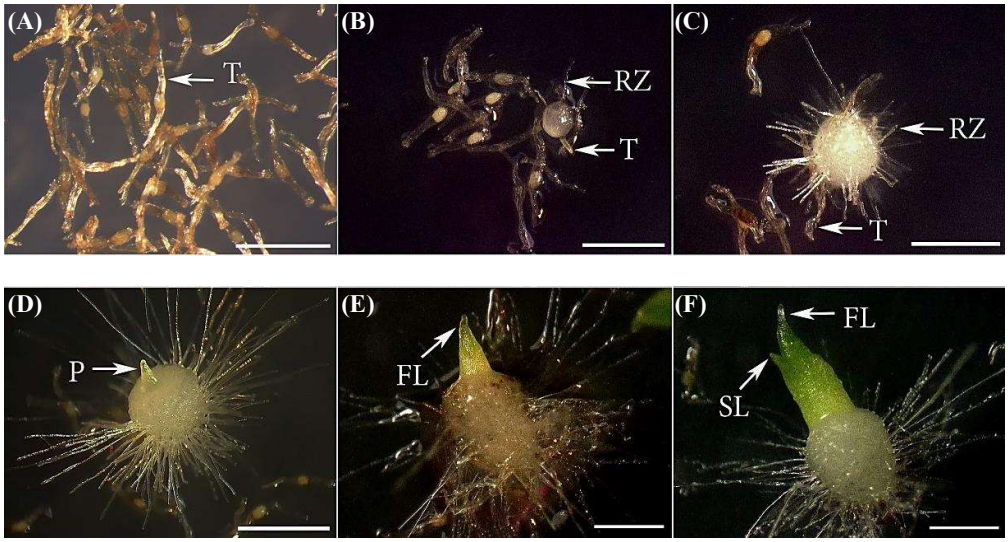

Figure 2 Stages of seed germination and protocorm growth of *H. rhodocheila* (orange form) on ½ VW medium; (A): Stage 0, (B): Stage 1, (C): Stage 2, (D): Stage 3, (E): Stage 4, (F): Stage 5, FL: first emerged leaf, RZ: rhizoids, SL: second emerged leaf, P: protomeristem, T: testa (scale bar= 1 mm).

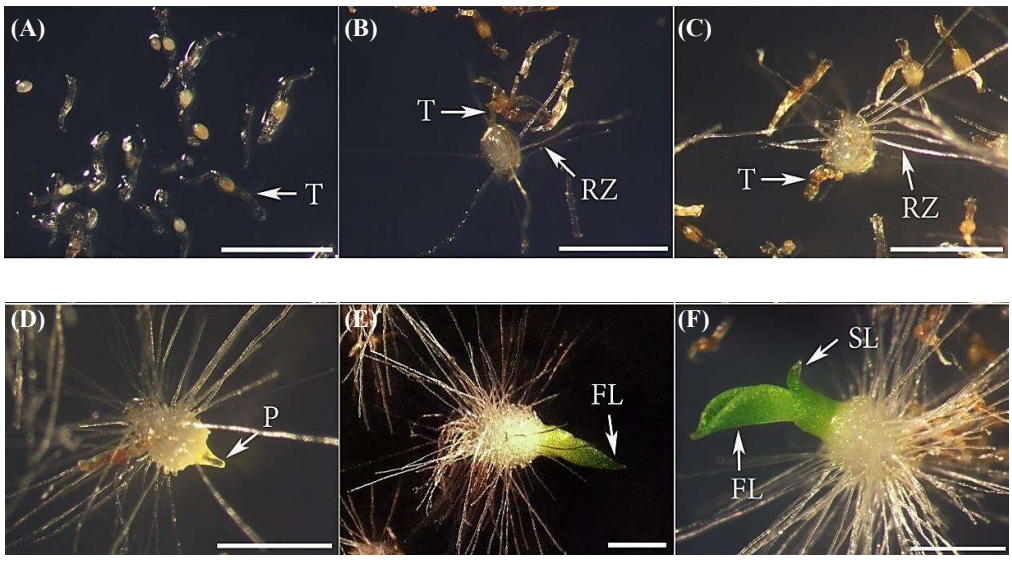

Figure 3 Stages of seed germination and protocorm growth of *H. rhodocheila* (pink form) on ½VW medium; (A): Stage 0, (B): Stage 1, (C): Stage 2, (D): Stage 3, (E): Stage 4, (F): Stage 5, FL: first emerged leaf, RZ: rhizoids, SL: second emerged leaf, P: protomeristem, T: testa (scale bar= 1 mm)**.**

Table 2 Influence of different media on germination rate index (GRI) at stage 1 and developmental rate index (DRI) at stages 2-5 for *H. rhodocheila* at 16 weeks.

Stages	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
Basal media	GRI (% per week)	DRI (% per week)	DRI (% per week)	DRI (% per week)	DRI (% per week)
Orange form					
MS	8.58±2.16^{ab}	0.21±0.24^{b}	0.00±0.00^{b}	0.00±0.00^{a}	0.00±0.00^{a}
½MS	6.67±1.98^{b}	0.37±0.32^{ab}	0.12±0.06^{b}	0.06±.023^{a}	0.00±0.00^{a}
VW	10.12±1.39^{ab}	0.34±0.12^{ab}	0.10±0.07^{b}	0.07±.038^{a}	0.02±0.03^{a}
½VW	12.95±1.41^{a}	0.79±0.32^{a}	0.70±0.21^{a}	0.08±.030^{a}	0.03±0.06^{a}
Pink form					
MS	20.48±4.13^{ab}	1.09±0.33^{ab}	0.74±0.11^{a}	0.37±0.03^{b}	0.03±0.03^{b}
½MS	12.29±2.06^{b}	2.48±0.94^{a}	0.88±0.26^{a}	0.38±0.13^{b}	0.19±0.02^{b}
VW	12.68±1.44^{b}	0.64±0.26^{b}	0.20±0.12^{b}	0.10±0.04^{b}	0.07±0.04^{b}
½VW	23.63±2.19^{a}	2.24±0.22^{ab}	1.19±0.15^{a}	0.84±0.18^{a}	0.57±0.11^{a}

Different letters in each column are significantly different at $p< 0.05$ (DMRT) for each flower color form. Each mean value is determined by stereomicroscopic examination.

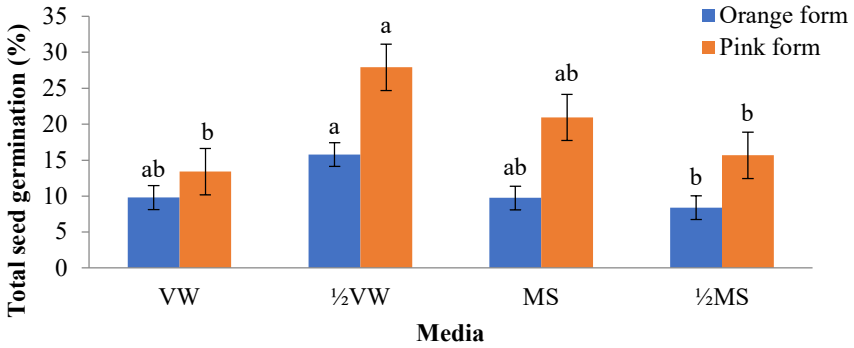

Figure 4 Total seed germination of *H. rhodocheila* on various basal media for 16 weeks. Different letters show significant differences according to DMRT ($p < 0.05$) for each flower color form. Each mean is determined by stereomicroscopic examination. Error bars represent standard error.

3.2 Influence of BAP, GA, and TDZ on seed germination and protocorm growth

The GRI and DRI percentages of both orange and pink forms on 10 tested media for 16 weeks after sowing are presented in Table 3. For *H. rhodocheila* orange form, the addition of BAP and GA gave higher GRI than the control, whereas the addition of TDZ gave a lower GRI. Seeds of the orange form had the highest GRI on the ½VW medium supplemented with 1 mg/L BAP (8.08±1.54% per week). Among all tested media, the highest DRI of stages 2-5 (3.42±0.45, 2.02±0.30, 1.66±1.53, and 0.86±0.56% per week) were recorded in the ½VW medium with 1 mg/L BAP added. Lower DRI for stages 2-5 were mostly found in the media with TDZ added. For the total seed germination at 16 weeks, the result showed that the ½VW medium with 1 mg/L BAP added had the highest value of 11.83±1.62% compared to the other media. Moreover, stage 5 protocorms of the orange form were reached sooner within 10 weeks. On the other hand, the addition of 5 mg/L TDZ gave the lowest total seed germination of 3.55±2.26% (Table 3).

The GRI of the pink form was highest (10.65±1.40% per week) on the ½VW media with 1 mg/L BAP added, and lowest (4.30±0.24% per week) on the media with 1 mg/L TDZ added. The addition of GA and TDA gave lower GRI than the control. The DRI in stage 2 (3.45±0.98% per week) and stage 3 (1.71±0.41% per week) were highest in the 5 mg/L BAP, while the highest DRI of stage 4 (1.07±0.32% per week) and stage 5 (0.90±0.20% per week) were obtained on the media with 1 mg/L BAP added (Table 3). Similar to the result of the orange form, the ½VW with TDZ showed lower GRI and DRI of all developmental stages than the control, even arrested in stage 4 on the media with 3 mg/L TDZ and stage 5 on the medium with 5 mg/L TDZ. Furthermore, it was found that stage 5 protocorms were reached sooner within 10 weeks in the ½VW media with 1 mg/L BAP than for the pink form (data not shown).

At the end of the experiment, the ½VW medium with 1 mg/L BAP proved to be a suitable media for protocorm development, where the highest percentage of germinated seeds was achieved for both the orange

and pink forms (Figure 5). The pink form had comparatively higher seed germination percentages than the orange form in all media tested.

Table 3 Influence of BAP, GA, and TDZ on germination rate index (GRI) at stage 1 and developmental rate index (DRI) at stages 2-5 for *H. rhodocheila* cultured on ½VW media for 16 weeks.

Stages	Stage 1	Stage 2	Stage 3	Stage 4	Stage 5
Basal media	GRI (% per week)	DRI (% per week)	DRI (% per week)	DRI (% per week)	DRI (% per week)
Orange form					
Control	4.68±1.58^{ab}	2.48±1.63^{ab}	1.24±0.24^{b}	0.93±0.24^{abcd}	0.49±0.07^{b}
1 (mg/L) BAP	8.08±1.54^{a}	3.42±0.45^{a}	2.02±0.30^{a}	1.66±1.53^{a}	0.86±0.56^{a}
3 (mg/L) BAP	5.85±1.00^{ab}	2.51±1.80^{ab}	1.17±0.29^{bc}	1.40±0.27^{ab}	0.55±0.12^{b}
5 (mg/L) BAP	6.17±1.04^{ab}	1.73±0.41^{bc}	1.04±0.16^{bcd}	1.27±0.21^{abc}	0.16±0.94^{c}
1 (mg/L) GA	6.41±1.92^{ab}	1.80±0.34^{bc}	0.76±0.30^{bcde}	0.74±0.36^{abcd}	0.10±0.06^{c}
3 (mg/L) GA	6.72±1.42^{ab}	1.61±0.49^{bc}	0.41±0.26^{cde}	0.43±0.29^{cd}	0.16±0.16^{c}
5 (mg/L) GA	6.80±1.70^{ab}	0.89±0.18^{c}	0.32±0.22^{de}	0.67±0.35^{bcd}	0.07±0.07^{c}
1 (mg/L) TDZ	3.40±1.10^{b}	0.72±0.31^{c}	0.11±0.11^{e}	0.19±0.19^{d}	0.10±0.10^{c}
3 (mg/L) TDZ	4.13±1.79^{b}	0.91±0.41^{c}	0.69±0.28^{bcde}	0.19±0.19^{d}	0.05±0.05^{c}
5 (mg/L) TDZ	3.08±1.57^{b}	0.70±0.19^{c}	0.34±0.21^{de}	0.32±0.18^{cd}	0.06±0.06^{c}
Pink form					
Control	10.11±0.80^{ab}	1.59±0.21^{bc}	0.95±0.23^{abc}	0.99±0.16^{a}	0.40±0.10^{bc}
1 (mg/L) BAP	10.65±1.40^{a}	2.83±0.55^{ab}	1.38±0.14^{a}	1.07±0.32^{a}	0.90±0.20^{a}
3 (mg/L) BAP	9.53±0.87^{ab}	2.22±0.33^{abc}	1.43±0.27^{a}	1.02±0.17^{a}	0.70±0.25^{ab}
5 (mg/L) BAP	10.24±1.17^{ab}	3.45±0.98^{a}	1.71±0.41^{a}	0.66±0.32^{ab}	0.26±0.17^{c}
1 (mg/L) GA	8.84±0.46^{abc}	2.11±0.58^{abc}	0.42±0.24^{c}	0.27±0.17^{bc}	0.10±0.10^{c}
3 (mg/L) GA	7.02±0.42^{cd}	1.37±0.32^{bc}	1.20±0.23^{ab}	0.16±0.16^{bc}	0.00±0.00^{c}
5 (mg/L) GA	7.79±0.46^{bc}	1.02±0.32^{c}	0.52±0.32^{bc}	0.25±0.25^{bc}	0.02±0.02^{c}
1 (mg/L) TDZ	4.30±0.24^{e}	1.56±0.44^{bc}	0.31±0.18^{c}	0.06±0.06^{bc}	0.04±0.04^{c}
3 (mg/L) TDZ	4.89±0.71^{de}	1.16±0.40^{c}	0.25±0.15^{c}	0.00±0.00^{c}	0.00±0.00^{c}
5 (mg/L) TDZ	4.63±0.43^{e}	0.75±0.12^{c}	0.20±0.11^{c}	0.02±0.02^{c}	0.00±0.00^{c}

Different letters in each column show significant differences at $p< 0.05$ (DMRT) for each flower color form. Each mean value is determined by stereomicroscopic examination.

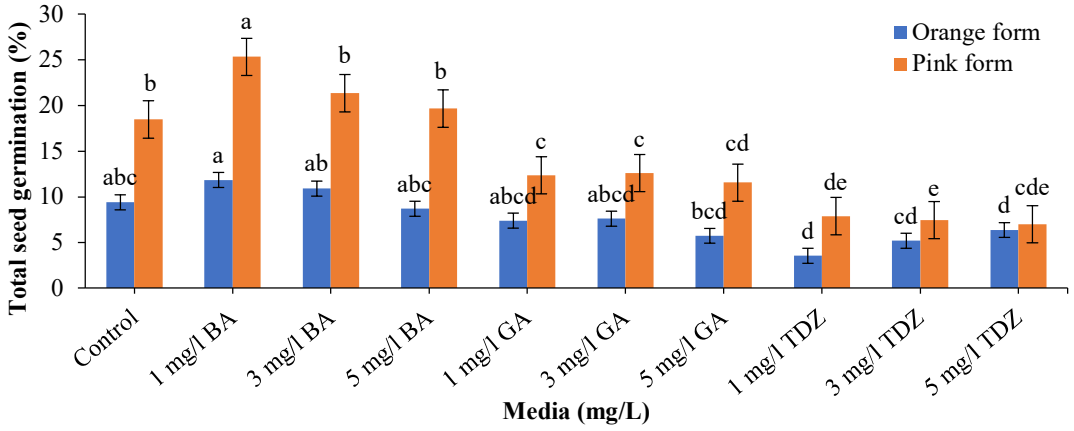

Figure 5 Effect of BAP, GA, and TDZ on total seed germination of *H. rhodocheila* at 16 weeks after inoculation. Different letters show significant differences according to DMRT ($p< 0.05$) for each flower color form. Each mean is based on stereomicroscopic observation. Error bars represent standard error.

4. Discussion

The results from the testing of four different basal media showed that *H. rhodocheila* could germinate on all four tested media but at different rates. The pink form had a higher germination percentage than the orange form that could be due to higher viability as shown with the tetrazolium test, which showed 56.71% viability for the

orange form and 71.96% viability for the pink form. A previous report by Piyatrakul *et al*. (2014) [8] obtained 2.46% on a VW formula with 15% coconut water and 1 g/L peptone 20 weeks after sowing, and germinated seeds developed to stage 2 only. In the present study, both orange and pink forms had the highest germination rates on the ½VW media with 15% coconut water, 15.78%, and 27.92%, respectively, and had developed to advanced stage (stage 5) protocorms within 16 weeks after sowing. This result indicates that a media with a lower nutrient concentration might be a suitable media for seed germination and developing advanced stage protocorms of this terrestrial orchid. Malmagen (1996) [21] reported that modified terrestrial orchid medium (MM) has been widely used, but the efficacy of this media differed widely across species. However, Saikun *et al*. (2020) [22] recently tested the viability and germination of seven *Habenaria* species from self-cross pollination and found that viability varied among species ranging from 15.15% -55.80%. They found that *H. dentata* and *H. rhodochila* (orange) had the highest viabilities of 55.80% and 54.63%, respectively, but seed viability did not determine seed germination for the tested species. The seed germination of these *Habenaria* species on MS varied from 0-36.60% and germinated within 90-99 days after sowing. *H. rhodocheila* orange and red forms germinated on MS at 21.00% within 94 days and 20.00% within 92 days, respectively. In contrast with this study result, stage 1 protocorms of the orange and pink forms were observed within four weeks and stage 5 were reached within 16 weeks.

Several studies about the effect of media on seed germination in other *Habenaria* species have been evaluated. Stewart and Kane (2006) [10] tested six asymbiotic media (Modified Lucke, MS, Lindemann, VW, Malmgren Modified, and Knudson C) for *H. macroceratitis*. They found that seed germination was greatest on both Modified Lucke and Knudson C after seven weeks of culture; but protocorm formation was increased on Malmgren Modified media after both seven and 16 weeks. For some terrestrial orchids in the *Pecteilis* genus, closely related to *Habenaria*, VWBM was the best medium for *P. gigantean* seed germination (96%) after 42 days of culture in comparison to other media, such as Bergeff Basal Medium (BGBM), Knudson 'C' Basal Medium (KCBM), Murashige and Skoog's Basal Medium (MSBM), and Fonnesbech Basal Medium (FBBM) supplemented with 15% coconut water [23], while *P. radiata* (Thunb.) Raf. had higher seed germination on MS (38.8%) compared to ½MS (24.3%) and ¼MS (10.7%) [16]. Thus, mineral nutrient requirements for seed germination and protocorm growth might differ in different species.

Furthermore, when testing for suitable plant growth regulators, it was found that adding BAP to ½VW medium enhanced the seed germination of the orange and pink forms, while the addition of GA and TDZ showed inhibitory effects. BAP at 1 mg/L was an appropriate dose promoting the leaf growth stage. Stewart and Kane (2006) [10] presented similar findings that show the application of cytokinins (kinetin and zeatin) at a dose of 1 µM enhanced seed germination and protocorm growth for *Habenaria macroceratitis*. Godo *et al*. (2010) [24] found that when applying BAP to the media stimulated germination of *Calather tricarminata*, but auxin had a less pronounced impact on increasing the germination of terrestrial orchids, which confirmed this study result. Similar results were also observed in *Orchis coriophora* L. [25], as well as in *Serapias vomeracea* (Burm.f.) Briq. [26].

The addition of GA and TDZ did not promote germination and seedling development in both forms, as it resulted in less germinated seeds than the control. Kim *et al*. (2019) [16] demonstrated an opposite response for germination in *Pecteilis radiata* (Thunb.) Raf., in which the greatest asymbiotic seed germination was seen in seeds cultivated on 1/2MS medium with 2 µM TDZ (90.2 %). Similar results to the earlier study were found in *Malaxis acuminate* [27] and *Rhynchostylis retusa* [28]. Furthermore, Bektaş and Somken (2016) [26] found that the longest shoot elongation amongst the plantlets of *S. vomeracea* (Burm.f.) Briq. was on the medium with 0.25 mg/L TDZ. GA3 has been shown to have a positive response in seed germination of *Comparettia falcata,* in a concentration-dependent manner [15], and the combination of GA3 and kinetin provided a positive synergistic response on seed germination and seedling growth. According to the findings of this study, a high hormonal concentration may contribute to an inhibitory effect. Even through plant growth regulators are commonly employed to stimulate orchid germination, orchid seed responses to plant growth regulators varied by genus and even species [29].

Many studies have successfully applied the asymbiotic technique for the germination of orchid seeds. Due to mechanical or physiological factors that influence seed dormancy, In vitro seed germination of some terrestrial orchids may fail or be poor. Certain temperature regimes, photoperiods, chemical or mechanical softening of the testa, or specific factors could be further employed to break seed dormancy. Furthermore, the seed germination of several terrestrial orchid species relies on mycorrhizal fungal association [30], thus specific studies should be conducted for each species to determine the optimum methods.

5. Conclusion

For the orange and pink forms of *H. rhodocheila* it has been shown that their seed germination and protocorm formation are affected by different culture media and plant growth hormones. The greatest germination and protocorm (stage 5) percentages were achieved from seeds in the ½VW medium that had been

treated with 1 mg/L BAP. This means that these conditions are the most effective ones for use with this terrestrial orchid.

6. Acknowledgments

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7. References

[1] Pridgeon AM, Cribb PJ, Chase MW, Rasmussen FN, editors. Genera *Orchidacearum*: orchidoideae, part 1. 2 vols. 1st ed. Oxford: Oxford University Press; 2001.
[2] Kurzweil H. The genus *Habenaria* (Orchidaceae) in Thailand. TFB. 2009;37(Special Issue):7-105.
[3] Teoh ES. Medicinal orchids of Asia. 1st ed. Cham: Springer International Publishing AG; 2016.
[4] Sinumporn P, Kawasaki TN, Fukai S. Development of interspecific hybrids between *Habenaria radiata* and *Habenaria rhodocheila* complex. Adv Hort Sci. 2020;34(1):3-10.
[5] Swarts ND, Dixon KW. Conservation methods for terrestrial orchids. 1st ed. Florida: J Ross publishing; 2017.
[6] Stewart SL, Zettler LW. Symbiotic germination of three semi-aquatic rein orchids (*Habenaria repens, H. quinqueseta, H. macroceratitis*) from Florida. Aquat Bot. 2020;72(1):25-35.
[7] Hossain MM, Kant R, Pham V, Winarto B, Zeng S, Silva JT. The application of biotechnology to orchids. Crit Rev Plant Sci. 2013;32(2):69-139.
[8] Piyatrakul P, Apavatjrut P. Effect of pod age on seed germination of *Habenaria rhodocheila* Hance in aseptic conditions. J Agric. 2004;20(3):230-234.
[9] Diengdoh R, Kumaria S, Tandon P, Das M. Asymbiotic germination and seed storage of *Paphiopedilum insigne*, an endangered lady's slipper orchid. S Afr J Bot. 2017;112:215-224.
[10] Stewart S, Kane M. Asymbiotic seed germination and In vitro seedling development of *Habenaria macroceratitis* (Orchidaceae), a rare Florida terrestrial orchid. Plant Cell Tiss Org. 2006;86(2):147-158.
[11] Gayatri MC, Kavyashree R, Usha SR, Dhananjaya SP. In vitro seed germination of *Pecteilis gigantea*: a rare and endangered orchid from Western ghats. Plant Cell Biotechnol Mol Biol. 2006;7(3):191-194.
[12] Dowling N, Jusaitis M. Asymbiotic In vitro germination and seed quality assessment of Australian terrestrial orchids. Aust J Bot. 2012; 60:592-601.
[13] Sotthikul C, Kaewpoowat C, Saimoon N. In vitro propagation of *Habenaria* hybrids. Acta Hortic. 2017;1155:293-300.
[14] Barrientos B, Fang JY. Influence of photoperiod and culture medium on the speed of asymbiotic seed germination and seedling development in *Spathoglottis plicata.* HortScience. 2019;54(9):1570-1575.
[15] Manrique JP, Lizarazo CF, Suarez SA. Evaluation of the effect of three growth regulators in the germination of *Comparettia falcata* seeds under In vitro conditions. In Vitro Cell Dev Biol Plant. 2005;41(6):838-843.
[16] Kim DH, Kang KW, Enkhtaivan G, Jan U, Sivanesan I. Impact of activated charcoal, culture medium strength and thidiazuron on non-symbiotic In vitro seed germination of *Pecteilis radiata* (Thunb.) Raf. S Afr J Bot. 2019;124:144-150.
[17] Giri L, Jugran A, Rawat S, Dhyani P, Andola H, Bhatt ID, et al. In vitro propagation, genetic and phytochemical assessment of *Habenaria edgeworthii*: an important Astavarga plant. Acta Physiol Plant. 2012;34(3):869-875.
[18] Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant. 1962;15(3):473-497.
[19] Vacin EF, Went FW. Some pH changes in nutrient solutions. Botanical Gazette. 1949; 110(4):605-613.
[20] Papenfus HB, Naidoo D, Posta M, Finnie JF, Staden JV. The effects of smoke derivatives on in vitro seed germination and development of the leopard orchid *Ansellia africana*. Plant Biol. 2015;18(2):289-294.
[21] Malmgren S. Orchid propagation: theory and practice. In: Allen C, editor. North American native terrestrial orchids: propagation and production. 1996 Mar 16-17; Washington, United States. Philadelphia: Biodiversity Heritage Library;1996. p. 63-71.
[22] Saikum C, Kunasakdakul K, Potapohn N. Crossability and In vitro seed germination of some terrestrial orchid species of genus *Habenaria* and *Pecteilis*. J Agric. 2020;36(1):47-58.
[23] Gayatri MC, Kavyashree R, Usha SR, Dhananjaya SP. In vitro seed germination of *Pecteilis gigantea*: a rare and endangered orchid from Western ghats. Plant Cell Biotechnol Mol Biol. 2006;7(3):191-194.
[24] Godo T, Komori M, Nakaoki E, Yukawa T, Miyoshi K. Germination of mature seeds of *Calanthe tricarinata* Lindl., an endangered terrestrial orchid, by asymbiotic culture in vitro. In Vitro Cell Dev Biol Plant. 2010;46(3):323-328.

[25] Bektaş E, Cuce M, Sokmen A. In vitro germination, protocorm formation, and plantlet development of *Orchis coriophora* (Orchidaceae), a naturally growing orchid species in Turkey. Turk J Bot. 2013;37(1):336-342.
[26] Bektaş E, Sokmen A. In vitro seed germination, plantlet growth, tuberization, and synthetic seed production of *Serapias vomeracea* (Burm.f.) Briq. Turk J Bot. 2016;40(6):584-594.
[27] Cheruvathur MA, Abraham J, Mani B, Thomas D. Adventitious shoot induction from cultured internodal explants of *Malaxis acuminata* D. Don, a valuable terrestrial medicinal orchid. Plant Cell Tiss Org. 2010;101(2):163-170.
[28] Thomas D, Michael A. High-frequency plantlet regeneration and multiple shoot induction from cultured immature seeds of *Rhynchostylis retusa* Blume., an exquisite orchid. Plant Biotechnol Rep. 2007;1(4):243-249.
[29] Arditti J, Ernst R. Micropropagation of orchids. 1st ed. New York: John Wiley & Sons, Inc.; 1993.
[30] Rasmussen N. Terrestrial orchids, from seed to mycotrophic plant. 1st ed. Cambridge: Cambridge University Press; 1995.