# TAXONOMY, REPRODUCTIVE BIOLOGY AND SEED GERMINATION OF *HABENARIA RHODOCHEILA* HANCE COMPLEX (ORCHIDACEAE)

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# อนุกรมวิธาน ชีววิทยาการสืบพันธุ์ และการงอกของเมล็ดกล้วยไม้ดินลิ้นมังกร *Habenaria rhodocheila* Hance และชนิดใกล้เคียง (Orchidaceae)

นายธีระ ธรรมวงศา



วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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# TAXONOMY, REPRODUCTIVE BIOLOGY AND SEED GERMINATION OF *HABENARIA RHODOCHEILA* HANCE COMPLEX (ORCHIDACEAE)

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ธีระ ธรรมวงศา : อนุกรมวิธาน ชีววิทยาการสืบพันธุ์ และการงอกของเมล็ดกล้วยไม้ดินลิ้นมังกร *Habenaria rhodocheila* Hance และชนิดใกล้เคียง (Orchidaceae) (TAXONOMY, REPRODUCTIVE BIOLOGY AND SEED GERMINATION OF *HABENARIA RHODOCHEILA* HANCE COMPLEX (ORCHIDACEAE)) อาจารยที่ปรึกษา : รองศาสตราจารย์ ดร.หนูเดือน เมืองแสน, 134 หน้า

คำสำคัญ: การวิเคราะห์ความสัมพันธ์ทางวิวัฒนาการ/รูปแบบการสืบพันธุ์/ขนาดประชากร/การเพาะเลี้ยงแบบสมชีพ

กล้วยไม้สกุลลิ้นมังกร (*Habenaria*) เป็นกล้วยไม้ดินที่มีความต้องการของตลาดสูง เนื่องจากดอกมีสีสันที่หลากหลาย จึงมีผู้นิยมลักลอบนำออกจากธรรมชาติเพื่อมาจำหน่าย ทำให้กลายเป็นพืชที่เสี่ยงต่อการสูญพันธุ์ งานวิจัยนี้มีวัตถุประสงค์เพื่อ 1) ทบทวนอนุกรมวิธานของกล้วยไม้ดินลิ้นมังกรและชนิดใกล้เคียง (*H. rhodocheila* Hance complex) 2) ศึกษาชีววิทยาการสืบพันธุ์ ประกอบด้วย ระบบการผสมพันธุ์ การออกดอกและติดผล และโครงสร้างขนาดประชากร และ 3) ประเมินการงอกของเมล็ดลิ้นมังกร (*H. rhodocheila*) และลิ้นมังกรชมพูร่องกล้า (*H. janellehayneiana*) ผลการทดลอง พบว่า การวิเคราะห์ทางสัณฐานวิทยาเชิงตัวเลขด้วยวิธีวิเคราะห์องค์ประกอบหลัก (Principal Component Analysis, PCA) และ เดนโดแกรม โดยใช้ลักษณะทางสัณฐานวิทยาของดอก 26 ลักษณะ ทำให้ประชากรลิ้นมังกรชมพูร่องกล้าแยกออกมา ด้วยลักษณะของยอดเกสรเพศเมียที่มีลักษณะเป็นก้านลู่เข้าหากัน สนับสนุนการระบุความแตกต่างของชนิดนี้ออกจากลิ้นมังกร นอกจากนี้ยังแยกกลุ่มของลิ้นมังกรประชากรที่ 03 ที่มีความยาวจงอยเล็กที่สั้นกว่าชนิดอื่น และลิ้นมังกรประชากรที่ 06 ที่มีก้านดอกและรังไข่ยาวกว่าประชากรอื่น และกลุ่มที่ไม่สามารถแยกออกจากกันได้ ซึ่งลักษณะของสีที่แตกต่างกันไม่สามารถใช้แยกกลุ่มทางอนุกรมวิธานได้ ส่วนความยาวจงอยเล็ก และความยาวรังไข่ อาจเป็นลักษณะที่ใช้แยกกลุ่มทางอนุกรมวิธานได้ แต่ยังต้องการตัวอย่างศึกษาเพิ่มเติม โดยเฉพาะตัวอย่างจากเขตการกระจายพันธุ์ในประเทศฟิลิปปินส์ เนื่องจากลิ้นมังกรเป็นกล้วยไม้ที่มีการกระจายพันธุ์กว้าง ผลการวิเคราะห์ความสัมพันธ์ทางพันธุกรรมโดยใช้การรวม 3 ยีน (Internal Transcribed Spacers (ITS) + *matK* + *rbcL*) ด้วยวิธี maximum-parsimony analysis พบว่า เครื่องหมายดีเอ็นเอของยีนทั้งสามร่วมกันสามารถจำแนกตัวอย่างพืชได้ 3 กลุ่ม โดยไม่สามารถแยกลิ้นมังกรชมพูร่องกล้าออกจากลิ้นมังกรได้ จึงยังไม่มีการเปลี่ยนแปลงสถานะทางอนุกรมวิธานในการศึกษาครั้งนี้ ส่วนผลการศึกษาระบบการสืบพันธุ์และโครงสร้างประชากรของลิ้นมังกรฟอร์มดอกสีส้มและลิ้นมังกรชมพูร่องกล้า ในพื้นที่อุทยานภูหินร่องกล้า จังหวัดพิษณุโลก พบว่ากล้วยไม้ดินทั้งสองชนิดมีการออกดอก 1-11 ดอกต่อช่อ และการติดผลในสภาพธรรมชาติอยู่ระหว่าง 42.55-52.21 % ไม่พบการติดผลหากไม่มีการถ่ายละอองเรณู แสดงว่าไม่มีการผสมตัวเองตามธรรมชาติ ในขณะที่การผสมเกสรด้วยมือมีการติดผล 100 % ซึ่งบ่งชี้ถึงความสามารถในการผสมตัวเองได้ และต้องการพาหนะในการช่วยผสมเกสร และไม่พบความสัมพันธ์ระหว่างขนาดของใบและการติดผล ในเรื่องของการทดสอบการงอกของเมล็ดแบบสมชีพร่วมกับเชื้อราที่แยกได้จากรากและไรโซมกล้วยไม้ดินทั้งสองชนิด จำนวน 32 ไอโซเลตเป็นเวลา 16 สัปดาห์ พบว่าเมล็ดกล้วยไม้ลิ้นมังกรชมพูร่องกล้า มีการงอกเมื่อเลี้ยงร่วมกับเชื้อราไอโซเลต I03 I08 และ I20 แตกต่างกันอย่างมีนัยสำคัญทางสถิติโดยมี

เปอร์เซ็นต์การงอกของเมล็ดสูงสุด เมื่อเลี้ยงร่วมกับราไอโซเลต I20 (14.08 %) ในขณะที่กล้วยไม้ลิ้นมังกรไม่พบการงอกของเมล็ดในการเพาะเลี้ยงแบบสมชีพ การระบุเชื้อรา 3 ไอโซเลต ที่ส่งเสริมการงอกของเมล็ด ด้วยการศึกษาความคล้ายกันของดีเอ็นเอบริเวณ ITS กับฐานข้อมูล GenBank พบว่าราไอโซเลต I03 มีความคล้าย *Colletotrichum* sp. ไอโซเลต 08 มีความคล้าย *Hypoxylon* sp. และไอโซเลต 20 มีความคล้าย *Aspergillus* sp. การทดสอบการงอกของเมล็ดแบบไม่อาศัยราในอาหาร 4 ชนิด พบว่าเมล็ดของกล้วยไม้ดินทั้งสองชนิด งอกภายใน 4 สัปดาห์หลังการเพาะเลี้ยง ที่ระยะเวลา 16 สัปดาห์ เมล็ดลิ้นมังกรชมพูร่องกล้าและลิ้นมังกรมีเปอร์เซ็นต์การงอกรวมสูงสุดบนอาหาร ½VW คือ 19.11 % และ 15.78 % ตามลำดับ ผลการทดสอบฮอร์โมน 3 ชนิดได้แก่ BAP, GA และ TDZ ที่ระดับความเข้มข้น 1, 3, และ 5 mg/L ในอาหาร 1/2VW ต่อการการงอกของเมล็ดพบว่า หลังจากเพาะเลี้ยง 16 สัปดาห์ เมล็ดลิ้นมังกรชมพูร่องกล้าและลิ้นมังกรที่เลี้ยงบนอาหาร ½VW ที่เติม BA 1 mg/l มีการงอกรวมสูงสุด (12.86 % และ 11.83 % ตามลำดับ) ข้อมูลที่ได้สามารถเป็นแนวทางในการอนุรักษ์และใช้ประโยชน์ของกล้วยไม้สองชนิดนี้ และกล้วยไม้ดินชนิดอื่น ๆ



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THEERA THUMMAVONGSA : THESIS TITLE. TAXONOMY, REPRODUCTIVE BIOLOGY AND SEED GERMINATION OF *HABENARIA RHODOCHEILA* HANCE COMPLEX (ORCHIDACEAE). THESIS ADVISER : ASSOC. PROF. NOODUAN MUANGSAN, Ph.D. 134 PP.

Keyword: Phylogenetic analysis/Breeding system/Population size structure/ Symbiotic seed germination

*Habenaria rhodocheila* Hance is a highly demanded terrestrial orchid due to the diversity of its floral color. Thus, it has been taken out from the natural for commercial purposes, becoming endanger species. The objectives of this thesis were to 1) taxonomically revise the species of the *H. rhodocheila* Hance complex, 2) compare reproductive biology including breeding system, flowering and fruit set, and population size structure of *H. rhodocheila* and *H. janellehayneiana*, and 3) compare symbiotic and asymbiotic seed germination of *H. rhodocheila* and *H. janellehayneiana*. The results revealed that the morphometric study of seven populations of *H. rhodochila* complex examined by PCA plot and dendrogram using 26 floral morphological characters *H. janellehayneiana* was separated with the convergent stigma, supporting the species circumscription differed from *H. rhodocheila.* Moreover, *H. rhodocheila* population number 03 and 06 were separated by rostellum length, and pedicel and ovary length, respectively. The other populations were mixed. It was indicated that color forms are not a taxonomic character for *H. rhodocheila.* Although, rostellum length, and pedicel and ovary length seem to be good taxonomic characters, but it is better to add more specimen especially from Philippines, because this species has very wide distribution. The phylogenetic analysis using a maximum-parsimony analysis method, combined (Internal Transcribed Spacers (ITS) + *matK* + *rbcL*) dataset identified four major clades. The result indicated that *H. janellehayneiana* is in the same group with *H. rhodocheila*. So, there is no taxonomic status change from this study. Comparative study of breeding system and population size structure in Phu Hin Rong Kla National Park, Phitsanulok province showed the number of flowers per inflorescence *of H. rhodocheila* (orange form) and *H. janellehayneiana* ranged from 1 to 11. Natural fruit set ranged from 42.55-52.21 %. No fruits was created by untouched (no pollination) of both species on caged individuals set fruit, however all manually self-pollinated flowers gave 100 % fruit set. This indicated that both species were genetically self-compatibility, non-autogamous species with no apomixis. There is no relationship between leave length and fruit set. Both orchid species are self-compatibility and

need vectors for pollination. The population size structure of both species showed the low number of seedlings and large individual, indicating low reproductive success. To conserve these populations, re-introduction with seedlings propagated by seeds and protection the large individual are crucial needed. A total of thirty-two fungal isolates were recovered from roots and tubers of both plant species. The seed germination test of *H. janellehayneiana* with fungal isolates I03, I08, and I20 differed significantly between treatments. The highest total seed germination percentage was on I20 (14.08 %). *H. rhodochila* had no germination when cultured with 12 fungal isolates. Fungal identifications based on DNA similarity of ITS to the GenBank database revealed that I03 was similar to the *Colletotrichum* sp., I08 was similar to the *Hypoxylon* sp., and I20 was similar to the *Aspergillus* sp. The asymbiotic seed germination of four tested media revealed that seeds of both plant species germinated within four weeks after inoculation. At 16 weeks, *H. janellehayneiana* and *H. rhodocheila* had the highest total seed germination percentages on ½VW media with 19.11 % and 15.78 %, respectively. The results of testing three hormones, BAP, GA and TDZ, at concentrations of 1, 3, and 5 mg/L demonstrated that *H. janellehayneiana* and *H. rhodocheila* cultured on ½VW added with 1 mg/l BA had the highest total germination, 12.86 % and 11.83 %, respectively. The results of this research may be applied for conservation and exploitation of these species and other terrestrial orchids species.



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Theera Thummavongsa

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# CHAPTER I
# INTRODUCTION

## 1.1 Background/Problem

*Habenaria* is one of the large terrestrial orchid genus with 880 species, belonging to subfamily Orchidoideae (Zhang and Gao, 2021), family Orchidaceae. The genus is well represented in Thailand with 46 species which are mainly found in the northern and north-eastern parts of the country (Kurzweil, 2009). *Habenaria rhodocheila* Hance complex is terrestrial orchids with showy flowers, which can be used for ornamental purpose as well as herbal usage for treating heartiness, swellings, traumatic injuries and relieving pain (Teoh, 2016). The current classification of the *H. rhodocheila* complex is unclear in terms of names, because of flower color and morphology variation. Over the years, new species have been classified out of the *H. rhodocheila*, such as *H. erichmichelii* Christenson. *H. militaris* Rchb. f., *H. pusilla* Rchb. f., *H. rhodocheila* subsp. *rhodocheila*, *H. roebbelenii* Rolfe, *H. xanthocheila* Ridl., *Smithanthe erichmichaelii* (Christenson) Szlach. & Marg., and *S. rhodochelia* (Hance) Szlach. & Marg. These names were accepted as the synonyms of *H. rhodocheila* (Govaerts, 2003; Averyanov, 2010; Pedersen *et al.*, 2011, Glichenstein, 2018). The classification of *H. rhodocheila* complex was mainly on morphological characters. In 2017 Choltco *et al.* (2017) named a new species of *Habenaria* (*H. janellehayneiana*, Choltco, Moloney, & Yong Gee.) from Phu Hin Rong Kla, Thailand whereas Kurzweil (2009, in Pedersen *et al.*, 2011) did not accept this population as a separated species. However, the morphological variation among populations of the *H. rhodocheila* complex whether it is a continuous or discrete variation of these species has not yet been reported. Therefore, the morphometrics of floral characteristics may reveal the relevant information for further species delimitation.

Developed molecular markers used in the identification and molecular systematics of Orchidaceae species have been well reported. DNA barcoding is a taxonomic tool with a high potential for species identification in Orchidaceae (Schindel and Miller, 2005). The plastid *matK*, *rbcL*, *trnH-psbA* and nuclear internal transcribed spacer (ITS) have been used as DNA barcodes in *Dendrobium* from mainland Asia and it was found that ITS and *matK* have distinct barcoding gaps and are the optimal DNA barcode based on all evaluation methods (Xu *et al.*, 2015). To identify species in

Zambian Edible Orchids, authors used the *rbcL*, *matK*, and nrITS DNA barcoding markers, and theis results revealed that 16 orchid species from six different genera could be identified using nrITS DNA barcoding. It was also shown that combining *rbcL* and *matK* was helpful in identifying tubers down to the genus or family level (Veldman *et al.*, 2018). The combination of *matK*+ITS was more effective at identifying species in *Holcoglossum* than *matK* or ITS individually (Xiang *et al.*, 2011). The *matK*, *rbcL*, and ITS were used in molecular systematic of subtribe Orchidae and Habenariinae combined with morphological characters (Jin *et al.*, 2014). Using these DNA markers, molecular evidence shall be useful for proving the *H. janellehayneiana* species acceptance and may reveal the evolutionary relationship of the *H. rhodocheila* complex.

The reduction of the orchid population in nature is an important issue. The studies of population size structure and reproductive biology were giving a real idea of the decline of this wild orchid population due to over-collection, and may also be a consequence of an evolutionary process such as natural selection or genetic drift (Tremblay *et al.*, 2005). Reproductive biology studies are therefore urgently needed for conservation management and applied in agriculture to develop into economic crops. The population size structure has been studied in many rare and endemics species, such as the three species of genus *Sirindhornia* (local endemics *S. mirabilis* and *S. pulchella* and the widespread *S. monophylla*). These three species have similar demographic characteristics, but different reproductive success and reproductive attributes (Srimuang *et al.*, 2010a; Srimuang *et al.*, 2010b). Some *Habenaria* species have a reproductive type of non-autogamy (Tremblay *et al.*, 2005; Jersakova *et al.*, 2006) and high reproductive success, ranging from 69.48 to 97.40 % (Pedron *et al.*, 2012). Pollinators for *Habenaria* involve different kinds of moths or butterflies (Lepidoptera) (Dressler, 1993; Singer and Cocucci, 1997; Zhang and Gao, 2017). However, some *Habenaria* (*H. malintana*) are agamospermy with 100 % fruit setting (Zhang and Gao, 2018). So far, the reproduction pattern of *H. janellehayneiana* has not yet been reported. The studies of the reproductive biology of these orchid species will provide information assisting the conservation of orchid species.

*In vitro* propagation of *Habenaria* species is often considered difficult because seed germination, growth, and development depend on several factors such as capsule maturity, macro- and microelement composition, culture method, and often the addition of nonspecific growth factors (Stewart and Zettler, 2002). Researches have been conducted via symbiotic culture, and found to promote the seed germination (Khamchatra *et al.*, 2016; Stewart and Kane, 2006b). A symbiotic seed

germination protocol for *H. macroceratitis* was also reported although protocorms did not develop to advanced leaf-bearing stages in this study (Stewart *et al.*, 2006a). Sangmanee *et al*. (2012) examined the effects of mycorrhizas on the growth of *H. erichmichelii* Christenson, and the results showed that media with *Humicola* sp. and *Oidiodendron* sp. gave the greatest average plant height. The approach of symbiotic seed germination may be critical to ensure that plants used in translocation include a suitable mycobiont. Moreover, commercial axenic production of *Habenaria* species has been achieved for some species (Kongsawad *et al.*, 2013). However, symbiotic seed germination of *H. rhodocheila* and *H. janellehayneiana* has not yet been performed.

The main objectives of this study were to investigate the relationship among populations of the *H. rhodocheila* complex and to prove the *H. janellehayneiana* species acceptance. Morphometric and molecular phylogenetic analysis, reproductive biology studies, as well as seed germination studies were conducted. The results from this research study will be useful for future orchid conservation and exploitation of these *Habenaria* species and other terrestrial orchid species.

## 1.2. Research objectives

The objectives of this thesis were:

1.2.1 To taxonomically revise the species of the *H. rhodocheila* Hance complex by morphometric and molecular phylogenetic analysis

1.2.2 To compare the reproductive biology of *H. rhodocheila* and *H. janellehayneiana*

1.2.3 To compare the symbiotic seed germination of *H. rhodocheila* and *H. janellehayneiana*

1.2.4 To compare the asymbiotic seed germination of *H. rhodocheila* and *H. janellehayneiana*

## 1.3 Research questions

Research questions of this thesis were:

1.3.1 How many taxa of *H. rhodocheila* Hance complex can be recognized?

1.3.2 What are the patterns of breeding system, population size structure, flowering and fruit set of *H. rhodocheila* and *H. janellehayneiana*?

1.3.3 What are the most efficient and promising mycorrhiza species/isolates for seed germination of *H. rhodocheila* and *H. janellehayneiana*?

1.3.4 What is the most suitable medium in promoting the seed germination of *H. rhodocheila* and *H. janellehayneiana*?

## 1.4 Research hypotheses

Research hypotheses of this thesis were:

1.4.1 One species of *H. rhodocheila* Hance complex with more than one varieties were recognized by using morphometric and phylogenetic analysis.

1.4.2 Because there is variation of flower color forms and habitats, the patterns of breeding system, population size structure, flowering and fruit set of *H. rhodocheila* and *H. janellehayneiana* were different.

1.4.3 Due to habitat differences, the most effective mycorrhiza species/isolates in promoting the symbiotic seed germination of *H. rhodocheila* and *H. janellehayneiana* were different.

1.4.4 Medium conditions significantly influence successful germination of orchid species. Symbiotic seed germination of *H. rhodocheila* and *H. janellehayneiana* differed among various media.

## 1.5 Scope and limitation of the study

1.5.1 Taxonomic revision of *H. rhodocheila* Hance with 6 color forms and *H. janellehayneiana* were carried out by morphometric analysis only 5 color forms of *H. rhodocheila* and *H. janellehayneiana* were carried out by phylogenetic analysis (Figure 1.1).

1.5.2 Reproductive biology including breeding system, flowering and fruit set, and population size structure of *H. rhodocheila* (orange form) and *H. janellehayneiana* were investigated.

1.5.3 Symbiotic and asymbiotic seed germination of *H. rhodocheila* (orange form) and *H. janellehayneiana* were investigated.


**Figure 1.1** *Habenaria rhodocheila* with various flower color forms. (A) red form 1, (B) pink form, (C) yellow form 1, (D) red form 2, (E) orange form, (F) yellow form 2, and (G) *H. janellehayneiana*. (Photos by T. Thummavongsa)

## 1.6 Expected results

1.6.1 The systematic relationships among the taxa in the *Habenaria rhodocheila* Hance complex in Thailand were revised by morphometric and phylogenetic analysis.

1.6.2 Knowledge of reproductive biology of *H. rhodocheila* and *H. janellehayneiana* were obtained.

1.6.3 Potential mycorrhiza fungi for symbiotic seed germination and protocorm development of *H. rhodocheila* and *H. janellehayneiana* were identified and used to aid future orchid propagation.

1.6.4 Suitable medium for asymbiotic seed germination and protocorm development of *H. rhodocheila* and *H. janellehayneiana* were identified.

1.6.5 The results from this thesis study might be used to aid future orchid conservation and exploitation of the *Habenaria* species and other terrestrial orchid species.

# CHAPTER II
# LITERATURE REVIEW

## 2.1 The genus *Habenaria* and taxonomy revision of *H. rhodocheila* Hance complex

The genus *Habenaria* comprises 880 species and is grouped into 32 sections (Kranzlin, 1893 in Pridgeon *et al*., 2001; Zhang and Gao, 2021), in which 46 species were found in the northern and north-eastern parts of Thailand. The species of Thai *Habenaria* are normally terrestrial and found in different forest types, ranging from dry to moist deciduous or evergreen forest, bamboo forest, pine forest, and open tree savanna, thriving from low altitudes to over 2000 m (Kurzweil, 2009; Pedersen *et al.*, 2011).

Based on morphological characteristics, Kurzweil (2009) revised the taxonomic classification of *Habenaria* in Thailand and proposed that potentially closely related species (refer as species complex) can be recognized as follow:

1) The *H. amplexicaulis* group
2) The *H. medioflexa* group
3) The *H. longitheca* group
4) The *H. dentata* group
5) The *H. rostellifera* group
6) The *H. marginata* group
7) The *H. hosseusii* group
8) The seven species of the *H. humistrata* group,
9) The *H. rhodocheila* group.

Taxonomy revision of *H. rhodocheila* complex has a history of identifying and synonyms as follows (Table 2.1).

**Table 2.1** Taxonomic revision of *H. rhodocheila* complex.

| Year | Name | Type of name | | References |
|---|---|---|---|---|
| | | Accepted | Synonyms | |
| 1866 | *H. rhodocheila* Hance | √ | | Hance (1866) |
| 1878 | *H. pusilla* Rchb.f | | √ | Rchb (1878) |
| 1886 | *H. militaris* Rchb.f | | √ | Rchb (1886) |

**Table 2.1** Taxonomic revision of *H. rhodocheila* complex (Continued).

| Year | Name | Type of name | | References |
|---|---|---|---|---|
| | | Accepted | Synonyms | |
| 1896 | *H. xanthocheila* Ridl. | | √ | Ridley (1896) |
| 1913 | *H. roebelenii* Rolfe | | √ | Rolfe (1913) |
| 1866 | *H. rhodocheila* Hance | √ | | Hance (1866) |
| 1878 | *H. pusilla* Rchb.f | | √ | Rchb (1878) |
| 1886 | *H. militaris* Rchb.f | | √ | Rchb (1886) |
| 1896 | *H. xanthocheila* Ridl. | | √ | Ridley (1896) |
| 1913 | *H. roebelenii* Rolfe | | √ | Rolfe (1913) |
| 1992 | *H. rhodocheila* Hance subsp. *philippinensis* (Ames) Christenson | √ | | Christensen (1992) |
| 2003 | *H. erichmichelii* Christenson | | √ | Christenson (2003) |
| 2017 | *H. janellehayneiana*, Choltco, Moloney, & Yong Gee. | √ | | Choltco *et al*. (2017) |

The general morphology characters of *Habenaria rhodocheila* is described by Pedersen *et al*. (2011) as follows; Terrestrial or epilithic, deciduous with obliged tuber. Smooth or elongate-papillose and 2-3 tubular cataphylls cover the stem up to 45 cm tall. Leaves; simple and spiral arrangement, with lanceolate-oblong blades up to 7-leaved, acute, mucronate, up to 24 by 3.8 cm, green or infrequently with greyish-green mottling, occasionally with reddish margins, margins papillose. Inflorescences; Lax, with up to 20 flowers. Rachises are up to 6 cm long, papillose, green or light pinkish, and predominantly papillose. Floral bracts are ovate-lanceolate, acuminate, and up to 30 by 7 mm in size, with reddish or denticulate margins. Flowers; 20–35 mm across, red, orange, pink, or yellow in color, and have a mild perfume. Sepals and petals are greenish with a red lip; lateral sepals are occasionally pale green; petals were originally described as green; spurs are whitish brown or orange; and light maroon, reddish green, or orange are infrequently seen. Gynostemium comes in a variety of colors, including orange, reddish, and whitish-yellow. Pollinia; orange, rostellum is whitish yellow or red, and stigma processes are whitish yellow or red. The ovary (containing the pedicel) can be up to 32 mm long, smooth, and orange or brownish in color. The sepals; subacute. The median sepal is upright and widely oval, measuring up to 15 by 10 mm in size.

Lateral sepals; obliquely oblong, reflexed, up to 12 by 6 mm, partially rolled-in, and basally joined with the lip. Petals; erect, creating a hood with the median sepal, elliptic-lorate, subacute, 1-veined, up to 13 by 3 mm, with a conspicuous rounded apical lobe on the front side, and elliptic-lorate, subacute, 1-veined, up to 13 by 3 mm. Lip; 3-lobed, measuring up to 35 by 23 mm, with a 28-mm long claw that is fairly papillose and has a collar in front of the spur entrance. Mid lobe; spathulate, measuring 9–19 by 7.5–19 mm, and is apically bifid, with a 3–7 mm deep incision and a minute lobule. Flabellate side-lobes, 9–21 by 4–9 mm, are typically oblong-ovate. Spur; cylindric, 30–50 mm long, and thickened at the apex. Gynostemium; length of 3–5 mm. Anther canals range in length from 4 to 8 mm. Anther; apical connective process. Central rostellum lobe; prominent and longer than the anther. Fruits; elliptic-fusiform, measuring up to 28.1–38 mm length and 4–5.2 mm wide, with a short stalk up to 6 mm long and a 3 mm apical beak (Figure 2.1).

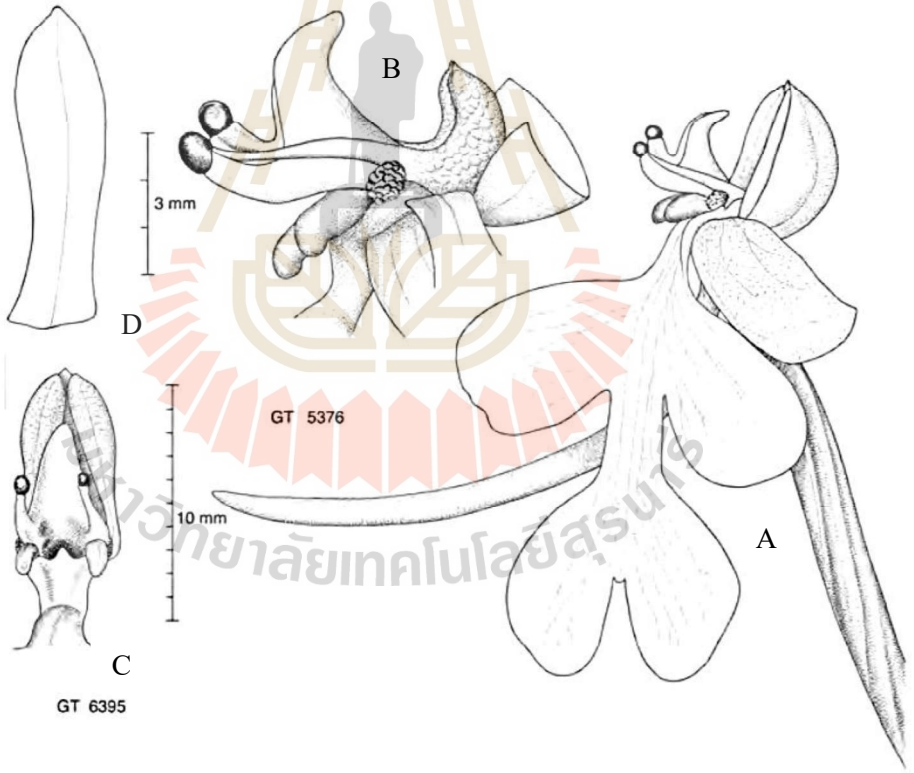


**Figure 2.1** Morphology of *Habenaria rhodocheila* Hance (A) flower, (B-C) gynostemium, (D) petal (Source: Pedersen *et al*., 2011).

## 2.2 Morphometric analyses of Orchidaceae

In orchids, morphological characterization is also critical in conservation and agribusiness breeding efforts (Kasutjianingati and Firgiyanto, 2018). Morphometric

approaches are widely employed in systematics and have a long history of usage in the study of plant development, population differentiation, and systematics (Bateman and Rudall, 2006). Several studies have used morphometrics to classify orchid species, including; Watthana (2006) analyze the morphological variation of *Pomatocalpa maculosum* complex (Orchidaceae) by Principal components analysis (PCA). The studies included 37 quantitative characters and 5 binary characters. There were four distinct species that could be identified, namely *P. diffusum, P. fuscum, P. marsupial,* and *P. maculosum.* And *P. maculosum* subsp. *maculosum* and *P. maculosum* subsp. *andamanicum* are two subspecies of *P. maculosum.* Pinheiro and Barros (2009). Using morphometric analysis to taxonomically revise the species of the *Brasiliorchis picta* complex (Orchidaceae). Morphometric multivariate analysis was analyzed using methods PCA, CVA, DA, and Cluster Analysis with UPGMA. A total of 340 specimens from six species were collected. A total of 340 specimens from six species were collected (*B. chrysantha, B. gracilis, B. marginata, B. picta, B. porphyrostele,* and *B. ubatubana*). The *B. gracilis* has the most morphological discontinuity, because of its diminutive size. Other species start to form groupings, but intermediate characteristics across species cause overlaps among species, difficult to discriminate between individuals. The differentiation of the species and lineages in this complex may be influenced by hybridization and geographic distribution. Warghat *et al.* (2012) reported the morphometric study of *Dactylorhiza hatagirea* in India. These groups are stably physically similar or dissimilar. Researchers used multivariate morphometric approaches such as principal component analysis (PCA), multidimensional scaling (MDS), and cluster analysis. The first two major components account for more than 75 % of population variation. PCA and MDS analysis yielded results that were equivalent to cluster analysis, which revealed significant phenotypic variation in morphological and horticultural traits that can be used in genetic improvement. To support this research, more useful information on the state of *D. hatagirea* populations was provided, which may improve the site's conservation value and settle taxonomic and nomenclatural issues in appropriate locations. Lustre *et al.* (2020) analyzed morphological characters of the *Habenaria parviflora* complex (Orchidaceae). Eighteen morphological characters were used to undertake multivariate and univariate morphometric analysis of 675 individuals from 65 communities across Brazil, Argentina, and Uruguay, representing 12 morphotypes. The group exhibits perplexing morphological patterns, implying that the complex does not correlate to a single species and that each morphotype does not correspond to a taxon. *H. parviflora*, only two taxa could be distinguished using a combination of characters: *H. paulensis* and

*H. ulei.* Flower size, leaf width, and opening angle of the sepals were always the major distinguishing characteristics in both species. In the investigations, there was a lot of overlap between the populations of the remaining morphotypes, making it impossible to create morphological groups with the features utilized. The number of flowers, ovary length, and angles between floral components, which were not previously employed in the taxonomy of this complex, are crucial traits for taxonomic recognition and can be examined further in future taxonomic studies of both the group and the genus.

Sandamali *et al.* (2020) reported that the morphometric analyses and phenetic relationships were performed on five *Dendrobium* (Orchidaceae) species in Sri Lanka. Cluster Analysis (CA) and PCA were used to analyze the data. Thirty-one morphological characters, six vegetative and twenty-five florals, were considered, with five being qualitative and twenty-six being quantitative. Two distinct clusters have emerged as a result of cluster analysis. As a result, the floral morphology of cluster 1, *D. nutans*, *D. panduratum*, and *D. aphyllum*, is strikingly similar. Except for leaf thickness, *D. crumenatum* and *D. heterocarpum* are shown to be comparable in cluster 2. The floral features, as opposed to vegetative characters, were found to play a substantial role in inferring relationships between these species. Furthermore, PCA demonstrated that qualitative characters had a greater impact on determining interspecific species distinction than quantitative characters. Hartati *et al.* (2021) studies morphological characteristics of *Phaius* spp. (Orchidaceae) in Indonesia. To characterize the physical appearance of *Phaius* orchids and to cluster the species based on their morphological similarities. *Phaius* has five different species (*P. tankervilleae, P. indigoferus, P. montanus, P. amboinensis*, and *P. callosus*). The collection and observation were based on 30 morphological features, which included stems, pseudobulbs, leaves, and flowers. The acquired data were transformed to binary data and dendrograms were generated using the Numerical Taxonomy and Multivariate Analysis System (NTSYS) Spc 2. 02 tool. The findings suggest that the five species of *Phaius* spp. have morphological similarities ranging from 63 % to 80 % based on morphological characterization. Sumbembayev *et al.* (2021) studied flower morphometry, four species of genus *Dactylorhiza* (Orchidaceae) in Kazakhstan; *D. incarnata, D. fuchsii, D. maculata*, and *D. salina*, using 17 metric parameters as representatives. The result of principal component analysis for species and populations showed a high degree of flower morphometric variability. Cluster analysis revealed the population diversity's structure. Data from the photographic processing of flower morphometry, the analysis of variance ANOVA, and the degree of variation at the population level were used to

create structural schemes for further taxonomic work on members of the genus *Dactylorhiza*.

In addition, combined morphological and molecular phylogenetic data results in more efficient, reliable identification and helps to build up more stable phylogenetic hypotheses. Raskoti and Ale (2019) reported the morphological characters and molecular phylogeny that were used to establish the systematic position of the genus *Habenaria* from Nepal. The new plant species was identified using DNA analysis and morphological treatment. This species was distinguished by its multiple growing callus-shaped tuber, shorter stature with a short stem, longer and wider oblanceolate leaves, peduncle with a foliaceous bract and only one sterile bract, lateral sepals oblong, obtuse, petal apex obtuse, lateral lobes of lip spreading upwards, spur not exceeding the ovary and pedicel, and spur not. Phillips and Bytebier (2020) performed taxonomy revision of the African orchid genus *Stenoglottis* and revised utilizing a mix of morphometric and phylogenetic analysis to resolve species-level relationships and uncover useful diagnostic characteristics. The recognition of all known *Stenoglottis* taxa was not supported by multivariate morphometrics. The delimitation of five species and two variants is supported by morphological and phylogenetic evidence, with floral spurs, labella, leaves, bracts, and auricles serving as trustworthy diagnostic characters.

## 2.3 Molecular and phylogenetic/Systematics of Orchidaceae

Phylogenetic data can be used to investigate relationships between species on a variety of scales, both below and above species rank. This data is based on molecular DNA sequencing data and matrices created by analyzing a wide range of morphological characteristic data. DNA barcodes and molecular systematics are used to study the identification and the phylogenetics of Orchidaceae.

### 2.3.1 Single locus barcodes

Molecular markers used to identify orchid species are as follows:

1) ITS region (Internal Transcribed Spacer) of nuclear ribosomal regions have been proposed as a changeable molecular marker for detecting genetic variation between genera, species, and even within species. Because nuclear genes (especially introns) and spacers contain many genetic changes, they are used to identify genetic diversity, define taxa, and research relationships in plants (Asahina *et al.*, 2010), and exhibit the highest variability (Mort *et al.*, 2007). The ITS is approximately 635-653 bp long (Chiang *et al.*, 2012; Wu *et al.*, 2012). The flanked portions of the ITS fragment, 5.8S in the middle and 18S, 26S at the two ends, are conserved sequences that can be used to build primers (Shneyer, 2009) (Figure 2.2).

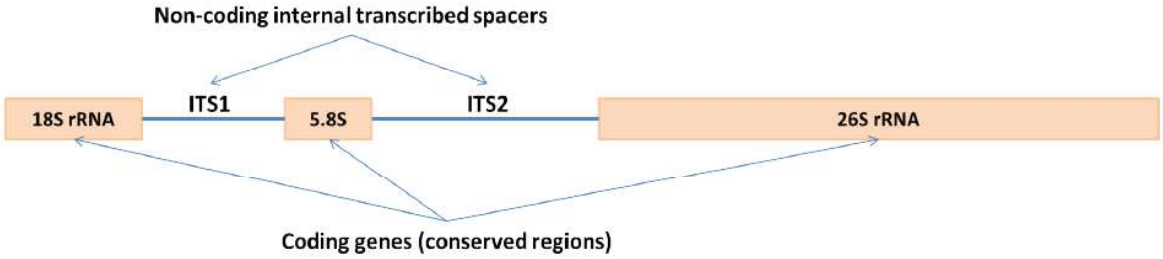


**Figure 2.2** ITS DNA region (Source: Vu *et al*., 2017).

At the species level, the ITS has a high resolution and they are also seen in cells with a lot of copies (Kress *et al*., 2005; Shneyer, 2009). Besides that, in comparison to plastid markers, the ITS with nuclear genes can give more complicated information about biparental inheritance (Chase and Fay, 2009). For example, the aligned sequence of the ITS length in *Holcoglossum* (Orchidaceae) was 567 bp and had 26 informative sites and 27 variable sites (Xiang *et al*., 2011). Based on 129 congeneric *Dendrobium* species and sets of sequences from both the experiment and the GenBank, the ITS barcode was shown to be the best DNA barcode, providing 100 % species resolution and 93 % based on sets of sequences from both the experiment and the GenBank. In the study, the single ITS barcode had a higher resolution value than other single barcodes such as *matK*, *rbcL*, *rpoB*, *rpoC1*, and even combination barcodes (Singh *et al*., 2012). In *Dendrobium* (Orchidaceae), the ITS was successfully implemented. This locus was used to investigate the evolutionary connection and differentiation of 11 medicinal *Dendrobium* spp. from one another, as well as from two adulterant species, *Pholidota articulata* and *Flickingeria comate* (Wu *et al*., 2012). Using nuclear ribosomal ITS sequence data, *G. speciosum* Blume, *G. wallisii* Rchb.f., *G. kinabaluense* Ames, *C. schweinf*., *G. pantherinum* Rchb.f., and *G. cominsii* Rolfe were completely identified at the species level in the *Grammatophyllum speciosum* complex (Orchidaceae) (Yokawa *et al.*, 2013). For phylogenetics analysis, the ITS tree comprising 16 *Paphiopedilum* species and two variants discovered in Vietnam showed significant Jack-knife support (Trung *et al*., 2013). In general, the ITS is worthwhile for barcoding projects; however, ITS2 can be used to boost resolution effects, or supplementary markers can be used as stated in the combination barcodes section (Vu *et al*., 2017).

2) *matK*: The maturase K protein is encoded by the *matK* gene. This is also a gene that is rapidly changing andhas the potential to be employed as an identification molecular marker in a variety of barcoding and phylogenetic studies for species discrimination. This region was assessed at significantly greater levels of

sequence variants (CBOL, 2009; Chase *et al*., 2007). The *matK* is the strongest barcode for Orchidaceae, according to large-scale orchid barcoding initiatives (Lahaye *et al.*, 2008). The *matK*, which is also recommended as a universal plant barcode, resolved 80.56 % of the species. For *Paphiopedilum*, *matK* had a species resolution of 100 % (Parveen *et al.*, 2012). The DNA barcodes, *matK* and *rbcL*, used for 20 endangered Orchidaceae species located in Mexico revealed that single *matK* enabled for identifying the majority of orchid species (Sosa *et al.*, 2013). Tallei *et al*. (2021) reconstructed the phylogenetic relationship of *Taeniophyllum* using DNA barcode markers. The *matK* gene was shown to be useful for DNA barcoding in *Taeniophyllum* orchids, butthe ITS sequence has yet to be proven. The effectiveness of *matK* as a barcode of *Paphiopedilum* in India had also been reported by Parveen *et al.* (2012), who demonstrated *matK*'s ability to discriminate natural hybrids.

For some taxonomic groupings, *matK* has a problem with homopolymer runs of mononucleotide repeats, resulting in low-quality bidirectional sequences (Asahina *et al*., 2010). Cuénoud *et al*. (2002) has solved this problem either by design improving the specific primer, pairs 390F and 1326R. The *matK* amplification rates in orchids are relatively high, up to 100 % in Oncidiinae (Wu *et al*., 2010) and *Dendrobium* (Xu *et al.,* 2015). And very high range in Indian *Paphiopedilum* (95.23 %) (Parveen *et al.*, 2012), and *Holcoglossum* (92.31 %) (Xiang *et al.*, 2011). The *matK* sequences perform well as DNA barcodes because to their high variation as a result of a faster pace of molecular evolution than other coding areas employed as barcodes, such as *rbcL* (Hilu *et al.*, 2003).

3) *rbcL*: The plastid genome is ribulose-1,5-bisphosphate carboxylase/ oxygenase large subunit (*rbcL*) gene. The *rbcL* is approximately 1,428 bp long (Kress *et al.*, 2005) and contains highly conserved areas (Chase *et al.*, 2007). In most investigations, *rbcL* amplification rates ranged from 90 to 100 % in orchids (Xiang *et al*., 2011; Singh *et al*., 2012; Xu *et al*., 2015). Highquality bidirectional sequences and universality were obtained using *rbcL* (Burgess *et al.*, 2011). Ho *et al*. (2020) compared two DNA barcodes (*matK* and *rbcL*) for classification of 21 jewel orchid accessions from Vietnam. The results revealed four different species, and the *rbcL* gene has a stronger potential for differentiation than either *matK* gene. Although, in some orchids, the weak variable of the *rbcL* region was able to discriminate well at the genus level, but not at the species level (Lahaye *et al*., 2008). The *rbcL*, on the other hand, was heavily considered in numerous combination barcodes (Vu *et al*., 2017).

4) Other molecular markers

- *trnH-psbA:* The *trnH-psbA* intergenic spacer is a noncoding intergenic spacer. It was recommended to be the most promising choice for a single-locus barcode for land plant identification because of its high interspecific variation, high length variation, and good priming sites (Kress and Erickson, 2007). The length of *trnH-psbA* across a wide range of plants is about 400 bp, which is fairly short and meets the barcode's criteria of being short enough for facile amplification (CBOL, 2009). PCR rates in orchids were likewise quite high, reaching 100 % in *Dendrobium* (Yao *et al*., 2009), *Oncidium* (Wu *et al*., 2010), and *Cymbidium* (Siripiyasing *et al*., 2012). In general, early on, *trnH-psbA* was highly regarded, but later on, due to numerous obvious flaws, it was viewed less favorably. The most common issue with *trnH-psbA* is that it was too difficult to align land plants in general (Kress *et al*., 2007).

- ITS2: ITS2 has lately gained popularity as a significant plant barcode, located between the ribosomal genes 5.8S and 28S of the ITS regions. The ITS2 spacer supplies structural features required for proper pre-rRNA processing and is thought to have a role in the regulation of active ribosomal subunit transcription (Chen *et al*., 2010). In *Dendrobium*, the length of ITS2 is just about 248 bp. Both PCR and sequencing success rates in ITS2 were quite high, generally 100 % in *Dendrobium* (Feng *et al*., 2015). In different genera, the success rates for using the ITS2 region to identify Orchidaceae taxa at the species level varied; 100 % in *Scaphyglottis*, 98.3 % in *Satyrium*, 91 % in *Dendrobium*, 81.8 % in *Dichaea*, 79.7 % in *Disa*, 79.6 % in Masdevallia, 76.6 % in *Paphiopedilum*, 76.1 % in *Telipogon*, 74.1 % in *Cymbidium*, 71.2 % in *Dendrochilum*, 69.3 % in *Cyrtochilum*, 65.9 % in *Phalaenopsis*, 65.1 % in *Oncidium*, 62.9 % in *Maxillaria*, 49.1 % in *Gomesa*, 31.1 % in *Diuris*, and 22.7 % in *Ophrys* (Yao *et al*., 2010).

- *ycf1:* any existing plastid candidate barcodes; *ycf1* encompasses the small single copy (SSC) and inverted repeat (IR) sections of the plastid genome. The IR region of *ycf1* is short (less than one kilobase in length) and preserved. In seed plants, however, the part of ycf1 in the SSC area displays a lot of sequence variability (Dong *et al*., 2015). The great diversity of *ycf1* suggests that it could be useful for DNA barcoding in land plants. For Orchidaceae, *ycf1* has been demonstrated to be useful in phylogenetic analysis (Sramko *et al*., 2014; Li *et al*., 2015). Because *ycf1* is too long, this valuable area received little attention for DNA barcoding or molecular systematic purposes at low taxonomic levels (5,709 bp in *Nicotiana tabacum*) (Done *et al*., 2012).

In addition, several molecular markers have been developed to identify Orchidaceae, including *atpF-atpH*, *rps16-trnQ*, *trnL-F*, and *psbK-psbI*. However, because of the numerous issues it confronts, it is not widely used (Vu *et al*., 2017).

**2.3.2 Using multi-locus barcodes for orchid identification**

In the study of universal barcodes for a wide variety of plants and orchids, it was evident that a single locus would not be enough in this position, both in terms of universality and editing capabilities, and multi-locus barcodes appear to be more powerful and efficient (Vu *et al.*, 2017). In 2007, Kress and Erickson (2017) first proposed the two-locus barcode *trnH-psbA*+*rbcL*, with species resolution enhanced to 85 % for angiosperms, compared to the greatest 82.6 % of *trnH-psbA*. Chase *et al.* (2007) first proposed the three-locus barcodes of *matK*+*rpoC1*+*rpoB* or *matK*+*rpoC1*+*trnH-psbA*, for 11 Mesoamerican orchid species, a combination of two or three *matK*, *rpoC1*, *rpoB*, and *trnH-psbA* loci was also recommended and the resolution findings revealed that *matK*+*rpoC1*+*rpoB* 100 %, *matK*+*rpoC1*+*trnHpsbA* and *matK*+*rpoB*+*trnH-psbA* 90.9 %, as the single *matK* 90.9 %. In 2009, the Consortium for the Barcode of Life (CBOL) first proposed the two-locus combination of *rbcL*+ *matK* as a plant barcode. This combination offered a viable solution to the difficult balance between universality, sequence quality, discrimination, and cost (CBOL, 2009). On three loci, *rbcL*, *matK*, and *trnH-psbA*. Kress *et al*. (2009) investigated whether using multilocus supermatrices to create phylogenetic hypotheses at the species level would increase resolution power. The results showed that the CBOL *rbcL*+ *matK* core-combining barcode only discriminated 92 % of the time, while *trnHpsbA*+ *rbcL* did 95 %. The *rbcL*+*matK*+*trnH-psbA*, a three-locus combination, discriminated 98 % of the time (Kress *et al.*, 2009). ITS2+*psbA-trnH* was strongly suggested as a core and complementary barcode by Chen *et al*. (2010). In *Holcoglossum*, the combination of *matK*+ITS was more effective at identifying species than *matK* or the ITS individually (Xiang *et al.*, 2011). Singh *et al*. (2012) found that *matK*+*rpoC1*+*rpoB* produced the greatest resolution (94.44 %) among other three locus barcodes on *Dendrobium* (Orchidaceae), only behind the ITS (100 %). They concluded that "barcodes, if based on a single or limited locus, would constitute specific taxa (Singh *et al.*, 2012). Both of the *Cymbidium* (Orchidaceae) possibilities in Thailand, 1) *matK*+*rpoC1*+*trnH-psbA* and 2) *matK*+*rpoB*+*trnH-psbA* have achieved 100 % species resolution of 19 *Cymbidium* (Siripiyasing *et al*., 2012).

Because intergenic spacers are known to be highly variable, Xu *et al*. (2015) reported the DNA barcodes (ITS, ITS2, *matK*, *rbcL* and *trnH-psbA*) studies in *Dendrobium* (Orchidaceae). The results showed that five barcodes can be easily amplified and sequenced with the currently established primers and ITS+*matK* is the

optimal barcode in all evaluation methods. Veldman *et al*. (2018) used DNA barcoding markers; *rbcL*, *matK* and nrITS to determine species in Zambian Edible Orchids. The results showed that a total of 16 orchid species in six different genera could be identified sing nrITS DNA barcoding. The *rbcL* combination with *matK* proved suitable to identify the tubers up to the genus or family level. Li *et al*. (2020) used combined sequences of nuclear ITS and plastid *matK* regions to support a new generic segregate; *Blepharoglossum*. The results of both Bayesian inference and maximum likelihood analysis agree that *Cestichis* is not monophyletic as it is currently defined. The *Blepharoglossum* clade, which corresponds to a well-known sect. *Blepharoglossum* has been removed from *Cestichis* and promoted to generic rank as a result of these findings. Li *et al.* (2021) studied the DNA barcoding for species identification, comparing the single or combined sequences among the 4 chloroplast genes (*matK*, *rbcL*, *ndhF* and *ycf1*). Based on genetic distance, phylogenetic analysis revealed that *ndhF* and *ycf1* sequences were capable of identifying orchids at the genus and species level in a single gene. The *matK* + *ycf1* and *ndhF* +*ycf1* were qualified for identification at the genus and species levels in the combined sequences. DNA barcode information is consistent and accurate, making it useful for academics. It lays the foundation for identification, evaluation, utilization and conserving Orchidaceae germplasm resources.

**2.3.3 Phylogenetic analysis of *Habenaria***

DNA barcodes and molecular systematics are used to study the identification and the phylogenetics of Orchidaceae. The phylogenetic studies of *Habenaria* were published in a new old-world tropical region. Inda *et al*. (2012) studied phylogenetics of tribe Orchideae including 103 species and 25 genera based on combined DNA matrices of three DNA regions including nrITS, mitochondrial *coxl* intron and plastid *rpll16* intron. The results support the recently recircumscribed generic concepts, evolution in the subtribe and classification and nomenclature the tribal and subtribal level. In 2013, Batista *et al.* used DNA sequences from the ITS region and the plastid *matK* gene toinvestigate the evolutionary connections of 151 *Habenaria* taxa from the Neotropics, Bonatea from the Old World, and selected groupings of African *Habenaria*. The Bayesian and parsimony trees were consistent, and the Neotropical species constituted a well-supported group in all analyses. The Neotropical clade and African *Habenaria* species in sections Dolichostachyae, Podandria, Diphyllae, Ceratopetalae, and Bilabrellae formed a well-supported "core *Habenaria* clade," which includes the genus's type species from the New World. The Neotropical clade is structure includes an African origin, and the low sequence divergence between Neotropical species suggests the genus's recent radiation in the

New World. *Bonatea* and *Habenaria* species are native to the Chlorinae and Multipartitae groups formed a well-supported clade that was sister to the "core *Habenaria* clade." There are at least 21 well-supported subgroups in the Neotropical clade. The genera Bertauxia, Kusibabella, and Habenella, which have been separated from New World *Habenaria*, are not monophyletic, a revision of the sectional classification rather than a generic division appears to be the most appropriate approach. The data do not corroborate the previously proposed wide generic fragmentation of *Habenaria*, but they do offer a framework for revising the infrageneric classification and researching the species' morphological development and geographical distribution in the New World (Batista *et al.,* 2013). In 2014, Jin *et al*. used molecular systematics based on plastid *matK*, *rbcL* and nuclear ITS and morphological characters for intricate taxonomic problems in the subtribe Orchidinae including Asian taxa of Habenariinae. Their results showed Orchidinae and Asian Habenariinae are monophyletic and Orchidinae is divided into distinct superclades (Jin *et al*., 2014).

In 2017 the phylogenetic relationships of terrestrial orchids, Subtribe Orchidinae (Orchidaceae, Orchidoideae), are illustrated, and generic delimitation within Orchidinae is discussed (Jin *et al*., 2017). The researchers employed seven DNA markers (five plastids and two nuclear), a large number of Orchidinae species (400 in 52 genera), and three phylogenetic analysis methods (maximum likelihood, maximum parsimony, and Bayesian inference). Orchidinae s.l. is a monophyletic family of orchids. *Satyrium* is a member of the Orchidinae s.l. family. Asian-European Orchidinae s.s. has two sisters: *Brachycorythis* and *Schizochilus*. *Sirindhornia* and *Shizhenia* are sister clades to the *Tsaiorchis-Hemipilia-Ponerorchis* alliance. The *Habenaria-Herminium-Peristylus* alliance has a sister, *Stenoglottis*. *Habenaria*, the biggest genus in the Orchidinae family, is polyphyletic, with two distinct clades: Asian-Australian and African–American–Asian. *Hemipilia*, *Ponerorchis* s.l., *Sirindhornia*, *Shizhenia*, and *Tsaiorchis* are sister genera to *Herminium* s.l. and Asian-Australian *Habenaria*, and the *Ponerorchis* alliance proposes to recognize five genera; *Hemipilia*, *Ponerorchis* s.l., *Sirindhornia*, *Shizhenia*, and *Tsaiorchis*. It seems reasonable to preserve Satyrium in Orchidinae, and splitting *Habenaria* into two genera based on morphological features and geographical distribution may be the least disruptive method. Batista *et al.* (2017) described phylogenetic relationships of four new species and one variety of *Habenaria* (Orchidaceae) in Brazil. *H. brachydactyla, H. irwiniana, H. minuta, H. pansarinii*, and *H. pansarinii* var. *minuscula* are among the species illustrated. *H. campylogyna* is proposed as a new name for the ill-conceived *H. humilis.* The five species are small,

slender plants with linear leaves concentrated at the stem's base, which distinguishes them from other Neotropical taxa. They are not closely related and do not belong to any of the previously discovered Neotropical subclades, according to molecular phylogenetic analyses based on ITS *matK*, *trnK* intron, and *rps16–trnK*. All of the new taxa were found in isolated locations, branching from bigger subclades' basal nodes, implying that these physical characteristics are plesiomorphic in the Neotropical clade. *Habenaria brachydactyla* is a genetically different lineage of the Neotropical *Habenaria* clade that reflects an early lineage.

In 2019, Raskoti and Ale studied molecular phylogeny of genus *Habenaria* from Nepal, using nuclear (ITS) and chloroplast (*matK*, *rbcL*) DNA sequence regions to infer its systematic position. There is also no significant difference for topologies of individual markers, according to the parsimony-informative. For the discussion of phylogenetic relationships, the combined data showed consistency index = 0.41 and retention index = 0.81. The topologies generated by maximum parsimony and Bayesian inference analyses were similar. For the discussion of evolutionary relationships, the Bayesian inference tree is shown with significant support (BSMP = 90 %, PP = 1.00). The phylogenetic tree suggests that various Asian clades of Old World *Habenaria*, including the nested *Pecteilis* are sister to *Herminium* + *Hsenhsua*. Clade III also has three well-supported subclades. Subclade I is made up of 32 species that live in tropical, subtropical, and temperate climates. Subclade II has seven alpine species, while subclade III contains three tropical and subtropical species. Clade IV is divided into six subclades, with the New World *Habenaria* intermixed with ten species from the Asian Old World *Habenaria* (support BSMP = 96 %, PP = 1.00). In New World, *Habenaria*, *Bonatea, Gennaria*, and *Diplomeris* are nested. *Peristylus* has been identified as sister to the subclade formed by clades I, II, III, and IV, with moderate support (BSMP = 71 %, PP = 0.82). The combined data revealed that the newly sampled taxon *H. sandiegoensis* (BSMP = 86 %, PP = 1.00), which is highly supported as sister and its allies (BSMP = 98 %, PP = 1.00), represents a different lineage within Clade III/Sublcade 1 (Raskoti and Ale, 2019).

## 2.4 Reproductive biology of Orchidaceae and *Habenaria*

The reproductive biology of a plant is important information for orchid conservation. The flowering phenology and the breeding system are important in the natural population of the flowering plants. The orchids have various life strategies for their own sake as ecological niche. Food-deceptive orchid such as *Orchis purpurea* had low fruit set with no relation to inflorescence size and individual density

(Jacquemyn and Brys, 2010). Many species are self-compatible such as *Sirindhornia mirabalis*, *S. monophyla* and *S. pulchella* (Srimuang *et al.*, 2010b) and *Habenaria davidii* and *H. fordii* (Zhang and Gao, 2017). The reproductive success of the narrow-distributed species may be lower than the wide distributed species (Srimuang *et al.*, 2010a). The juvenile number in some population may relate to density of adults, while fruit set was significantly lower in highly disturbed fragment (Parra *et al*., 2011).

For the genus *Habenaria*, Pedron *et al*. (2012) reported the pollination biology and breeding system of four sympatric *Habenaria* species (*H. johannensis*, *H. macronectar*, *H. megapotamensis*, and *H. montevidensis*) from southern Brazil. *H. montevidensis* is pollinated with butterflies (Hesperiidae, especially of the genus *Urbanus*), *H. johannensis* is pollinated by the moths *Manduca rustica* and *M. sexta*, *H. macronectar* is pollinated by the moths *Eumorpha labrusca* and *M.* cf. *lucetius*, and *H. megapotamensis* is pollinated by moths of *M.* cf. *lucetius*. All studied species are self-compatible. They also had high reproductive success (from 69.48 to 97.40 %). A case study by Watthana and Pedersen (2008) reported that the threatened *Dendrobium scabrilingue* Lindl from Salawin Wildlife Sanctuary in Mae Hong Son province, northern Thailand had low flowering (18.5 %), indicating that the type of breeding system is an allogamous, and needs vectors for pollination. The lager plants had 100 % flowering and fruit set and need to be conserved in the natural habitat to maintain genetic diversity. Moreover, there were high number of seedlings in the population, suggesting that there is no need to do re-introduction with seedlings. Other field studies of *Habenaria* species include *H. hieronymi* (Singer and Cocucci, 1997), *H. fordii* (Zhang and Gao, 2017), and *H. aitchisonii* (Zhang and Gao, 2017). However, *Habenaria* is also notorious for having a very low fruit set (Ikeuchi *et al.*, 2015; Singer and Cocucci, 1997; Thien and Utech, 1970).

Williams and Watthana (2011) reported that *Habenaria rhodocheila* from Thailand was found to be pollinated by swallowtail butterflies (Papilionidae: Papilio) *Papilio helenus*. Recently, Zhang *et al*. (2021) found a congeneric Thailand pollinator to be the pollinator of *H. rhodocheila* in Guangxi Province, south China. Chen *et al*. (2020) reported the pollination of *H. rhodocheila* in South China. Two diurnal swallowtail butterflies, *Papilio helenus* and *P. nephelus* (Papilionidae), were discovered to be effective pollinators (Figure 2.3). Experiments with hand pollination revealed that this species was self-compatible but pollinator-dependent. Self-pollinated fruits, on the other hand, generated considerably fewer seeds with bigger embryos than cross-pollinated fruits, indicating considerable inbreeding depression. Fruit set was higher than pollinium removal rates in this orchid species, which indicates a high level

of pollination effectiveness in a species with friable pollinia. In the genus *Habenaria*, shifts from moth to butterfly pollination parallel other orchid lineages, revealing the possibility for pollinator-mediated floral feature selection. Both bagged intact flowers and emasculated (anthers removed) blooms failed to produce fruits in the breeding system. The fruit set of butterflies visited; organically pollinated flowers was 83.33 %. Fruit set was 100 % in both self-pollination and cross-pollination series. However, after cross-pollination, the proportion of seeds with big embryos in fruits was much larger (62.26 %) than in the self-pollination series (33.32 %). The proportion of seeds with big embryos in spontaneously pollinated fruits (42.67 %) was significantly greater than in self-pollinated fruits but significantly lower than in cross-pollinations mediated by hand. In cross-pollinated fruits, the proportion of seeds with aborted embryos was much lower than in self-pollinated and naturally pollinated fruits. The index of inbreeding was 0.219 (Chen *et al.*, 2020). The flowering period of *H. rhodocheila* population in Thailand was half a month longer than that of the Guangxi populations, according to phenology, floral characteristics, and pollinator behavior (Zhang *et al.*, 2021).


**Figure 2.3** Habitats and pollinators of *Habenaria rhodocheila* in South China. (A) Plants growing on rocks, (B) A flower of plant, (C) The pollinium of plant (D-I) *Papilio nephelus,* P= pollinium; C, caudicle; Vs = Viscidium disk; St= Stigma; L= Labellum; Sp = Spur (Source: Chen *et al*., 2020).

## 2.5 Seed germination and seed development of Orchidaceae

### 2.5.1 Stage of seed germination

In order for an orchid seed to germinate *in situ*, it must be in close proximity to a suitable fungus. The microporous gaps between the integument plates allow fungal hyphae to penetrate the seed. Once inside the seed coat, the hyphae pass through the embryo's suspensor region and into the adjacent cortical cells walls, where they form coli-like formations (pelotons). The presence of pelotons indicates that the

orchid and the invading fungus are compatible. When the meristematic cells within the seed divide and proliferate, they produce a protocorm. As the protocorm grows, new cells infiltrate the space between the seed epidermis and the existing cortex, eventually growing to form the outer cortex and serving as the protocorm is principal point of infection. Rhizoids, also known as trichomes, are outward protrusions from the protocorm that serve as a hyphal activity interface between the germinated seedling and the culture media. At this stage, the protocorm has nearly doubled in size, with roots and leaves emerging (Swarts and Dixon, 2017). Stewart and Zettler (2002) reported symbiotic germination stages of *Habenaria repens*, *H. quinquiseta*, and *H. macroceratitis*. A dissection microscope was used to evaluate seed germination, and protocorm development. On a scale of 0–5, developmental growth phases were rated; stage 0 = no germination, stage 1 = production of rhizoid, stage 2 = rupture of the testa, stage 3= appearance of the shoot, stage 4 = the emergence of leaf from shoot region, and stage 5 = elongation of leaf (Figure 2.4).

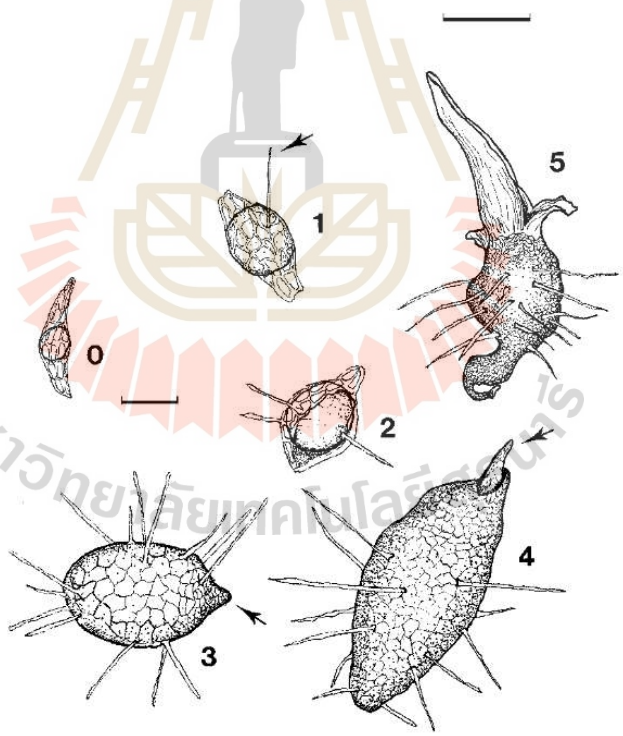


**Figure 2.4** *Habenaria* seed germination and protocorm developmental growth stages. (0) Stage 0 no germination, (1) Stage 1 Production of rhizoid, (2) Stage 2 rupture of the testa, (3) Stage 3 appearance of the shoot, (4) Stage 4 emergence of leaf from shoot region, (5) Stage 5 elongation of leaf, scale bar = 1 mm. (Source: Stewart and Zettler, 2002).

### 2.5.2 Orchid mycorrhiza association

#### 2.5.2.1 Orchid mycorrhizal fungi (OMF)

In nature, orchids establish mycorrhizal symbioses with fungi, which affect seed germination, protocorm growth, and adult nutrition. In addition, orchids absorb mineral nutrients and even chemical substances from contacts with orchid mycorrhizal fungi. As a result, orchid mycorrhizal fungi have a wide range of characteristics and play an important function in the orchid life cycle (Li *et al*., 2021). The fungi colonize orchid tissues with orchid mycorrhizal characteristics such as peloton development (intracellular hyphal coils) and the absence of cortical tissue necrosis (Rasmussen and Rasmussen, 2014). Diversity of orchid mycorrhizal fungi, Basidiomycota from the form-genus *Rhizoctonia*, are the most common orchid mycorrhiza fungi, the formation of loose clumps of hyphae, which look as poorly developed sclerotia or resting bodies, is a persistent feature of orchid mycorrhizal mycelium in culture and, some cases, hyphae with sepatae or the creation of monilioid cells (Figure 2.5), which are bead-like structures (Currah *et al*., 1997; Agustini *et al*., 2016; Swarts *et al*., 2017).

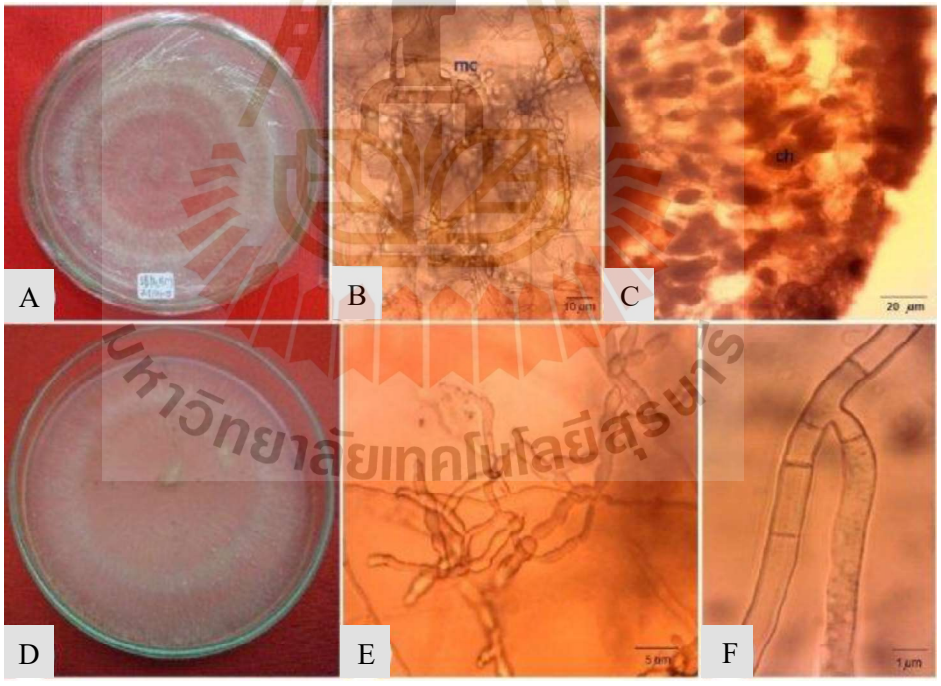


**Figure 2.5** Rhizoctonia- like fungi isolates. A- C fungi isolate from *Dendrobium lancifolium*, (A) isolate on PDA, (B) monilioid cells, (C) hyphae-coil in root section, D-C fungi isolate from *Calanthe triplicate* (D) isolate on PDA, (E) monilioid cells, (F) hyphae with sepatae (Source: Agustini *et al*., 2016).

The most prevalent orchid mycorrhizal fungi, which belong to the Basidiomycota, are known as Rhizoctonia, and include the Tulasnellaceae and Ceratobasidiaceae. The majority of these three fungal taxa have ecological niches ranging from saprotrophy, or the use of decaying matter, to entomophagy, or the use of living things (Dearnaley *et al.*, 2012; Swarts *et al.*, 2017; McCormick *et al.*, 2018). There is dispute about whether nutrition transfer from the fungus to the plant (particularly carbon) occurs across intact pelotons or via lysis of these structures (Figure 2.6), two non-mutually exclusive mechanisms (Dearnaley *et al.*, 2012).

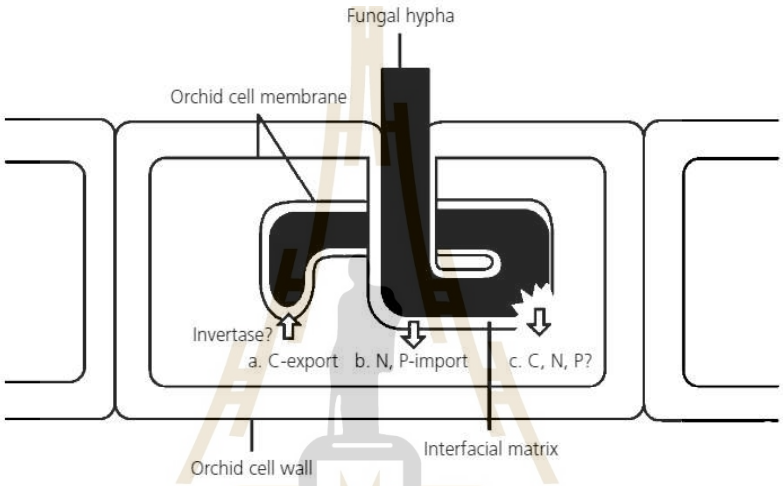


**Figure 2.6** A possibility of nutrition transfer of mycorrhizal fungi in adult green orchid tissues. (a) Carbon (C) from the orchid cytoplasm is transferred to the interfacial matrix, and then to the fungal hypha. Before sucrose is taken up by the fungus, invertase may cleave it into glucose and fructose, (b) Phosphorus (P) and nitrogen (N) from the hypha are transferred to the interfacial matrix and then to the orchid cytoplasm, (c) Fungal C, N, P, and other nutrients are released into the orchid cytoplasm by peloton lysis (Source: Dearnaley *et al.*, 2012).

Habitats have an indirect impact on orchid species cohabitation and extensive distribution through influencing the composition, structure, and richness of Orchid mycorrhizal fungi communities (Li *et al.*, 2021). Different habitats shape orchid mycorrhizal fungi communities, which influence orchid growth and development (bioregulation) through boosting nutrient absorption (biofertilization) (Figure 2.7). Orchid mycorrhizal fungi associated with the same orchid species in different habitats, other orchids co-occurring in the same habitat, or individuals from most orchid species in the same habitat might vary to some extent (Jacquemyn *et al.*, 2021; Duffy *et al.*, 2019).

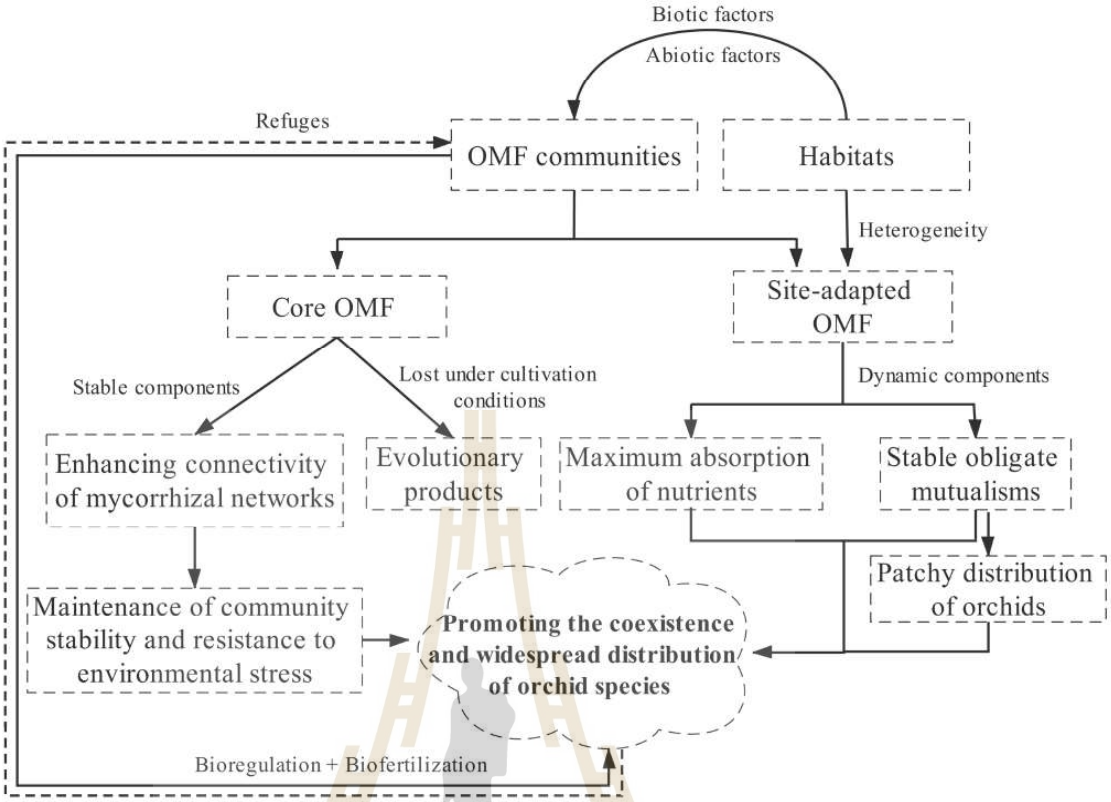


**Figure 2.7** A framework demonstrating how habitats affect Orchid mycorrhizal fungi (OMF) communities, which in turn affect orchid species cohabitation and wide distribution (Source: Li *et al.,* 2021).

### 2.5.2.2 Orchid non-mycorrhizal fungi

In addition, to mycorrhizal fungi that are commonly found in orchids, there are also fungi that are non-mycorrhizal, associated with orchids. Over 110 genera are covered, with 76 of them belonging to Ascomycetes, which have far more diversity and occurrence frequency than orchid mycorrhizal fungi (Sudheep and Sridhar, 2012; Ma *et al*., 2015). The reports of orchid non-mycorrhizal fungi associated with tropical orchids are Xylariales (Govinda Rajulu *et al*., 2016), Helotiales and Capnodiales (Oliveira *et al*., 2014). The fungal genera such as *Colletotrichum*, *Fusarium*, and *Trichoderma* are commonly detected in the roots of tropical orchids (Salazar-Cerezo *et al*., 2018; Sisti *et al*., 2019; Sarsaiya *et al*., 2020). Some orchid non-mycorrhizal fungi have been shown to support orchids growth and development. Fusarium fungi isolated from *Cypripedium reginae* have been shown to stimulate early seed germination in *C. reginae* (Vujanovic *et al*., 2000). And *Colletotrichum* fungi have shown to promote the growth of adults *Dendrobium* spp. (Shah *et al*., 2019; Sarsaiya *et al*., 2020). *In vitro* studies on the roles of non-mycorrhizal fungi during the life cycle of orchids are

currently restricted. Some non-mycorrhizal fungi active chemicals may benefit orchids by enhancing their resilience to abiotic stressors and thereby promoting their adaptability to various environmental circumstances (Ma *et al*., 2015). Waterman *et al*. (2011) suggested that some non-mycorrhizal fungi may degrade local substrates and supply nutrients to orchids.

**2.5.3 Symbiotic seed germination and seed development**

The seed of all wild orchids have required for mycorrhizal fungi association to facilitate germination and development (Khamchatra *et al*., 2016). Although the relationship between the need and the specificity of the fungus in question varies greatly between taxa. Due to the nature of the dust-like seeds that are characteristic of all orchids, mycorrhizal-assisted nourishment is important (Swarts and Dixon, 2017). *In vitro* orchid seed germination and protocorm growth has been classified into stages in a variety of leafy epiphytic and terrestrial orchids, including;

In epiphytic orchids, Alomia *et al*. (2017) studied the viability of seeds and symbiotic seed germination in *Vanilla* spp. from Colombia. The results showed high percentage of *Ceratobasidium* germination (approximately 80 %) co-cultured with fungi isolated from root of adult plants. The *Tulasnella* isolate promoted high germination percentage close to 60 %. Hoang *et al*. (2017) studied *Dendrophylax lindenii* (Orchidaceae) seed germination and protocorm development, as well as asymbiotic and symbiotic culture with one of two mycorrhizal strains obtained from ghost orchid roots. Results showed that the development of the ghost orchid embryo and protocorm was divided into seven stages. When seeds were grown with the fungal strain Dlin-394, they germinated at a higher rate than when they were cultured with strain Dlin-379. The fungus Dlin-394 was identified as a putative ghost orchid germination mycobiont, promoting seed germination and protocorm development considerably. Some study reported of host-specificity of symbiotic mycorrhizal fungi. *Dendrobium devonianum* was inoculated with a fungus (FDd1) isolated from naturally occurring *D. devonianum* protocorms, as well as two other germination-enhancing fungi (FDaI7 and FCb4) from *D. aphyllum* and *Cymbidium mannii*, respectively. When compared to the control treatment, all three fungal strains had a substantial boosting effect on seed germination and protocorm development. The most successful fungal strain was FDd1, followed by FDaI7 and FCb4. Seeds transformed into protocorms in the absence of lighting, but protocorms did not develop into seedlings unless they were exposed to light. The results supported the concept that orchid seedling mycorrhizae are extremely host-specific. The fungal strain was isolated from five *D. devonianum* protocorms and classified as an *Epulorhiza* species.

In terrestrial orchids, Sangmanee *et al*. (2012) examined the effects of mycorrhizas on the growth of *H. erichmichelii* Christenson and found that media with *Humicola* sp. and *Oidiodendron* sp. gave the greatest average plant length at 12 weeks after planting. Stewart and Kane (2006b) reported symbiotic seed germination of *Habenaria macroceratitis*, a rare Florida terrestrial orchid. They described efficient symbiotic seed germination, discussed the fungal specificity and explained the effects of three photoperiods (0/24 h, 16/8 h, 24/0 h L/D) on symbiotic seed germination *in vitro*. Six fungal isolates were obtained from vegetative and flowering plants. The percentage of symbiotic seed germination was 65.7 % and the development of protocorm was most advanced (Stage 2) with fungal mycobiont Hmac-310 (*Epulorhiza* sp.). Kamchatra *et al*. (2016) studied asymbiotic and symbiotic seed germination of *Paphiopedium vilosum* from Thailand and found that asymbiotic seed germination on MS, VW, and Thomale GD (TH) media were not obtained after 16 weeks. The symbiotic germination result with 7 fungal isolates showed that 3 isolates (PVCP01, PVCP05, and PVCP06) substantially increased the germination percentage higher than control. The fungal isolate PVCP01 significantly increased the germination rate index (GRI) and the development rate index (DRI), PVCP05 and PVCP06 isolates, on the other hand, could only increase seed germination and protocorm development to stage 2. Based on morphological characters and nuclear ribosomal transcribed spacer (ITS) sequences of the ITS1 and ITS4 primers, the PVCP01 was identified as *Tulasnella* sp., PVCP05 as *Ceratobasidium* sp., and PVCP06 as *Flavodon* sp. Symbiotic seed germination has applications in horticulture and species recovery, and is regarded as an efficient orchid conservation strategy (Batty *et al*., 2008; Otero *et al*., 2013).

**2.5.4 Asymbiotic seed germination and seed development**

In nature, orchids cannot use their limited lipid reserves, therefore they break down starch or photosynthesis. Orchid seeds may turn green as a result of water intake, which produces swelling, but in the absence of fungal infection, they will not develop further. As a result, they do not germinate well in nature (less than 1 %) (Mondal and Banerjee, 2017). Following the development of the Knudson 'B' and 'C' media, orchid seeds were successfully germinated *in vitro* (Knudson, 1922). Several reports have described orchid species propagation. Orchid seed germination without the presence of fungal symbionts (asymbiotic cultivation) is simplest method for creating vast volumes of seedlings (Stewart and Kane, 2006a). This has been used successfully and widely in numerous orchids for both commercial and conservation purposes (Decruse *et al*., 2013; Chen *et al*., 2015). This technique has seen a lot of development, for example; Asymbiotic germination of an endangered orchid, *Paphiopedilum insigne*. In

modified Burgeff medium (BG1), seeds germinated 88.5 % of the time, with initiation taking 26 days. Plant growth regulators such as 5 M kinetin (KN) + 10 M indole-3-acetic acid (IAA) were added to 1/2 MS medium and influenced the seedlings stage-wise development in a short period of time (Diengdoh *et al*., 2017). Zeng *et al*. (2012) studied asymbiotic seed germination and seedling development of *Paphiopedilum wardii*, an endangered terrestrial orchid on 1/2 MS + 0.5 mg/L NAA, 10 % coconut water, and 1.0 g/L activated charcoal, and obtained 65.33 % seed germination, when compared to a 16-hour photoperiod, 45-day darkness considerably increased germination percentage (75.67 %). Kokaklang *et al*. (2014) reported a maximum of 3.25 % germination of *H. rhodocheila* on modified VW medium (added coconut water 15 % and peptone 1 g/L) after 4 months. Piyatrakul (2014) studied factors influencing germination and seedling development of *H. rhodocheila* (pink from), and found a maximum of 5.48 % germination on modified VW medium (CMU1+ NAA 0.1 mg/L + BA 1 mg/L) after 20 weeks. Stewart and Kane (2006a) compared the effects of six asymbiotic media including Modified Lucke (ML), Murashige & Skoog (MS), Lindemann (LN), Vacin & Went (VW), Malmgren Modified (MM), and Knudson C (KC), four exogenous cytokinins including benzyladenine (BA), zeatin (Zea), kinetin (Kin), 6- (γ, γ-dimethylallylamino) purine (2iP), and three photoperiods including 0/24, 16/8, 24/0 h L/D on seed germination and early protocorm development of *H. macroceratitis*. The highest seed germination was observed on both LM (89.1 %) and KC (89.2 %) after seven weeks of culture. The highest protocorm development was found on MM after 7 and 16 weeks. Zeatin and Kinetin at 1 µM concentration enhanced seed germination at 58 % and 47.2 %, respectively. Seed germination of 91.7 % and protocorm development at stage 4 was observed in the absence of light at 0/24 h L/D.

# CHAPTER III
# MATERIALS AND METHODS

## 3.1 PART I: Plant Taxonomy; A taxonomic revision of *H. rhodocheila* Hance complex using morphological and molecular phylogenetic analysis.

### 3.1.1 Plant materials

Total seven populations, six flower color forms of *H. rhodocheila* Hance and *H. janellehayneiana* were collected from different locations of Thailand (Table 3.1). For molecular phylogenetic analysis, 5 populations of *H. rhodocheila* including 01, 02, 03, 05 and 06 were avialable for analysis. I included a close relative taxa, *H. carnea* (Kurzsweil, 2009) as an outgroup species, collected from Khuan kalong District, Satun Province. *Habenaria dentata* (Sw.) Schltr, and *H. longicorniculata* J. Grahm Lindl. were used as outgroup, and their DNA sequences were downloaded from GenBank (https://www.ncbi.nlm.nih.gov/genbank/).

**Table 3.1** Flower color forms and locations/sources of *H. rhodocheila*, *H. janellehayneiana, H. carnea*, *H. dentata*, and *H. longicorniculata*.

| No | Species/flower color form | Location/sources |
|---|---|---|
| 01 | *H. rhodocheila*/orange | Nakhon Thai District, Phitsanulok Province |
| 02 | *H. rhodocheila*/yellow 1 | Phu Phan District, Sakon Nakhon Province |
| 03 | *H. rhodocheila*/pink | Phu Phan District, Sakon Nakhon Province |
| 04 | *H. rhodocheila*/red 1 | Bung Kan District, Bung Kan Province |
| 05 | *H. rhodocheila*/yellow 2 | Khao Khitchakut District, Chanthaburi Province |
| 06 | *H. rhodocheila*/red 2 | Pak Chong District, Nakhon Ratchasima Province |
| 07 | *H. janellehayneiana* | Nakhon Thai District, Phitsanulok Province |
| 08 | *H. carnea* | Khuan kalong District, Satun Province |
| 09 | *H. dentata* | GenBank (MT500674.1, MT533578.1, KF296645.1) |
| 10 | *H. longicorniculata* | GenBank (JN114561.1, MH935820.1, JN005522) |

### 3.1.2. Morphological study

The habit and flower characters of each *H. rhodocheila* forms and *H. janellehayneiana* was illustrated from spirit or dry herbarium specimens, while the

gynostemium was examined from liquid-preserved materials using a stereoscopic microscope and a digital caliper for measurement. Flowering time and habitat were recorded based on herbarium labels and field observation. Thirty-two morphological terminology was based on Stearn (1992) and Simpson (2006). Morphological characters (Table 3.2) were examined and analyzed using Principal Coordinate Analysis (PCA) with BioVinci V3.0 and SPSS V16.0 statistical package. Non-variable characters were excluded in the analysis.

**Table 3.2** List of morphological characters intended to be used for morphometric analysis of *H. rhodocheila* and *H. janellehayneiana*.

| No | Variables | State |
|---|---|---|
| 1 | Shoot length | Continuous (mm) |
| 2 | Leaf length | Continuous (mm) |
| 3 | Leaf width | Continuous (mm) |
| 4 | Leaf margin | 1. entire; 2. lobbed |
| 5 | Leaf apex | 1. acute; 2. Obtuse |
| 6 | Flower number | number |
| 7 | Dorsal sepal length | Continuous (mm) |
| 8 | Dorsal sepal width | Continuous (mm) |
| 9 | Lateral sepal length | Continuous (mm) |
| 10 | Lateral sepal width | Continuous (mm) |
| 11 | Lateral sepal apex | 1. acute; 2. obtuse |
| 12 | Petal length | Continuous (mm) |
| 13 | Petal width | Continuous (mm) |
| 14 | Petal margin | 1. entire; 2. lobbed |
| 15 | Petal color | 1. orange; 2. red; 3. pink; 4. yellow |
| 16 | Lip length | Continuous (mm) |
| 17 | Lip width | Continuous (mm) |
| 18 | Lip claw length | Continuous (mm) |
| 19 | Side-lobe of lip length | Continuous (mm) |
| 20 | Side-lobe of lip width | Continuous (mm) |
| 21 | Ratio between side-lobe of lip length and width | Continuous (ratio) |
| 22 | Epichile length | Continuous (mm) |
| 23 | Epichile width | Continuous (mm) |

**Table 3.2** List of morphological characters intended to be used for morphometric analysis of *H. rhodocheila* and *H. janellehayneiana* (Continued).

| No | Variables | State |
|---|---|---|
| 24 | Ratio between side-lobe width and epichile width | Continuous (ratio) |
| 25 | Sinus deep of epichile | Continuous (mm) |
| 26 | Sinus width of epichile | Continuous (mm) |
| 27 | Ratio between sinus deep and width of epichile | Continuous (ratio) |
| 28 | Spur length | Continuous (mm) |
| 29 | Rostellum length | Continuous (mm) |
| 30 | Rostellum width | Continuous (mm) |
| 31 | Stigmatophore | 1. divergent; 2. convergent |
| 32 | Pedicel length | Continuous (mm) |

### 3.1.3 Phylogenetic analysis

#### 3.1.3.1 DNA extraction

DNA was extracted from plants using the Genomic DNA Isolation Kit (Plant), (Bio-Helix, Taiwan), following the manufacturer's protocols. 1) Sample Preparation : 50 mg of fresh plant tissue or 25 mg of dry plant tissue were used. Using a mortar and pestle, sample was ground to a fine powder under liquid nitrogen. 2) Lysis : 500 µl of Buffer PL and 0.5 µl of RNase A (50 mg/mL) were added before the sample was ground. After completely dissolving the sample was transferred to a 1.5 mL microcentrifuge tube. Incubated for 30 minutes at 75 °C (Every 10 minutes, inverted the tube). Centrifuged at 14,000 x g for 5 minutes, and transferred a new 1.5 mL microcentrifuge tube with the supernatant. 3) DNA Binding : To the clear supernatant from the previous step, added the same volume of isopropanol and vortex for 5 seconds (e.g., add 350 µL Isopropanol to the 350 µL supernatant). After that, put a column PC in a 2 mL Collection Tube, transferred the mixture to the column PC, and centrifuged for 30 seconds at 14,000 x g, removed the flow-through and placed the column PC back into the collection tube. 4) Wash : the column PC with 400 µL of the buffer W1, centrifuged at 14,000 x g for 30 seconds. Removed the flow-through and placed the column PC back into the same collection tube, added 600 µL of the Buffer W2 (Ethanol added) into the column PC, centrifuged for 30 seconds at 14,000 x g. Discarded the flow-through and placed the column PC back into the same collection Tube. To remove any remaining Buffer W2, centrifuged for 2 minutes at 14,000 x g again. 5) DNA Elution:

transferred 1.5 mL microcentrifuge tube with the dried column PC. In the center of the column matrix, added 50-200 µL of the Pre-Heated Buffer BE or TE into the center of the column matrix, let aside for 3 minutes at 75 °C. Centrifuged at 14,000 x g for 2 minutes to elute the purified DNA. Extracted DNA was stored in -20 °C.

**3.1.3.2 PCR amplification and sequencing**

Two plastid barcodes (the coding genes *matK* and *rbcL*) and a nuclear internal transcribed spacer (ITS) were amplified and sequenced using universal primers (Table 3.3). The selected DNA regions was amplified by using a standard polymerase chain reaction (PCR). The PCR mixtures (25 µL) each contained approximately 10 ng (1–2 µL) of template DNA, 12.5 µL of 2×PCR mix (0.005 units/µL Taq DNA polymerase; 4 mM $MgCl_2$; and 0.4 mM dNTPs), 0.2 µL of each primer and 6.5–7.5 µL of $ddH_2O$. The sequencing reactions were performed using the Applied Biosystems Prism Big Dye Terminator Cycle Sequencing Kit (Foster City, CA).

**3.1.3.3 Sequence download**

All sequences of ITS, *matK*, and *rbcL* of *H. dentata* and *H. longicorniculata* from NCBI were downloaded. The downloaded sequences from NCBI were excluded according to the following three criteria: i) length less than 300 bp; ii) lacking voucher specimens; and iii) vouchers without specific names. *H. dentata* was used as outgroup and *H. longicorniculata* was used as sistergroup for the tree-based analysis following the procedure described by Xu *et al*. (2015). Sequence edits and alignment with bioEdit 7.2, at least three individual plants for each species form were used.

**3.1.3.4 Phylogenetic analysis**

Total twenty eight accessions were used for analysis. Sixteen assessions of six *H. rhodocheila* populations, three accessions of *H. janellehayneiana*, and three accessions of *H. carnea* were newly sequenced. Six accessions were downloaded from GenBank, three accessions of *H. dentata* and three accessions of *H. longicorniculata*. Maximum pasomony (MP) was employed with MEGA-X software version 10.1 to construct the phylogenetic tree. Unordered and equally weighted of all characters were set before analysis (Fitch, 1971). The data set was analysed by the heristic search method with bisection-reconnection (TBR) branch swapping. Consensus tree shall be presented. The bootstrap method (Felsenstein, 1985) with 10,000 replications and fast step searching was used to evaluate clades for internal support.

**Table 3.3** Primers used for PCR and sequencing (DNA Barcodes in *Dendrobium*) (Xu *et al*., 2015).

| Name | | Gene Primer | Product Size (bp) |
|---|---|---|---|
| *rbcL* | 1F | ATG TCA CCA CAA ACA GAA AC | 1,297 |
| | 1360R | CTT CAC AAG CAG CAG CTA GTT C | |
| *matK* | 390F | CGA TCT ATT CAT TCA ATA TTT C | 833 |
| | 1326R | TCT AGC ACA CGA AAG TCG AAG T | |
| ITS | 17SE | ACG AAT TCA TGG TCC GGT GAA GTG TTC G | 857 |
| | 26SE | TAG AAT TCC CCG GTT CGC TCG CCG TTA C | |

## 3.2 PART II: Reproductive biology including breeding system, flowering and fruit set, and population size structure of *H. rhodocheila* and *H. janellehayneiana*.

### 3.2.1 Plant materials/Study species and plots

*Habenaria rhodocheila* (orange form) and *H. janellehayneiana* on Phu Hin Rong Kla National Park, Phitsanulok province were studied in this work. Four 50x50 meter sampling plots of *H. rhodocheila* and *H. janellehayneiana* were established in the field at Phu Hin Rong Kla National Park including plot 1: *H. janellehayneiana* in Mhundaeng waterfall (1,196 m above sea level), plot 2: *H. janellehayneiana* in Romklaoparadorn waterfall, plot 3: *H. rhodocheila* in Lanhinpum (1,310 m above sea level), and plot 4: *H. rhodocheila* in Lanhintake (1,104 m above sea level). Data concerning flowering time and habitat were based on herbarium labels and field observation. Morphological terminology was based on Flora of Thailand. Specimens were preserved in SUT herbarium.

### 3.2.2 Experiment

#### 3.2.2.1 Breeding system and fruit set

Totally 36 floral buds from five plants of each species from their natural habitat were covered with fine-meshed nylon net. To test for autogamy through spontaneous self-pollination (and for apomixis) in the caged individuals, 12 flowers of each plant species were left untouched. To test for genetic self-compatibility, manual self-pollination and cross-pollination of 12 flowers of both species were used as referred by Dafni's method (1992).

**3.2.2.2 Population size structure, flowering and fruit set**

In this study, the length of the longest leaf, the shoot length, and the number of flowers in each inflorescence of *H. rhodocheila* and *H. janellehayneiana* in natural habitats were recorded. For describing population size structure in the plots, the length of the longest leaf and the shoot length was used as a general indicator of plant size and referred all individuals to arbitrarily defined species-specific size classes as vegetative or flowering (Table 3.4). Using the program SPSS for Windows 22, Pearson's correlation coefficient was calculated to test for correlations between leaf length and inflorescence length, and number of flowers per in inflorescence.

Seeds from dehisced capsules of two species were collected and dried in silica gel. Assessment of the number of seeds per capsule were done under a low-power binocular microscope. The mean number of seeds per capsule, mature seeds per capsule and sterile seeds per capsule were calculated. The program SPSS version 16, and independent T-Tests was used in order to test for differences in relative seeds per capsule between the two species.

**Table 3.4** Size class category of leaf length for *H. janellehayneiana* and *H. rhodocheila*.

| Size class | Leaf length (cm) |
|---|---|
| 1 | 0.1-2.0 |
| 2 | 2.1-4.0 |
| 3 | 4.1-6.0 |
| 4 | 6.1-8.0 |
| 5 | 8.1-10.0 |
| 6 | 10.1-12.0 |
| 7 | 12.1-14.0 |
| 8 | 14.1-16.0 |
| 9 | 16.1-18.0 |
| 10 | 18.1-20.0 |

## 3.3 PART III: Symbiotic and asymbiotic seed germination and protocorm development of *H. rhodocheila* and *H. janellehayneiana*.

**3.3.1 Plant materials**

*Habenaria rhodocheila* (orange form) and *H. janellehayneiana* on Phu Hin Rong Kla National Park, Phitsanulok province were studied. For seed collection,

mature undehisced capsules of *H. rhodocheila* and *H. janellehayneiana* plants from 7-8 weeks -old after flowering were stored in paper envelopes and desiccated under silica gel for 3-4 days until the dehiscence of capsules. The viability of seeds was determined within 7 days using tetrazolium test (1 % TTC). Brown and dried seeds from dehisced capsules were stored in sterile Eppendorf tubes at 4 ℃ until use for the seed germination experiment. Before germination test, it was needed to test 1 % TTC again.

**3.3.2 Experiment**

**3.3.2.1 Symbiotic seed germination**

**1) Fungal isolation**

Root samples without any disease were collected from natural habitat. Each root segment was placed into a sterile plastic bag and refrigerated (4 ℃) within 24-48 h after collection until use. In laboratory, the roots of *H. rhodocheila* and *H. janellehayneiana* were rinsed in tap water to remove debris and cut in a 1 cm segment. The segments were sterilized in 0.5 % NaOCl for 5 minutes and finally rinsed in sterile distilled water for three times. The surface- sterilized segments were transferred to a sterile Petri dish and immersed in sterile water. The segment was cut longitudinally and pelotons were removed from a cortical cell using a dissecting needle under a stereomicroscope. The peloton was washed with five changes of sterile distilled water. Each peloton was then placed on a Petri plate containing Potato dextrose agar (PDA) supplemented with 100 µg/mL of streptomycin and 100 µg/mL tetracycline. Plates were incubated at 25 ℃ in the dark and observed periodically until fungal colonies developed. Fungal mycelia of each colony were sub-cultured onto Potato Dextrose Agar plates.

**2) Fungal identification**

The fungal isolates were first identified as mycorrhizal fungi using morphological characteristics and the methods outlined by Currah *et al*. (1987, 1990), Zelmer and Currah (1995) and Zelmer *et al*. (1996). Molecular identification of fungi isolates was performed by amplifying ITS sequences (Table 3.5). DNA was prepared from 7 to 14-day-old fresh culture and extracted with the Genomic DNA Isolation Kit (Plant), (Bio-Helix, Taiwan). The primers ITS1 and ITS4 (White *et al*., 1990; Table 4) were used for ITS amplification (Table 3.5). PCR amplification was performed in a total volume of 35 µL which includes 100 ng genomic DNA, 1xPCR master Mix Kit (OnePCR™ Ultra, BIO-HELIX CO., LTD., Taiwan) and 100 nM of each primer. The amplification was carried out in a thermocycler (Takara, Japan) with a PCR profile 94 ℃ for 5 minutes, followed by 30 cycles of denaturation at 94 ℃ for 1 minute, annealing at 55 ℃ for 1

minute and extension at 72 °C for 1 minute, followed by 5 minutes of final extension at 72 °C. Direct sequencing was performed on both strands of DNA using PCR primers mentioned above by Macrogen, Inc. (Seoul, Korea). The sequences were compared with database sequences using BLAST (National Center for Biotechnology Information; NCBI).

**Table 3.5** Primers used for PCR amplification and sequencing of fungal isolates (White *et al*., 1990).

| Name | Gene Primer | Product Size (bp) |
|---|---|---|
| ITS1 (F) | TCC GTA GGT GAA CCT TGC GG | 290 |
| ITS4 (R) | TCC TCC GCT TAT TGA TAT GC | |

**3) Testing the fungal isolates' capacity to promote germination**

The effect of fungal isolates on promoting *H. rhodocheila* (orange form) and *H. janellehayneiana* symbiotic seed germination *in vitro* were evaluated using a modified method of Stewart and Kane (2006b). Seeds were surface sterilized as described by Batty *et al*. (2001). Seeds in a filter paper pocket were immersed into two changes of sterile distilled water for 2 minutes, followed by 0.5 % (V/V) NaOCl for 10 minutes and finally in three changes of sterile distilled water. Approximately 50-100 surface sterilized seeds were sown on the surface of a one-eighth strip of a sterile Whatman no. 1. Filter paper was placed onto media in Petri dish containing 20 mL of sterile 1/10 Oat Meal Agar (OMA) (pH 5.5). The plates were inoculated with a 5-mm-diameter plug of each fungal inoculum taken from the actively growing hyphal edge 7 days after culturing on PDA. Uninoculated plates were used as a control. The number of replicates for each treatment were four. Petri dish plates were sealed with parafilm and stored in the dark period for 2 weeks and light period for 14 weeks at 25 °C. Randomly sampled protocorms were examined microscopically to confirm the mycobiont structures under a compound light microscope.

**3.3.2.2 Asymbiotic seed germination**

**1) Testing media capacity to promote germination and protocorm development**

The influence of basal medium on seed germination and protocorm development were evaluated by sowing seeds on four basal media: (1) Murashige and Skoog (MS; Murashige and Skoog, 1962), (2) ½MS, (3) Vacin and Went (VW; Vacin and Went, 1949), and (4) ½VW. For each treatment, approximately 100 seeds were surfaces sterilized in a solution containing 90 mL of sterile, 10 % of Haiter for 1 minute, finally

in three changes of sterile distilled water. The seeds were cultured in 20 mL of solidified medium. All experiments consisted of three independent replicates with nine culture plates per replicate incubated at 25 °C under a 16-h light/8-h dark photoperiod provided by cool white fluorescence lamps for 32 weeks.

**2) Testing 6-benzyl aminopurine (BAP), gibberellic acid (GA) and thidiazuron (TDZ) capacity to promote germination and protocorm development**

Following an initial trial, 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) were all media used in this experiment and were further modified by the addition of 2 % sucrose, 0.8 % agar and 15 % coconut water. The pH of the medium was adjusted to 4.8 before sterilization and were autoclaved at 121 °C for 20 minutes. Three replicate plates per experiments were incubated at 25 °C under a 16-h light/8-h dark photoperiod cycle condition for 32 weeks.

**3.3.2.3 Data collection**

Seed germination and protocorm development for both the asymbiotic and symbiotic experiments were observed every 4 weeks for 8 months under a stereomicroscope. The germination and the developmental stages were scored on a 1-5 incremental growth scale (Table 3.6, Figure 3.1) (Stewart and Zettler, 2002). Percentages of seed germination and protocorm development for each developmental stage were calculated from the total number of viable seeds.

The germination rate index (GRI) and developmental rate index (DRI) were determined as described in Papenfus *et al*. (2015):

$$GRI = \frac{G1}{1} + \frac{G2}{2} + \ldots \frac{GX}{X.}$$

Where G1 is the germination percentage x 100 at the first count after sowing; G2 is the germination percentage x 100 at the second count after sowing; and so on.

$$DRI = \frac{D1}{1} + \frac{D2}{2} + \ldots \frac{DX}{X.}$$

Where D1 is the percentage of stage 2-5 developed protocorms x 100 at the first count after sowing; D2 is the percentage of stage 2-5 developed protocorms x 100 at the second count after sowing; and so on.

**3.3.2.4 Statistical analysis**

The experimental design was established in a completely randomized design (CRD). A one-way analysis of variance (ANOVA) was performed to test for significance, with SPSS V16.0 statistical package (SPSS Inc., Chicago, USA) and the means compared by Duncan's multiple range test (P=0.05)

**Table 3.6** Developmental stages of symbiotically cultured seeds and protocorms, adapted from Stewart and Zettler (2002).

| Stage | Description |
|---|---|
| 0 | no germination, testa intact |
| 1 | production of rhizoid(s) by embryo (= germination) |
| 2 | rupture of the testa by enlarging embryo |
| 3 | appearance of the shoot (= protomeristem) |
| 4 | emergence of leaf from shoot region |
| 5 | elongation of leaf |

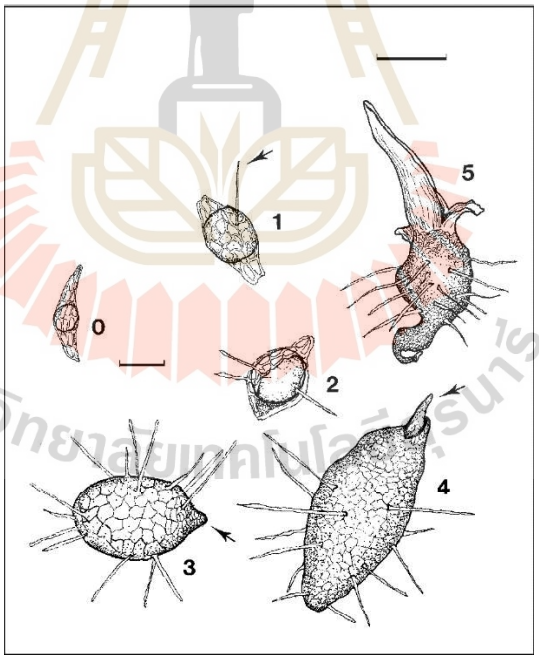


**Figure 3.1** Seed germination and protocorm developmental growth stages of *Habenaria*. Stage 0 = no germination; (1) Production of rhizoid by embryo as denoted by the arrow (= germination); (2) rupture of the testa by enlarging embryo; (3) appearance of the shoot (= protomeristem) as denoted by the arrow; (4) emergence of leaf from shoot region as denoted by arrow; (5) elongation of leaf (scale bar = 1 mm) (Source: Stewart and Zettler, 2002).

# CHAPTER IV
# RESULTS AND DISCUSSION PART I
# PLANT TAXONOMY; A TAXONOMICAL REVISION OF *HABENARIA RHODOCHEILA* HANCE COMPLEX USING MORPHOLOGICAL AND MOLECULAR PHYLOGENETIC ANALYSIS

## 4.1 Morphometric analysis

Morphometric methods are commonly used in systematics, and they have a long history of application in the study of plant development, population differentiation, and systematics (Bateman and Rudall, 2006). In PCA analysis, 70 individuals from *H. rhodocheila* complex populations were studied by using 32 morphological characters. The first axis accounted for 62.93 % of total variation, whereas the second axis accounted for 31.96 % (Figure 4.1A). From both vegetative and reproductive characters, the isolation of numerous samples from other individuals was insignificant. The analysis using only 26 floral characters, the PCA plot revealed an arrangement of four groups of the studied species (Figure 4.1B). The first axis accounted for 46.15 % of total variations, whereas for 25.10 % of total variation in the second axis. There are 4 discriminated groups; group 1 stigmatophore convergent (population *H. janellehayneiana*); group 2 pedicel length between 34.8-36.5 mm (population *H. rhodocheila* 06); group 3 rostellum length between 3.2-3.7 mm (population *H. rhodocheila* 03); group 4 non-separated populations *H. rhodocheila* 01, 02, 04 and 05. The cluster analysis was constructed using 32 morphological characters shown in Figure 4.2A. The dendrogram depicts the results of the morphometric measures in a graph form, the population is not divided by the dendrogram. The results of the study indicated that the use of flower traits can be used to effectively separate populations with stigmatophore, pedicel length and rostellum length (Figure 4.2B). Floral structure of 7 populations of *Habenaria rhodocheila* complex is shown in Figure 4.3. The result analysis also indicated that *H. janellehayeiana* having the taxonomic character, the position of the stigmatophores convergent vs. divergent, is congruent with species circumscription by Choltco *et al.* (2017). The other two discriminated characters, pedicel length and rostellum length, also reflected the population 03 and 06 separated out from the other populations. Meanwhile, the other four populations in

this study pictured broad variation of *H. rhodocheila,* supporting the species circumscription by Kurzweil (in Pedersen *et al.,* 2011). Exceptionally, the population 03 and 06 were separated with shorter rostellum length and longer pedicel and ovary length. The result of this study at least showed the preliminary of morphological character variations with noticeable distinct population. Since *H. rhodocheila* is widely distributed species, it needs more samples to add for morphometric analysis especially from the Phillippines where the accepted subspecies occur.

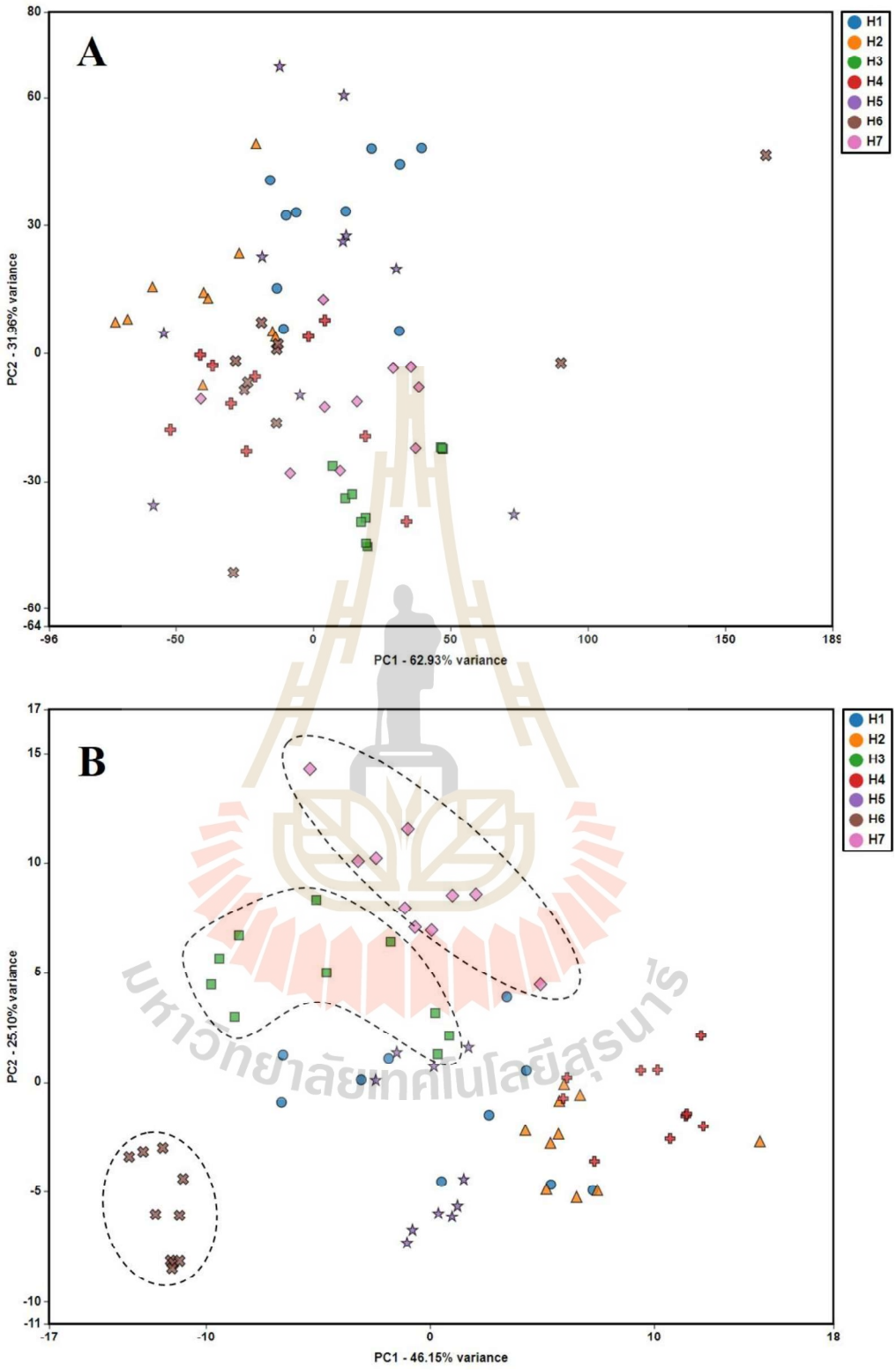


**Figure 4. 1** The Principal coordinates plots for individual plants of *Habenaria rhodocheila* complex. (A) All characters, and (B) Floral characters, H1 = *H. rhodocheila* (01), H2 = *H. rhodocheila* (02), H3 = *H. rhodocheila* (03), H4 = *H. rhodocheila* (04), H5 = *H. rhodocheila* (05), H6 = *H. rhodocheila* (06), H7 = *H. janellehayneiana* (07).

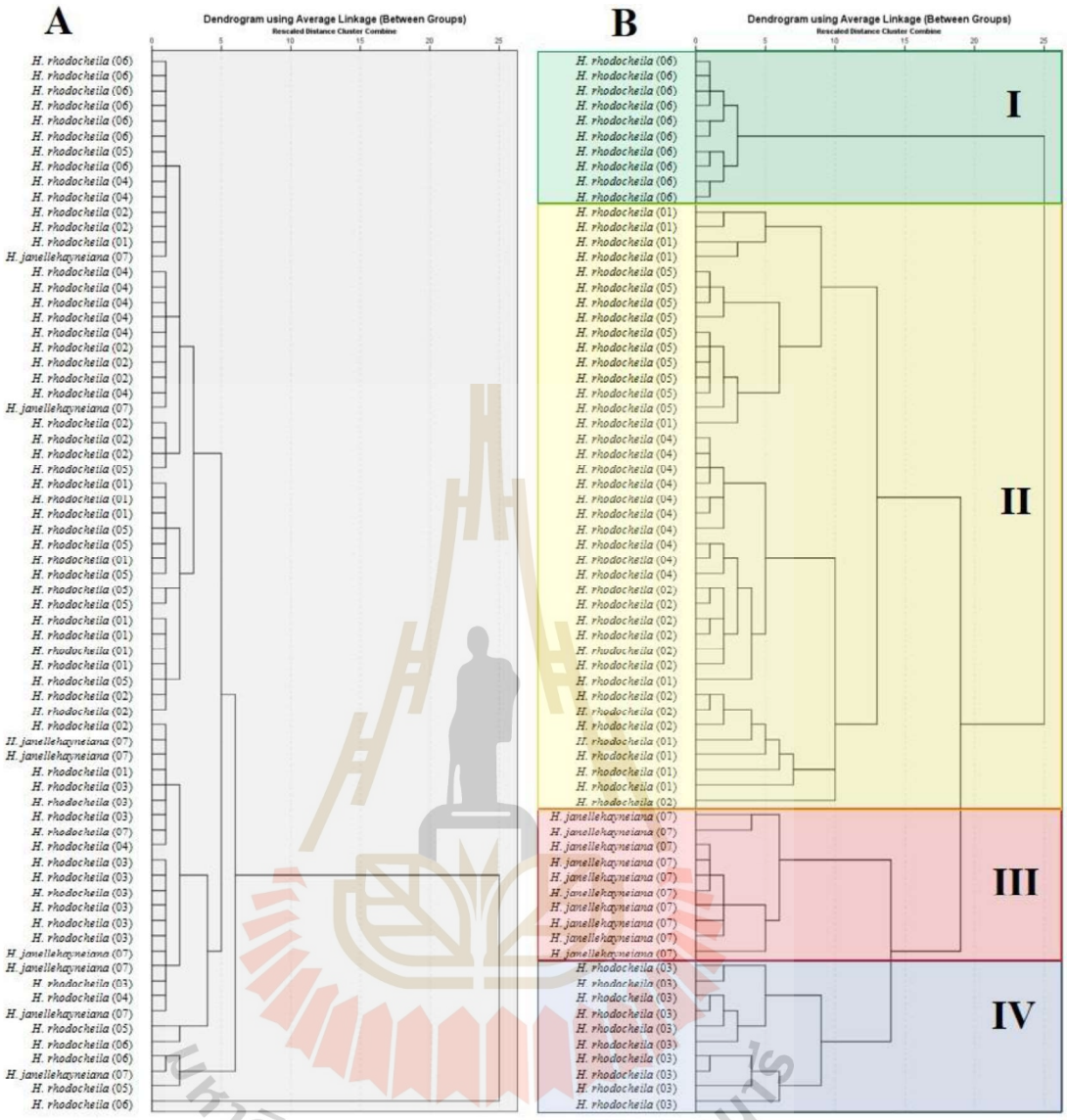


**Figure 4. 2** Dendrogram showing the phenetic relationships of 7 populations of *Habenaria rhodocheila* complex. (A) based on all characters and (B) based on floral characters, H1 = *H. rhodocheila* (01), H2 = *H. rhodocheila* (02), H3 = *H. rhodocheila* (03), H4 = *H. rhodocheila* (04), H5 = *H. rhodocheila* (05), H6 = *H. rhodocheila* (06), H7 = *H. janellehayneiana* (07).


**Figure 4.3** Floral structure of 7 populations of *Habenaria rhodocheila* complex. (A) *H. rhodocheila/*orange (01), (B) *H. rhodocheila/* yellow 1 (02), (C) *H. rhodocheila/*pink (03), (D) *H. rhodocheila/*red 1 (04), (E) *H. rhodocheila/*yellow 2 (05), (F) *H. rhodocheila/*red 2 (06), and (G) *H. janellehayneiana* (07), bar = 1 cm.

## 4.2 Molecular phylogenetic study

### 4.2.1 Sequences and alignment

In this study, twenty-four newly sequences were included. The six sequences of ITS, *matK* and *rcbL* were the first reporting of *H. janellehayneiana* and *H. carnea*. The lengths of the sequences consisted of 795 bp for the ITS region, 840 bp for *matK* and 959 bp for *rbcL*, and 2,594 bp of the alignment of the combined ITS and plastid genes. The features of each aligned data division are summarized in Table 4.1. The ITS is about 635-653 bp in length (Chiang *et al.*, 2012; Wu *et al.*, 2012). The flanking segments of the ITS fragment, 5.8S in the center and 18S, 26S at both ends, are conserved sequences that can be used to create primers (Shneyer, 2009). The ITS length of *Holcoglossum* (Orchidaceae) was 567 bp, including 26 informative sites and 27 variable sites in the aligned sequence (Xiang *et al*., 2011). The *matK* is about 1,442 bp in length (Li *et al.*, 2020). In this study, the length of 840 bp *matK* was used. However, in other orchids it is also shorter than normal, for example 924 bp in *Holcoglossum* (Xiang *et al*., 2011), 833 bp in *Dendrobium*, approximately 630 bp in New World *Habenaria* (Batista *et al.*, 2013). The ITS was also recommended as a universal plant barcode, resolved 80.56 percent of the species and had a species resolution of 100 % for *Paphiopedilum* (Parveen *et al*., 2012). The sequence variations in the *matK* region were found to be considerably higher (CBOL, 2009; Chase *et al.*, 2007). The *rbcL* is 1,428 bp long and comprises highly conserved sections (Kress *et al*., 2005; Chase *et al.*, 2007). In this study, the length of 959 bp of *rbcL* was used, while 623 bp has been used in some Orchidaceae (Sosa *et al*., 2012). The *rbcL* was used to obtain high-quality bidirectional sequences and universality (Burgess *et al.*, 2011). In most studies, *rbcL* amplification rates in orchids ranged from 90 % to 100 % (Xiang *et al*., 2011; Singh *et al*., 2012; Xu *et al*., 2015), which is comparable to high universality.

**Table 4.1** The statistics derived from the parsimony analysis of the chloroplast and nuclear data sets.

| Information | ITS | *matK* | *rbcL* | Combined |
|---|---|---|---|---|
| Aligned sites | 795 | 840 | 959 | 2594 |
| Number of most parsimonious trees | 67 | 48 | 653 | 779 |
| Consistency index (CI) | 0.75 | 0.86 | 0.91 | 0.88 |
| Retention index (RI) | 0.78 | 0.91 | 0.89 | 0.8 |

**4.2.2 Phylogenetic analyses**

All phylogenetic trees were constructed using a maximum-parsimony analysis method with 10,000 bootstrap replicates. The efficiency of the barcodes utilized was demonstrated by the results of the species clustering.

1) Phylogeny inferred from ITS data

A maximum-parsimony analysis retrieved 67 most parsimonious trees (MPTs), consistency index = 0.75 and retention index = 0.78 (Table 4.1). The consensus tree (Figure 4.4) indicates two distinct clades: (i) a clade of five selected populations of *H. rhodocheila*, *H. janellehayneiana*, and *H. carnea*, and (ii) a clade of *H. dentata* and *H. longicorniculata*. *Habenaria janellehayneiana*, *H. rhodocheila* and *H. carnea* have not been revealed any resolution among ingroup species.

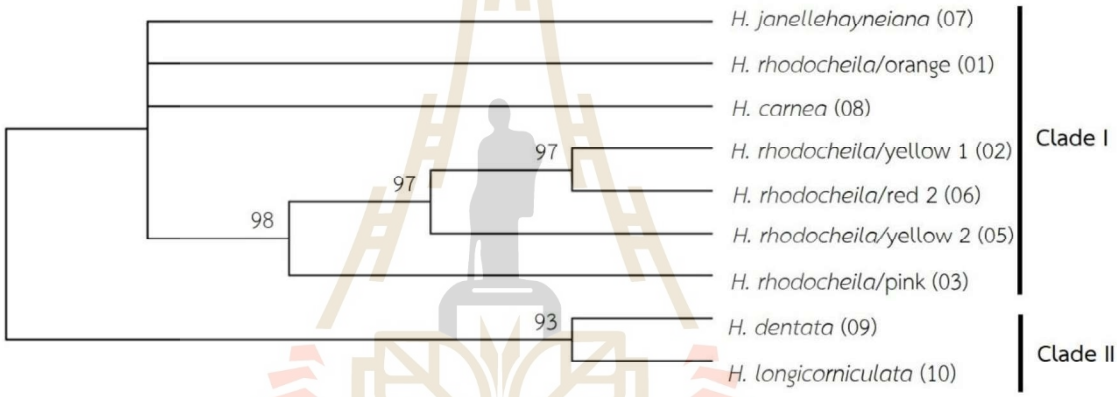


**Figure 4.4** Consensus tree of *Habenaria rhodocheila* complex based on the ITS markers, supported by more than 50 % of the bootstrap trees.

2) Phylogeny inferred from *matK* data

The *matK* gene gives better resolution than ITS gene. A maximum-parsimony analysis retrieved 48 MPTs, consistency index of 0.86 and retention index of 0.91. The consensus tree (Figure 4.5) indicates three major clades with relationships to one another: (i) a clade of five selected populations of *H. rhodocheila*, and *H. janellehayneiana*, (ii) a clade of *H. carnea*, and (iii) a clade of *H. dentata* and *H. longicorniculata*. However, *H. janellehayneiana* is grouped with *H. rhodocheila.*

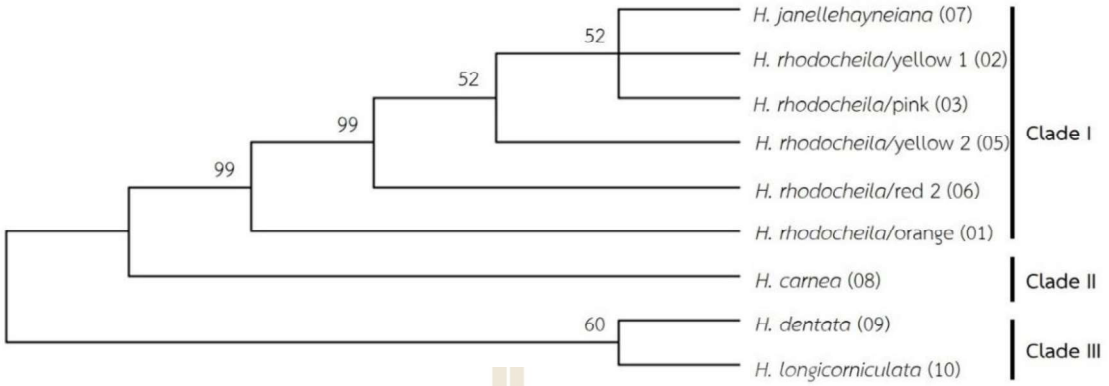


**Figure 4.5** Consensus tree of *Habenaria rhodocheila* complex based on the *matK* markers, supported by more than 50 % of the bootstrap trees.

3) Phylogeny inferred from *rbcL* data

A maximum-parsimony analysis retrieved 653 MPTs, consistency index of 0.91 and retention index of 0.90. The consensus tree (Figure 4.6) indicates four major clades with relationships to; (i) a clade of three selected populations of *H. rhodocheila* (01, 02, and 06), *H. janellehayneiana*, and *H. carnea*, (ii) a clade of *H. rhodocheila*. (03), (iii) a clade of *H. rhodocheila* (06), and (iv) a clade of *H. dentata* and *H. longicorniculata*.

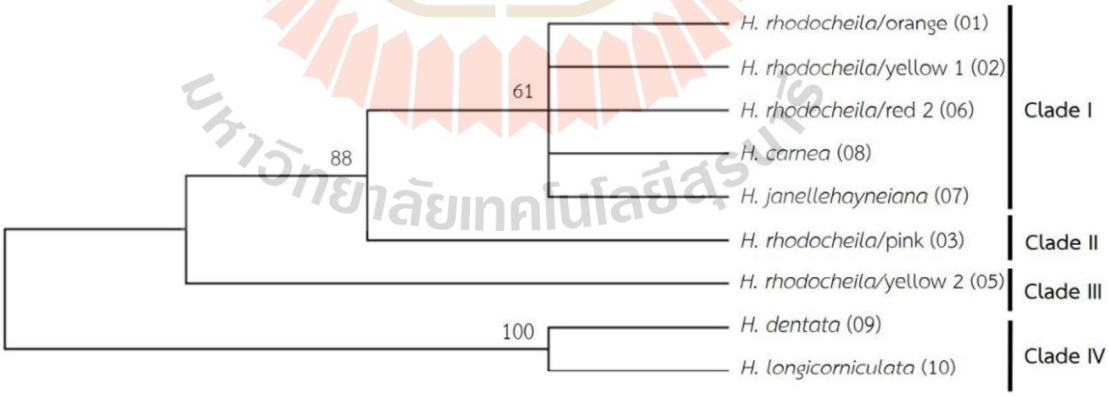


**Figure 4.6** Consensus tree of *Habenaria rhodocheila* complex based on the *rbcL* markers, supported by more than 50 % of the bootstrap trees.

4) Phylogeny inferred from Combined (ITS + *matK* + *rbcL*) data

A maximum-parsimony analysis retrieved 779 MPTs, consistency index of 0.88 and retention index of 0.87. The Consensus tree (Figure 4.7) indicates three

major clades with relationships to; (i) a clade of five selected populations of *H. rhodocheila*, *H. janellehayneiana*, and *H. carnea*, and (ii) a clade of outgroup, *H. dentata* and *H. longicorniculata*. Combined with ITS, *matK*, and *rbcL* DNA sequence data indicated that *H. janellehayneiana* is not separated lineage and mixed within *H. rhodocheila.*

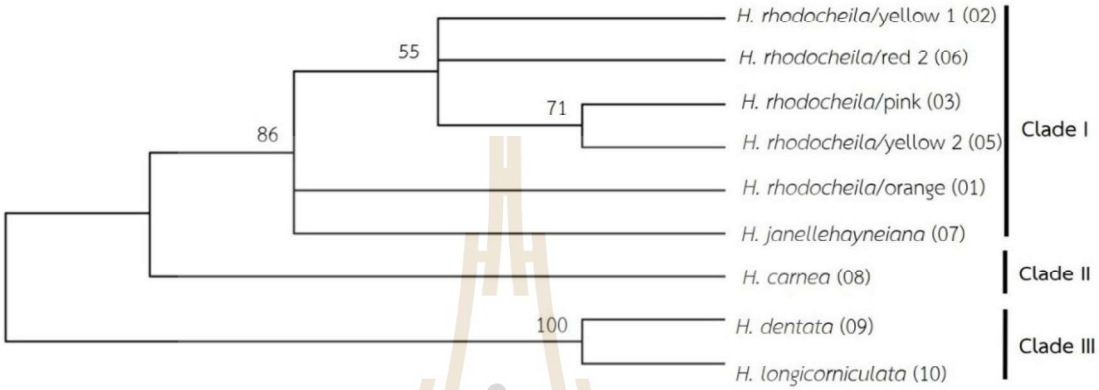


**Figure 4.7** Consensus tree of *Habenaria rhodocheila* complex based on the combined nuclear (ITS), and plastid (*matK*, *rbcL*) markers, supported by more than 50 % of the bootstrap trees.

The combined genes in this study gave a moderated resolution. Like previous reports, this study confirmed that combined genes is more effective for identify and classify the orchids (Xiang *et al.*, 2011; Singh *et al.*, 2012; Veldman *et al.*, 2018).

# CHAPTER V
# RESULTS AND DISCUSSION PART II
# REPRODUCTIVE BIOLOGY OF *HABENARIA RHODOCHEILA* AND *H. JANELLEHAYNEIANA*

## 5.1 Breeding systems

The breeding systems of the *Habenaria rhodocheila* (orange form) and *H. janellehayneiana* were carried out at Phu Hin Rong Kla National Park, Phitsanulok province. The result showed no fruit set of the no-pollination treatment of *H. rhodocheila* and *H. janellehayneiana* incaged individual (Figure 5.1), while all the manually self-pollinated flowers set fruit at 100 %. This indicated that both orchid species were genetically self and cross compatible, non-autogamous species, in which no apomixis occurs (Table 5.1). The lack of fruit production in the no-pollination treatment indicated that both orchid species need insects for pollination. The finding that the breeding system of these species as self-compatibility, non-autogamous species, and no apomixis, similar to other *Habenaria* species such as *H. gourlieana, H. hieronymi, H. limprichtii, H. monttevidensis, H. parviflora, H. petelotii, H. rupicola* (Tremblay *et al*., 2005; Zhang and Gao, 2021). Orchids have various breeding systems, for examples many species are self-compatible such as *Sirindhornia mirabalis*, *S. monophyla* and *S. pulchella* (Srimuang *et al*., 2010b), and *Habenaria davidii* and *H. fordii* (Zhang and Gao, 2017).


**Figure 5.1** Inflorescence of orchid (A) *Habenaria janellehayneiana*, (B) *H. rhodocheila*, natural habitats were covered with nylon net, (C) Fruit set of *H. janellehayneiana*, natural pollination (arrow), (D) Fruit set of *H. rhodocheila*, natural pollination (arrow), (E) Fruit set of *H. janellehayneiana*, untouched (arrow), and (F) Fruit set of *H. rhodocheila*, untouched.

The proportion of mature seeds (large embryos produced) of *H. janellehayneiana* was 92.30 % in natural condition, 59.39 % in self-pollination, and

64.53 % in cross-pollination which the natural fruits set had the highest number and are statistically significant differences ($p < 0.05$). *Habenaria rhodocheila* was 74.30 % in natural condition, 67.45 % in self-pollination, and 80.44 % in cross-pollination, statistically significant differences ($p < 0.05$) (Table 5.1). It is doubtful in different number of seed production in each pollination, possibly may be number of pollen depositing on the stigma. It is interested to conduct more study to compare the number of pollen deposition on the stigma and embryo formation.

According to previous study by Chen *et al.* (2020), hand pollination of *H. rhodocheila* in South China was found to be self-compatible, but it depended on the pollinator. This study showed the same breeding systems, including *H. janellehayneiana*. The mature seeds produced in self-pollinated fruits (33.32 %) was lower than in naturally (42.67 %) and cross-pollinated (62.26 %) fruits, likely indicating significant inbreeding depression. High fruit set in *H. rhodocheila* could be attributed to a combination of the rewarding nectar, bright easily detected flowers, and the dependable frequency and fidelity of the two species of pollinators (Chen *et al.*, 2020). However, some *Habenaria* have high fruit set such as *H. aitchisonii* (86.5 % fruits set) and *H. fordii* (79.40-89.76 % fruits set) (Xiong *et al.*, 2019; Zhang and Gao, 2017).

**Table 5.1** Type of pollination, fruit set, and seeds per capsules of *H. janellehayneiana* and *H. rhodocheila*.

| Species | Type of pollination (N) | Fruit set (%) | Total seeds (Mean ±SD) | Mature seeds (%) | |
|---|---|---|---|---|---|
| | | | | Range | Mean ±SD |
| *H. janellehayneiana* | Untouched (12) | 0 | 0 | 0 | 0 |
| | Natural-pollination (30) | 50 | 13,287±1,949$^{b}$ | 92.00-92.56 | 92.30±0.48 |
| | Self-pollination (12) | 100 | 25,338±2,552$^{a}$ | 55.99-64.46 | 59.39±4.48 |
| | Cross-pollination (12) | 100 | 9,432±1,048$^{c}$ | 63.57-65.11 | 64.53±0.84 |
| *H. rhodocheila* | Untouched (12) | 0 | 0 | 0 | 0 |
| | Natural- pollination (30) | 53.33 | 22,591±2,437$^{ab}$ | 72.97-75.11 | 74.30±1.16 |
| | Self-pollination (12) | 100 | 16,755±1,199$^{b}$ | 64.33-71.43 | 67.45±3.62 |
| | Cross-pollination (12) | 100 | 23,463±4,494$^{a}$ | 80.19-80.76 | 80.44±0.29 |

## 5.2 Flowering and fruit set

The flowering period of both orchid species occurs in the rainy season, *Habenaria janellehayneiana* from late July to September and *H. rhodocheila* flowering from mid-July to September (Table 5.2). All *Habenaria* individuals consistently produced one inflorescence. *Habenaria janellehayneiana* individuals reached the average height shoot length and leaf length almost 16.00 and 14.10 cm, respectively. The number of flowers per inflorescence ranged from 1 to 11. For *H. rhodocheila* the average height shoot length and leaf length were almost 19.80 and 15.00 cm, respectively. The number of flowers per inflorescence ranged from 1 to 15 (Table 5.3). Overall average fruit set, in each plot are 2.97±3.04 (1-11), 2.48±2.50 (1-10), 3.68±3.74 (1-15), 1.31±1.99 (1-7) in plot1, plot 2, plot 3, and plot 4, respectively (Table 5.3).

The percentage of fruit set of *H. janellehayneiana* was between 43.68 - 47.66 % while of *H. rhodocheila* between 41.22 - 70.00 %. *Habenaria* orchids have a high fruit set of ~ 85 %, due to the high efficiency of pollen transfer (Xiong *et al.,* 2015) and pollination was between 63.8 - 83.6 % (Pedron *et al.*, 2012). This genus is pollinated by butterflies (Pedron *et al.*, 2012; Tanaka, 2014). However, some *Habenaria* (*H. malintana*) can be agamospermy and give the ~100 % of fruit set (Zhang and Gao, 2018). According to the results of *H. rhodocheila* plot 4, the number of plants and flowers were lower than in plot 3, but the fruit set was higher, showing more success change dut to more pollinators. Lower fruit set of orchids probably related to insufficient pollen transfer resulting from pollinator scarcity and limited resources (Zimmerman and Aide, 1989; Ackerman and Montalvo, 1990; Vale *et al*., 2011). The reproductive success of the narrow-distributed species may be lower than that of the widely distributed species (Srimuang *et al*., 2010a). The most used parameter for determining the reproductive success of Orchidaceae are fruits per inflorescence (Humana *et al*., 2008; Pansarin and Amaral, 2009). In this study, using the fruit set as a reproductive success factor, showed that the fruit set of narrow distributed species is not different from the wide distributed species. The environment of each population, especially pollinator population may have more influenccy.

**Table 5.2** The observation time and habitat of *H. janellehayneiana* and *H. rhodocheila* at the study sites.

| Species | Period | Plot | Latitude | Longitude | Altitude (m) | pH | Moisture (%) | Type of forest | Light (%) |
|---|---|---|---|---|---|---|---|---|---|
| *H. janellehayneiana* | 10 Aug-10 Sep 2017 | 1 | 16.5709 | 101.0355 | 1,239 | 5.62±0.23 | 46.67±0.20 | MEF | 0-25 % |
| | | 2 | 16.5947 | 101.0050 | 1,243 | 5.66±0.18 | 50.00±1.41 | MEF | 0-25 % |
| *H. rhodocheila* | 5 Aug-5 Sep 2017 | 3 | 16.5926 | 101.0004 | 1,325 | 42.2±0.19 | 73.75±0.02 | HEF | 26-50 % |
| | | 4 | 17.0025 | 100.5924 | 1,120 | 5.25±0.12 | 58.13±0.01 | HEF | 26-50 % |

MEF = moist evergreen forest; HEF =Hill evergreen forest

**Table 5.3** Shoot length, leaf length, number of flowers, flowering, and natural fruit set of *H. janellehayneiana* and *H. rhodocheila* at the study site.

| Species | Plot | Shoot length (cm) | | Leaf length (cm) | | Number of flowers /Inflorescences | | Total number of flowers | Number of capsules | % Fruit set |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Length range | Mean ±SE | Length range | Mean ±SE | Number range | Mean ±SE | | | |
| *H. janellehayneiana* | 1 (n=184) | 1.10-16.00 | 7.14± 2.80 | 5.50-9.00 | 11.73± 2.49 | 1-11 | 2.97± 3.04 | 321 | 153 | 47.66 |
| | 2 (n=84) | 0.60-13.90 | 4.58± 2.27 | 2.60-14.10 | 8.07± 2.39 | 1-10 | 2.48± 2.50 | 87 | 38 | 43.68 |
| *H. rhodocheila* | 3 (n=68) | 0.90-19.80 | 10.26± 4.20 | 1.30-14.50 | 9.42± 2.30 | 1-15 | 3.68± 3.74 | 376 | 155 | 41.22 |
| | 4 (n=65) | 1.00-10.50 | 3.24± 2.95 | 3.10-15.00 | 8.23± 2.53 | 1-7 | 1.31± 1.99 | 70 | 49 | 70.00 |

## 5.3 Population size structure

In the study sites, *H. janellehayneiana* was found on stone boulders in the waterfall, evergreen forest, whereas *H. rhodocheila* was found on sandstone boulders in hill evergreen forest (Figure 5.2). The size-class dispersion in percent of a total number of individuals and percent of fertile individuals for each size class was recorded, by using leave length as age representation. It was found that the data of both species showed a normal distribution. The *H. janellehayneiana* plot 1 showed that size class 7 has the highest number of flowering individual at size class 6, and plot 2 showed the most flowering at size class 4. The *H. rhodocheila,* plots 3 and 4 showed the most flowering at size class 5 as shown in Figure 5.3 A-D. The study of the population size structure of both orchid species has the ability to produce flower from size class 2 (2-4 cm).

The correlation between the leaf length and fruit set using Pearson's correlation coefficient analysis confirmed that the leaf length of both plants was not positive significantly ($r<0.5$). The relation between age and fruit set depends on species (Winkler and Hietz, 2001) The population and plant sizes are determinant factors for the future survival of orchids as they can affect the growth and reproduction of orchids (Tremblay 2006; Jacquemyn *et al*., 2010). Large population and plant size of orchids had survival rate higher compared to the small population and small plant size (Gregg and Kery, 2006). This study showed a very low number of seedlings in every populations of both species indicating low reduction success. The re-induce return seedlings should be required for this population. As same as the large individuals, with potentially producing flower and fruits are a very low number. It needs to protect the mature plants in these populations. Thus, to maintain the natural population in this area, both strategies are urgently required.


**Figure 5.2** Study sites of *Habenaria janellehayneiana* and *H. rhodocheila*. (A-B) plots 1 at Mhundaeng waterfall, (C-D) plot 2 at Romklaoparadorn. The *H. rhodocheila* (E-F) plot 3 at Lanhinpum, and (G-H) plot 4 at Lanhintak at Phu Hin Rong Kla National Park, Phitsanulok province.

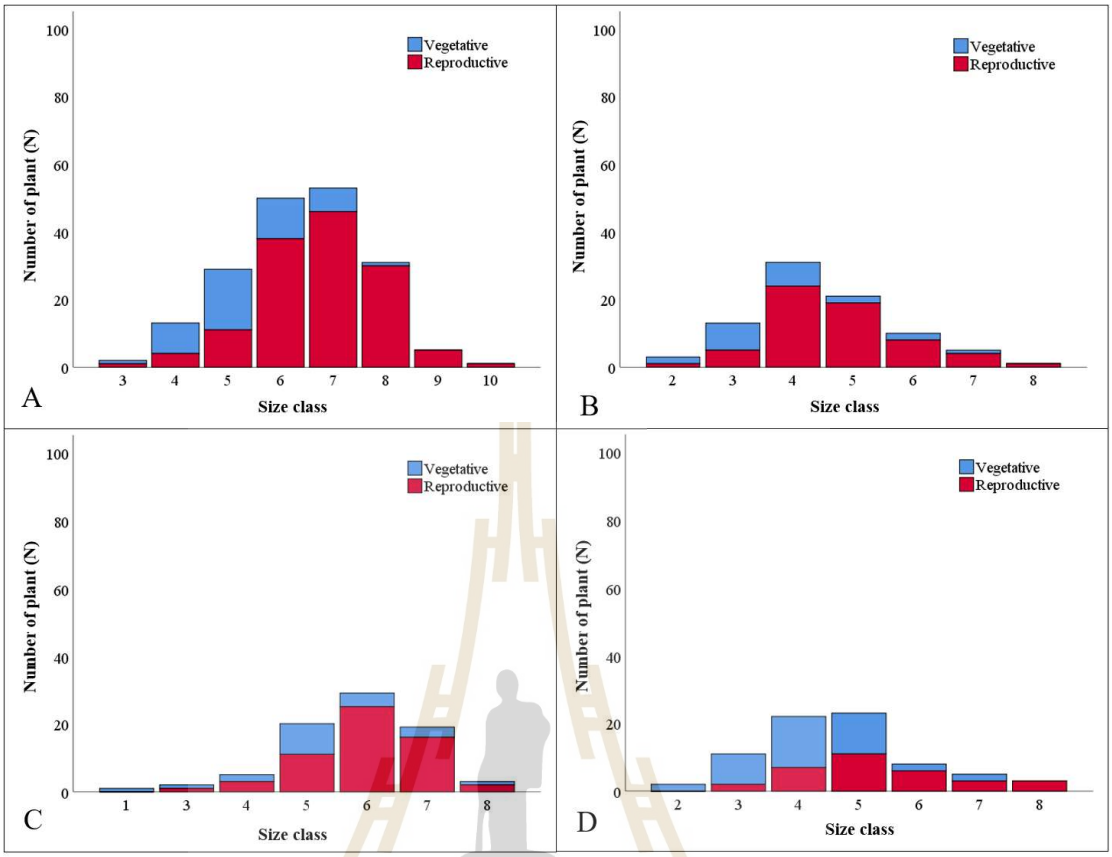


**Figure 5.3** The population size structure, comparison the number of vegetative and flowering plants by using the leaf length as a basis for grouping of *Habenaria janellehayneiana* (A) plot 1, (B) plot 2, and *H. rhodocheila,* (C) plot 3, (D) plot 4.

# CHAPTER VI
# RESULTS AND DISCUSSION PART III
# SYMBIOTIC AND ASYMBIOTIC SEED GERMINATION AND PROTOCORM DEVELOPMENT OF *HABENARIA RHODOCHEILA* AND *H. JANELLEHAYNEIANA*

## 6.1 Seed viability

The results of seed viability testing, *Habenaria janellehayneiana* and *H. rhodocheila* had the percentages of viability 14.89 % and 56.71 %, respectively (Table 6.1).

**Table 6.1** The seed size and viability with TTC test of *H. janellehayneiana* and *H. rhodocheila*.

| Species | Seed size (mm±SD) | | TTC stainability (%) |
|---|---|---|---|
| | Wide | length | |
| *H. janellehayneiana* | 0.14±0.05 | 0.21±0.03 | 14.89±1.77 |
| *H. rhodocheila* | 0.11±0.03 | 0.19±0.03 | 56.71±1.23 |

## 6.2 Symbiotic seed germination and protocorm development: testing fungal isolates capacity to promote germination and protocorm development.

### 6.2.1 Fungal isolation

In total, thirty-two fungal isolates were recovered from the roots and tubers of both plants, twenty isolates of *H. janellehayneiana* and twelve isolates of *H. rhodochila*. The morphological characters on PDA are white, light purple to yellow observed after 7 days of culture in Figure 6.1-6.3.

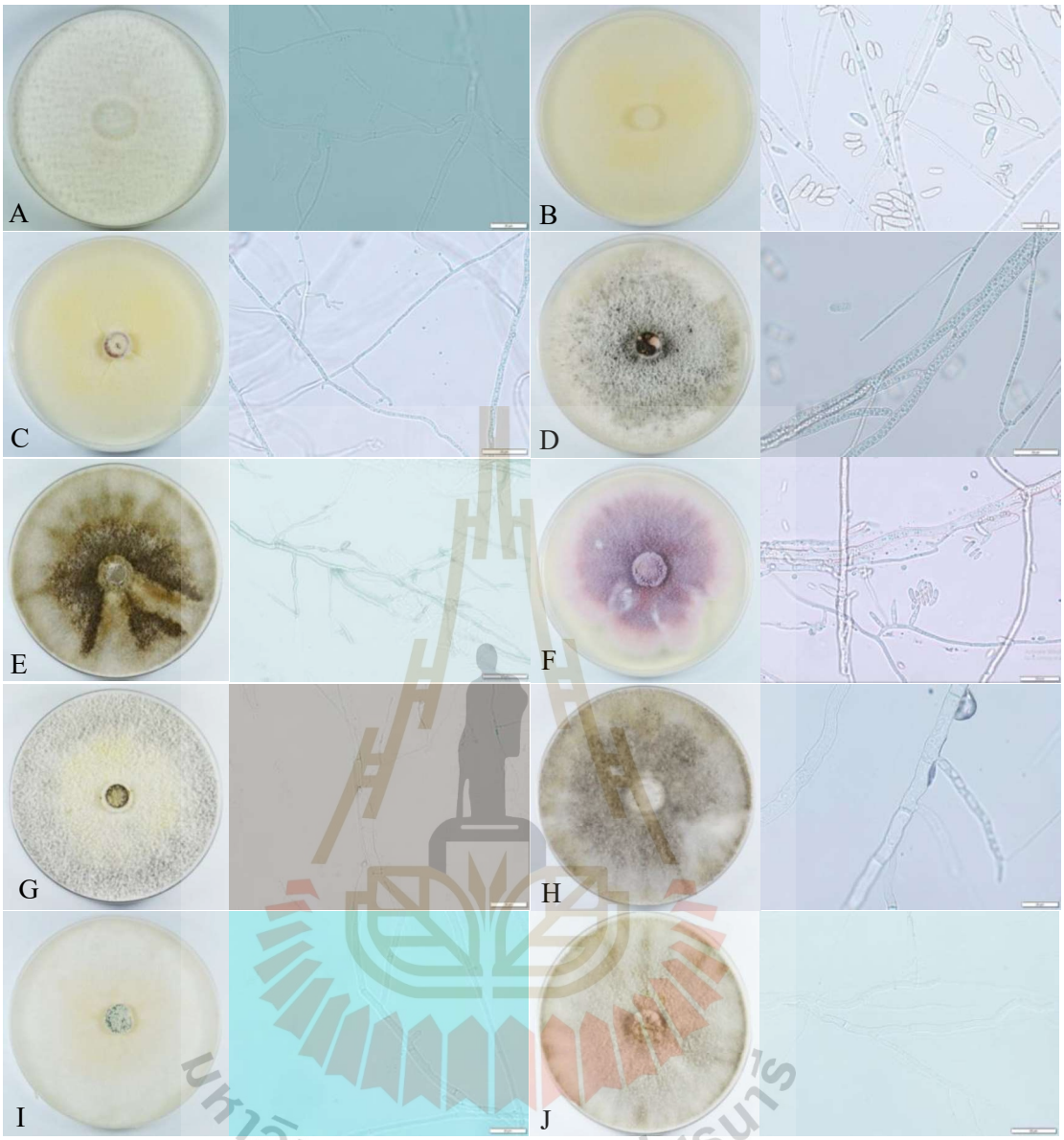


**Figure 6.1** Cultural characters of fungal isolates from *Habenaria janellehayneiana* on Potato Dextrose Agar observed after 7 days of culture, (A) I1, (B) I2, (C) I3, (D) I4, (E) I5, (F) I6, (G) I7, (H) I8, (I) I9, (J) I10, bar = 20 µm.

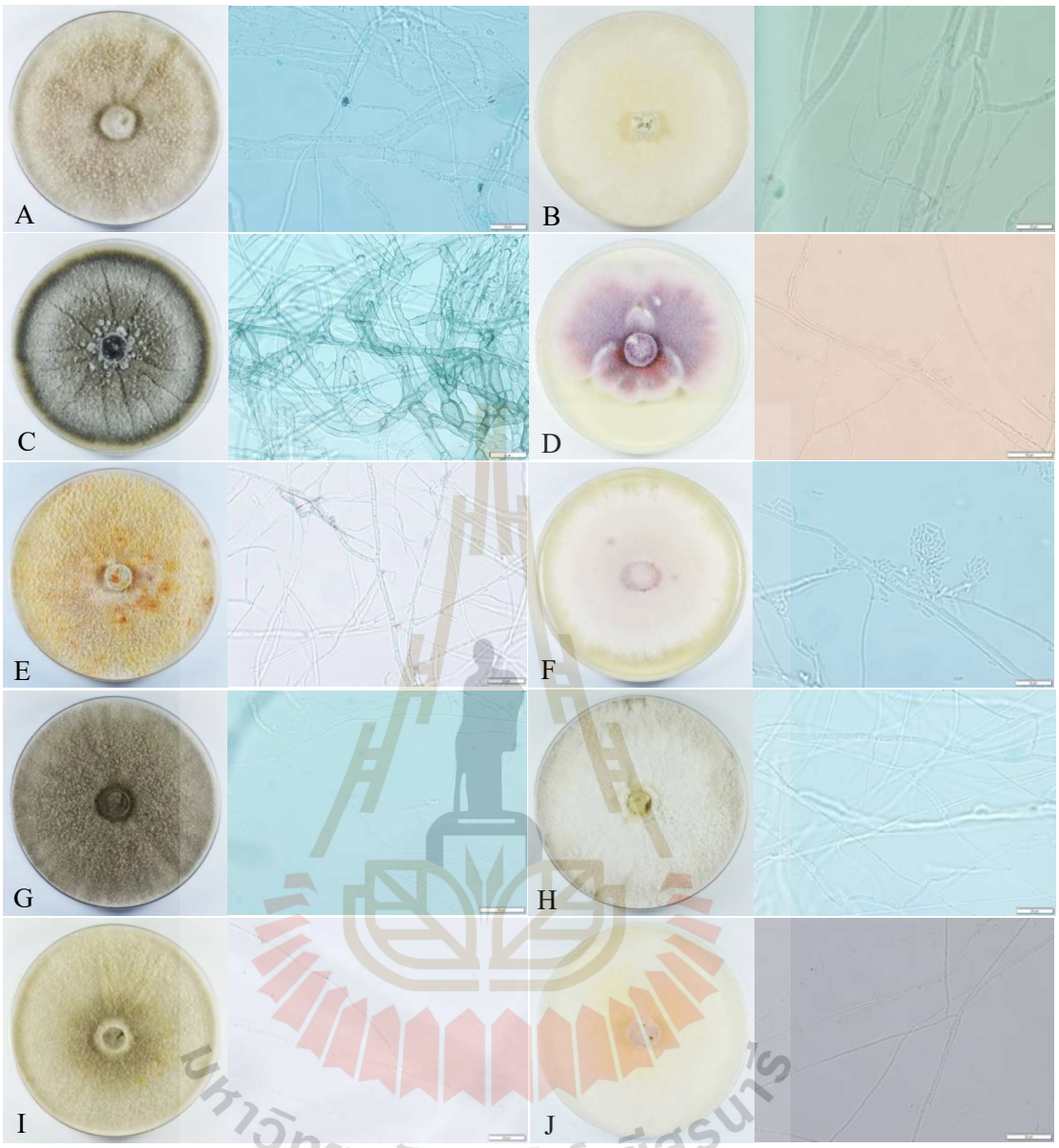




**Figure 6.2** Cultural characters of fungal isolates from *Habenaria janellehayneiana* on Potato Dextrose Agar observed after 7 days of culture, I11-I20. (A) I11, (B) I12, (C) I13, (D) I14, (E) I15, (F) I16, (G) I17, (H) I18, (I) I19, (J) I20, bar = 20 µm.

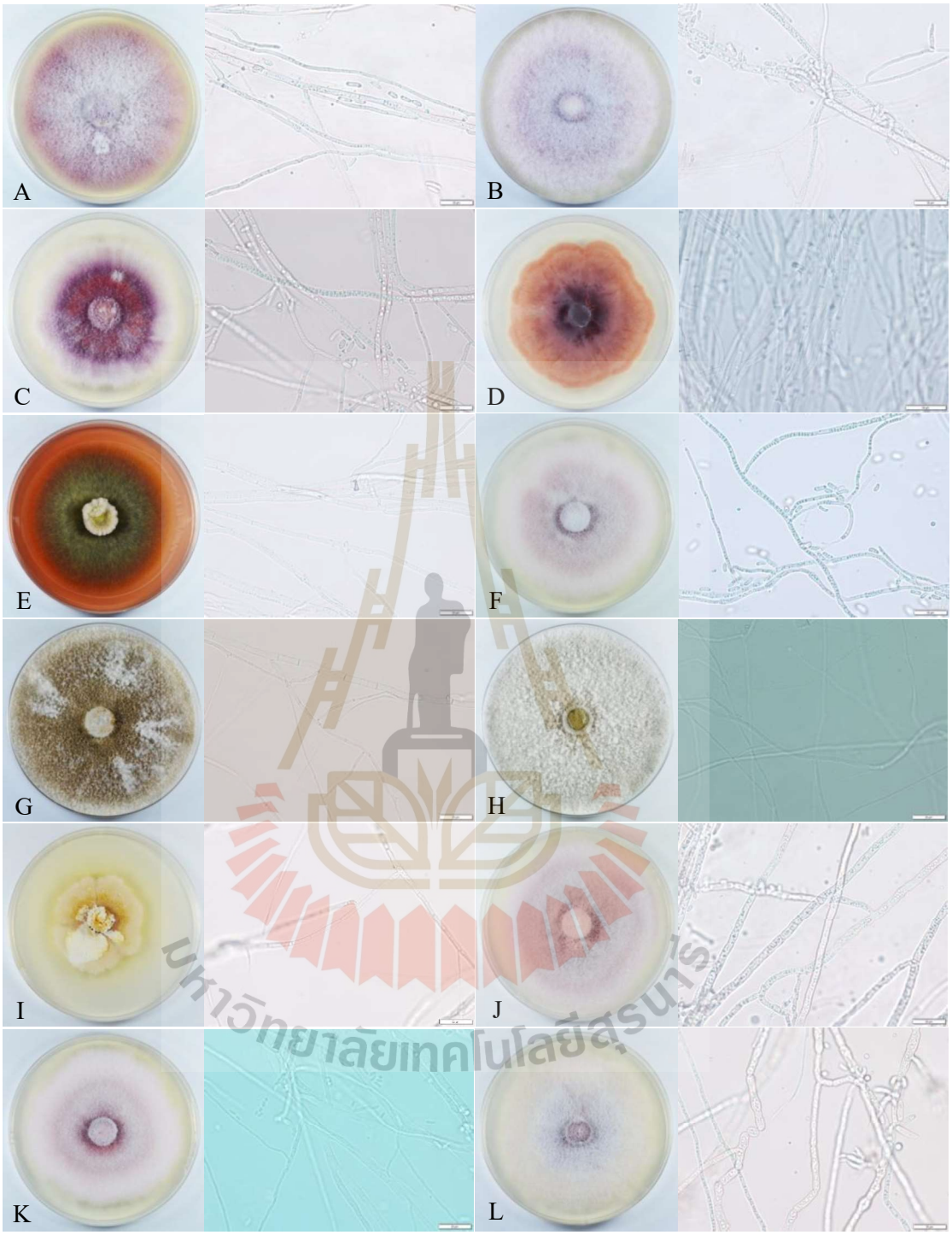


**Figure 6.3** Cultural characters of fungal isolates from *Habenaria rhodochila* on Potato Dextrose Agar observed after 7 days of culture, I21-I32. (A) I21, (B) I22, (C) I23, (D) I24, (E) I25, (F) I26, (G) I27, (H) I28, (I) I29, (J) I30, (K) I31, (L) I32, bar = 20 µm.

### 6.2.2 Testing the fungal isolates' capacity to promote germination

In total of thirty-two fungal strains, twenty isolates were used to test their ability to promote seed germination of *H. janellehayneiana* and twelve isolates were used to test their ability to promote seed germination of *H. rhodochila*. After 4 weeks of incubation, seeds of *H. janellehayneiana* in 3 treatments (I03, I20, and control) showed the broken testa and seed germination. Seeds treated with isolate I20 had highest seed germination (4.46 %) and with I08 showed seed germination in stage 1 after 14 weeks of incubation (Figure 6.4 A - C). After 16 weeks of incubation, I03 and I08 strains supported seed germination up to stage 2, while I20 and the control supported seed germination up to stage 3. The percentage of germination rate index (GRI) of stage 1 differed significantly between treatments. The highest seed germination was observed for strains I20 (64.08 %), followed by I03 (10.3 %) and control (6.81 %), respectively (Figure 6.5). The strain I20 had the highest developmental rate index (DRI) to stage 2 and stage 3 (0.99 and 0.18 %, respectively) (Table 6.2). The total seed germination percentages of *H. janellehayneiana* at 16 weeks of sowing differed significantly between treatments.

After 16 weeks of incubation, no germination was observed in the seeds of *H. rhodochila* (Table 6.3). Although, I tested seed germination three times, all treatments showed the unbroken testa, seeds covered with fungal hyphae and some seeds turning black color (Figure 6.4D). These results suggest that different endophytic fungi may play a role in promoting seed germination and protocorm development. Similar to other terrestrial orchids, the *Tulasnella* strains isolated from adult plants of *Arundina graminifolia* were less efficient at supporting germination than strains isolated from seedlings; they promoted protocorm formation, but no further development, while a *Tulasnella* strain isolated from seedlings had high seed germination and facilitated development seedlings of *A. graminifolia* to the advanced seedling stage (Meng *et al.*, 2019). Information on the differences among fungi in their ability to promote seedling development is an important factor influencing survival and persistence for orchid reintroduction or ex situ conservation projects (Reiter *et al.*, 2016). Although the assumption that mycorrhizal differentiation is the main factor driving these patterns. Differences in local environmental conditions such as light/shade, moisture, substrate chemistry, texture competitors and antagonists, may be invoked to explain these seed germination patterns (Rasmussen *et al.*, 2015). Symbiotic germination has become a popular and useful method for orchid seed germination (Chutima *et al.*, 2011). Many studies support the use of symbiotic seed germination as being more efficiency than asymbiotic condition. For example, Alomia

*et al*. (2017) showed that the viability of seeds and symbiotic seed germination in *Vanilla* spp. demonstrated high percentage of *Ceratobasidium* germination (approximately 80 %) co-cultured with fungi isolated from adult plant roots. The *Tulasnella* isolates promoted germination at a rate close to 60 %. Endophytic fungi were recovered from the roots of the *Pecteilis susannae* (L.) Rafin., a threatened terrestrial orchid, when seeds were co-cultured with *Epulorhiza* (CMU-Aug 013), the rate of seed germination was the highest (86.2 %) (Chutima *et al.*, 2011). Symbiotic seed germination was used to promote the germination of endangered epiphytic slipper orchid, *Paphiopedilum villosum* (Lindl.) Stein., The GRI and DRI inoculated with fungal isolates PVCP01 (*Tulasnella* sp.), PVCP05 (*Ceratobasidium* sp.), and PVCP06 (*Flavodon* sp.) was significantly higher than uninoculated control treatments in the basic media (MS, VW, Thomale GD, and TH) (Khamchatra *et al.*, 2016).

**Table 6.2** Effect of twenty-two fungal isolates on germination and development of *Habenaria janellehayneiana* seeds for 16 weeks after sowing.

| Developmental stages | Stage 1 | Stage 2 | Stage 3 | Stage 4 | Stage 5 |
|---|---|---|---|---|---|
| Treatment | GRI (% per week) | DRI (% per week) | DRI (% per week) | DRI (% per week) | DRI (% per week) |
| 1/10 OMA | 6.81±1.13$^{c}$ | 0.44±0.23$^{ab}$ | 0.06±0.06$^{ab}$ | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I01 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I02 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I03 | 10.3±0.43$^{b}$ | 0.46±0.21$^{b}$ | 0.00±0.00$^{b}$ | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I04 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I05 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I06 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I07 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I08 | 0.98±0.18$^{d}$ | 0.09±0.09$^{ab}$ | 0.00±0.00$^{b}$ | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I09 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I10 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I11 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I12 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I13 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I14 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I15 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I16 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |

**Table 6.2** Effect of thirty-two fungal isolates on germination and development of *Habenaria janellehayneiana* seeds for 16 weeks after sowing (Continued).

| Developmental stages | Stage 1 | Stage 2 | Stage 3 | Stage 4 | Stage 5 |
|---|---|---|---|---|---|
| Treatment | GRI (% per week) | DRI (% per week) | DRI (% per week) | DRI (% per week) | DRI (% per week) |
| 1/10 OMA + I17 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I18 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I19 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I20 | 14.00±0.96[a] | 0.99±0.68 [a] | 0.18±0.11 [a] | 0.00±0.00 | 0.00±0.00 |

**Table 6.3** Effect of twelve fungal isolates on germination and development of *Habenaria rhodochila* seeds for 16 weeks after sowing.

| Developmental stages | Stage 1 | Stage 2 | Stage 3 | Stage 4 | Stage 5 |
|---|---|---|---|---|---|
| Treatment | GRI (% per week) | DRI (% per week) | DRI (% per week) | DRI (% per week) | DRI (% per week) |
| 1/10 OMA | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I21 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I22 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I23 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I24 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I25 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I26 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I27 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I28 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I29 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I30 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I31 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| 1/10 OMA + I32 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |

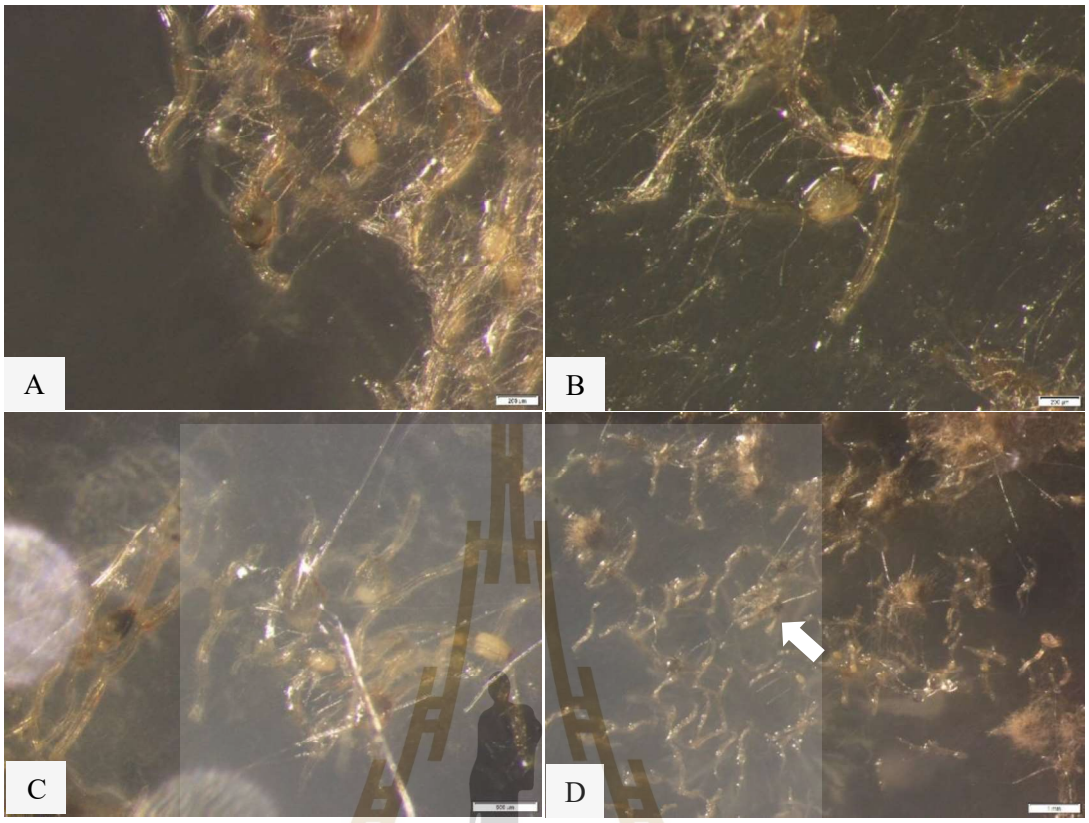


**Figure 6.4** Symbiotic seed germination and protocorm developmental stages of *Habenaria janellehayneiana* treated with fungal isolates; (A) Stage 1, bar = 200 µm; (B) Stage 2, bar = 200 µm; (C) Stage 3, bar = 500 µm; and *H. rhodochila* (D) black color seeds (arrow), bar = 1 mm.

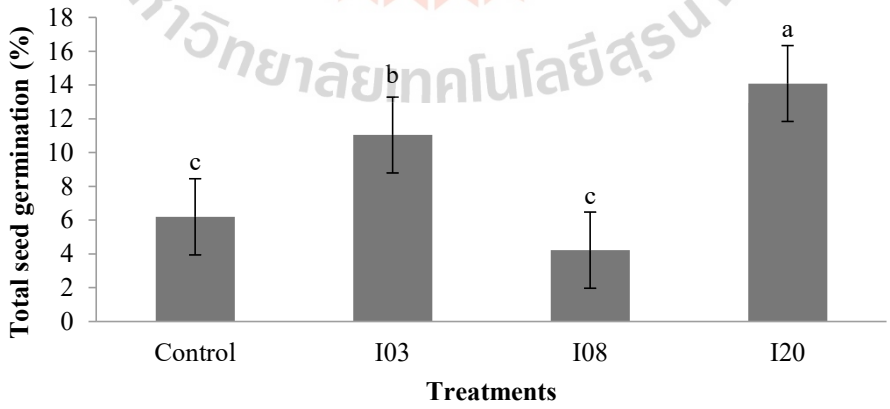


**Figure 6.5** Total seed germination of *Habenaria janellehayneiana* on various fungal isolates for 16 weeks. Different letters show significant differences according to DMRT ($p < 0.05$) for each plant.

**6.2.3 Fungal identification**

The ITS regions of the fungal DNA were sequenced and compared with available sequences at the NCBI based on BLAST sequence similarity and identity, only three fungal isolates of *H. janellehayneiana* promoted seed germination and protocorm development. I03 is *Colletotrichum* sp. (92.61 % identity) belonging to the Glomerellaceae family. I04 was a member of the family Hypoxylaceae, *Hypoxylon* sp. (84.97 % identity). I20 belongs to the Trichocomaceae family, *Aspergillus* sp. (97.92 % identity) (Table 6.4).

**Table 6.4** Molecular identification of endophytic fungi isolated from wild *Habenaria janellehayneiana* plants.

| Isolate | Identity (%) | BLAST search result (Accession no./ taxonomic affiliation) |
|---|---|---|
| I03 | 92.61 | *Colletotrichum* sp. (KC110619.1)/Glomerellales |
| I08 | 84.97 | *Hypoxylon* sp. (GQ334438.1)/Xylariales |
| I20 | 97.92 | *Aspergillus* sp. (MN017795)/Eurotiales |

In this study, I isolated fungal strains from two *Habenaria*, *H. janellehayneiana* that grew on stone boulders in the waterfall, evergreen forest, and *H. rhodocheila* that grew on sandstone boulders in hill evergreen forest. Fungi with diverse morphological characteristics were discovered in both plant species. Only three isolates from *H. janellehayneiana* promoted germination and protocorm development. Despite the fact that the three isolates of fungi are classified as non-mycorrhizal fungi, *Colletotrichum*, *Hypoxylon* and *Aspergillus* fungi have been reported as endophytes found in some orchids (Li *et al*., 2021; Sudheep and Sridhar, 2012). For example, Me *et al*. (2018) reported on twenty-one *Colletotrichum* strains isolated from *Dendrobium* spp. in China and Northern Thailand. Thirty-six strains of endophytic *Colletotrichum* species isolated from *Bletilla ochracea* (Tao *et al*., 2013) enhanced the growth of adult individuals belonging to *Dendrobium* spp. (Shah *et al*., 2019). Sudheep and Sridhar (2012) described eight *Aspergillus* taxa isolated from *Bulbophyllum neilgherense* and seven taxa isolated from *Vanda testacea* in India.

In addition, *Colletotrichum* fungi are generally found in the roots of various orchids from tropical and temperate zones (Tao *et al*., 2013; Salazar *et al*., 2018; Sarsaiya *et al*., 2020). The endophytic fungi *C. fioriniae* can be used as natural protectants against insect herbivory (Marcelino *et al*., 2008). *Colletotrichum*

*gloeosporioides* has been shown to protect *Theobroma cacao* against Phytophthora pathogens (Mejia *et al*., 2008; Rojas *et al*., 2010).

## 6.3 Asymbiotic seed germination and protocorm development

### 6.3.1 Testing media capacity to promote germination and protocorm development

The effect of basal media on germination and protocorm development of *H. janellehayneiana* was examined. The results showed that these seed began germinating (stage 1) 4 weeks after sowing in all asymbiotic treatment (Figure 6.6), in which the ½VW media had the highest seed germination (3.33 %). All tested media showed protocorm development in stage 2 after 6 weeks of sowing, and the ½VW media had the highest protocorm development (2.67 %). Stage 3 and stage 4 protocorm development were visible in all tested media at the 8 and 10 weeks of sowing, respectively. In addition, the ½VW media had the highest protocorm development (2.44 and 1.33 %, respectively). In all tested media treatments, *H. rhodocheila* seeds began germinating 4 weeks after sowing (Figure 6.7) with the VW media having the highest seed germination rate (2.20 %). The ½VW and ½MS media showed protocorm development in stage 2 after 6 weeks of sowing with the ½VW media showing the most protocorm development (0.41 %). In 8 weeks of sowing, stage 3 protocorms were visible on the ½VW media (0.41 %), stage 4 protocorms were observed on the ½VW and ½MS media in 14 weeks of sowing (0.20 and 0.12 %, respectively), and stage 5 protocorms revealed on ½VW and VW media in 16 weeks of sowing (0.20 and 0.15 %, respectively).

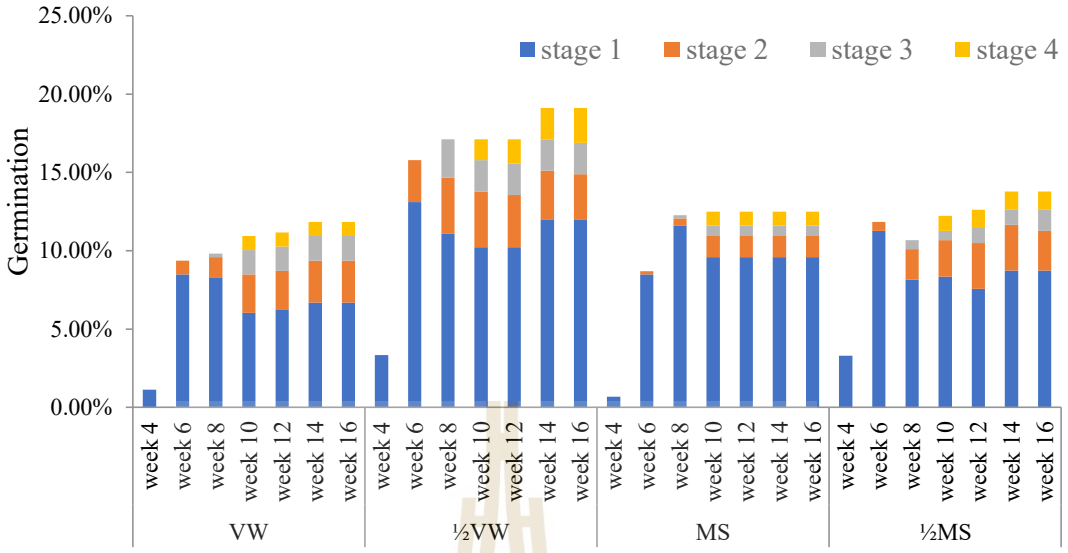


**Figure 6.6** Comparison of seed germination and protocorm development of *Habenaria janellehayneiana* on various basal media for 16 weeks.

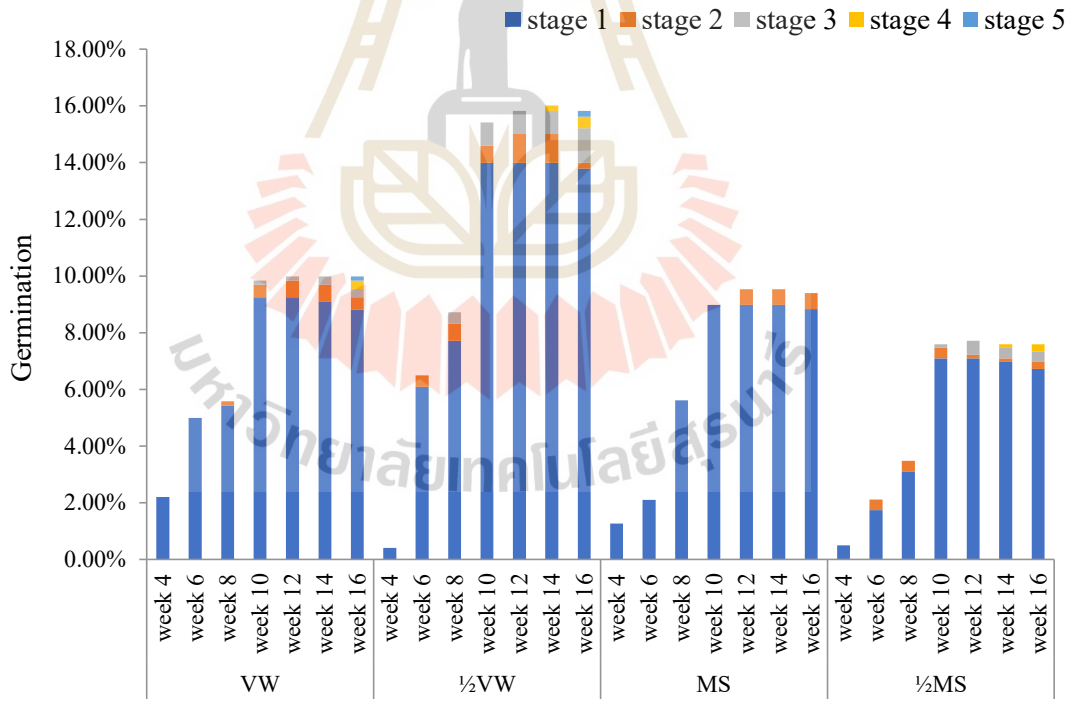


**Figure 6.7** Comparison of seed germination and protocorm development of *Habenaria rhodocheila* on various basal media for 16 weeks.

The results of different media on GRI at stage 1 and DRI at stages 2-5 for *H. janellehayneiana* and *H. rhodocheila* at 16 weeks are shown in Table 6.5. Germination

of *H. janellehayneiana* in basal media was the highest total seed germination percentage on ½VW media (15.70 %), followed by ½ MS (13.10 %), MS (12.10 %) and VW (9.52 %), respectively. All testing media showed protocorm development up to stage 4. The ½VW supported an advanced protocorm developmental stage 4 with giving the highest DRI (1.12 % per week). Germination of *H. rhodocheila* in basal media was the highest total seed germination percentage on ½VW media (12.95 %), followed by VW (10.12 %), MS (8.58 %), and ½MS (6.67 %), respectively. DRI values were significantly different in stage 3 on ½VW. The VW and ½VW media supported seed germination and advanced protocorm development up to stage 5. The ½VW media enhanced protocorm development upto stage 5 with the highest DRI (0.03 % per week). The seeds developed to stage 4 in the ½ MS and only stage 2 in the MS media.

**Table 6.5** Influence of different media on germination rate index (GRI) at stage 1 and developmental rate index (DRI) at stages 2-5 for *Habenaria janellehayneiana* and *H. rhodocheila* at 16 weeks.

| Stages<br>Basal media | Stage 1<br>GRI (% per week) | Stage 2<br>DRI (% per week) | Stage 3<br>DRI (% per week) | Stage 4<br>DRI (% per week) | Stage 5<br>DRI (% per week) |
|---|---|---|---|---|---|
| *H. janellehayneiana* | | | | | |
| VW | 9.52±0.63$^{b}$ | 2.21±0.58 | 1.09±0.31 | 0.58±0.34 | 0.00±0.00 |
| ½VW | 15.70±1.83$^{a}$ | 3.75±1.22 | 1.80±0.77 | 1.12±0.48 | 0.00±0.00 |
| MS | 12.10±1.49$^{ab}$ | 1.05±0.63 | 0.49±0.16 | 0.62±0.44 | 0.00±0.00 |
| ½MS | 13.10±2.13$^{ab}$ | 2.46±0.83 | 0.70±0.26 | 0.71±0.18 | 0.00±0.00 |
| *H. rhodocheila* | | | | | |
| VW | 10.12±1.39$^{ab}$ | 0.34±0.12$^{ab}$ | 0.10±0.07$^{b}$ | 0.07±.038 | 0.02±0.03 |
| ½VW | 12.95±1.41$^{a}$ | 0.79±0.32$^{a}$ | 0.70±0.21$^{a}$ | 0.08±.030 | 0.03±0.06 |
| MS | 8.58±2.16$^{ab}$ | 0.21±0.24$^{b}$ | 0.00±0.00$^{b}$ | 0.00±0.00 | 0.00±0.00 |
| ½MS | 6.67±1.98$^{b}$ | 0.37±0.32$^{ab}$ | 0.12±0.06$^{b}$ | 0.06±.023 | 0.00±0.00 |

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.

The overall seed germination percentages of *H. janellehayneiana* at 16 weeks of sowing were the highest on ½VW media (19.11 %), followed by ½MS (13.79 %), MS (12.50 %) and VW (11.83 %), respectively (Figure 6.8). After 14 weeks, they had progressed to stage 4, while stage 5 was observed after 18 weeks on ½VW media. The different growth stages of *H. janellehayneiana* are shown in Figure 6.9. The total seed

germination percentages of *H. rhodocheila* at 16 weeks of sowing were the highest on ½VW media (15.82 %), followed by VW (9.99 %), MS (9.40 %), and ½MS (7.59 %), respectively. They had developed to stage 5 after 16 weeks. The different growth stages of *H. rhodocheila* are shown in Figure 6.10.

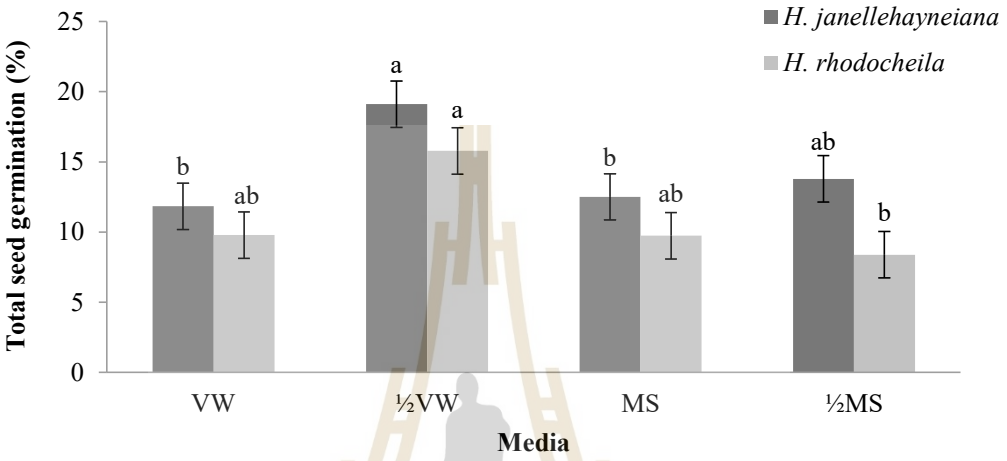


**Figure 6.8** Total seed germination of *Habenaria janellehayneiana* and *H. rhodocheila* on various basal media for 16 weeks. Different letters show significant differences according to DMRT ($p < 0.05$) for each plant.

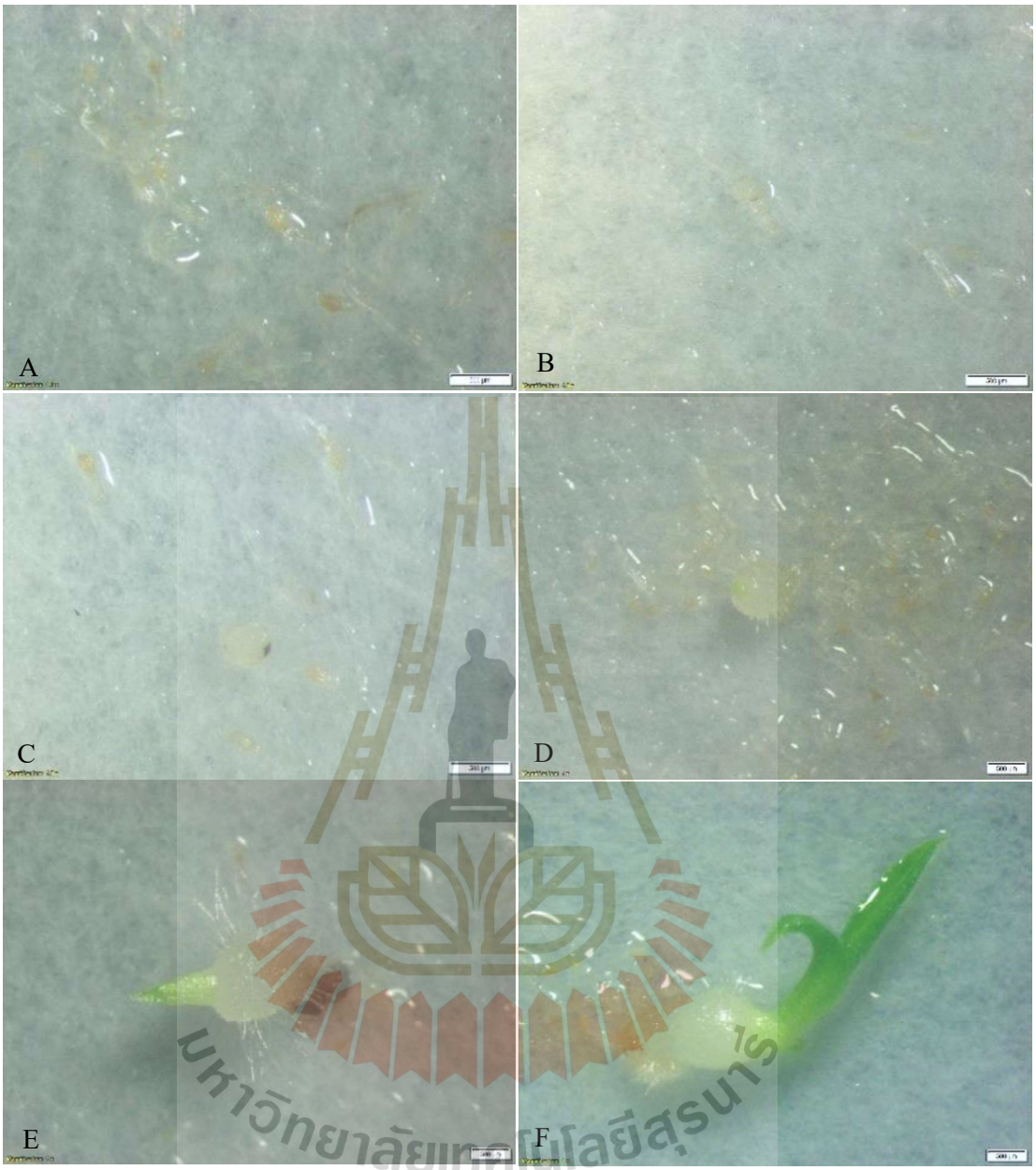


**Figure 6.9** Seed germination and protocorm developmental stages of *Habenaria janellehayneiana* on ½VW media, (A) Stage 0, bar = 500 µm; (B) Stage 1, bar = 500 µm; (C) Stage 2, bar = 500 µm; (D) Stage 3, bar = 500 µm; (E) Stage 4, bar = 500 µm; (F) Stage 5, bar = 500 µm.

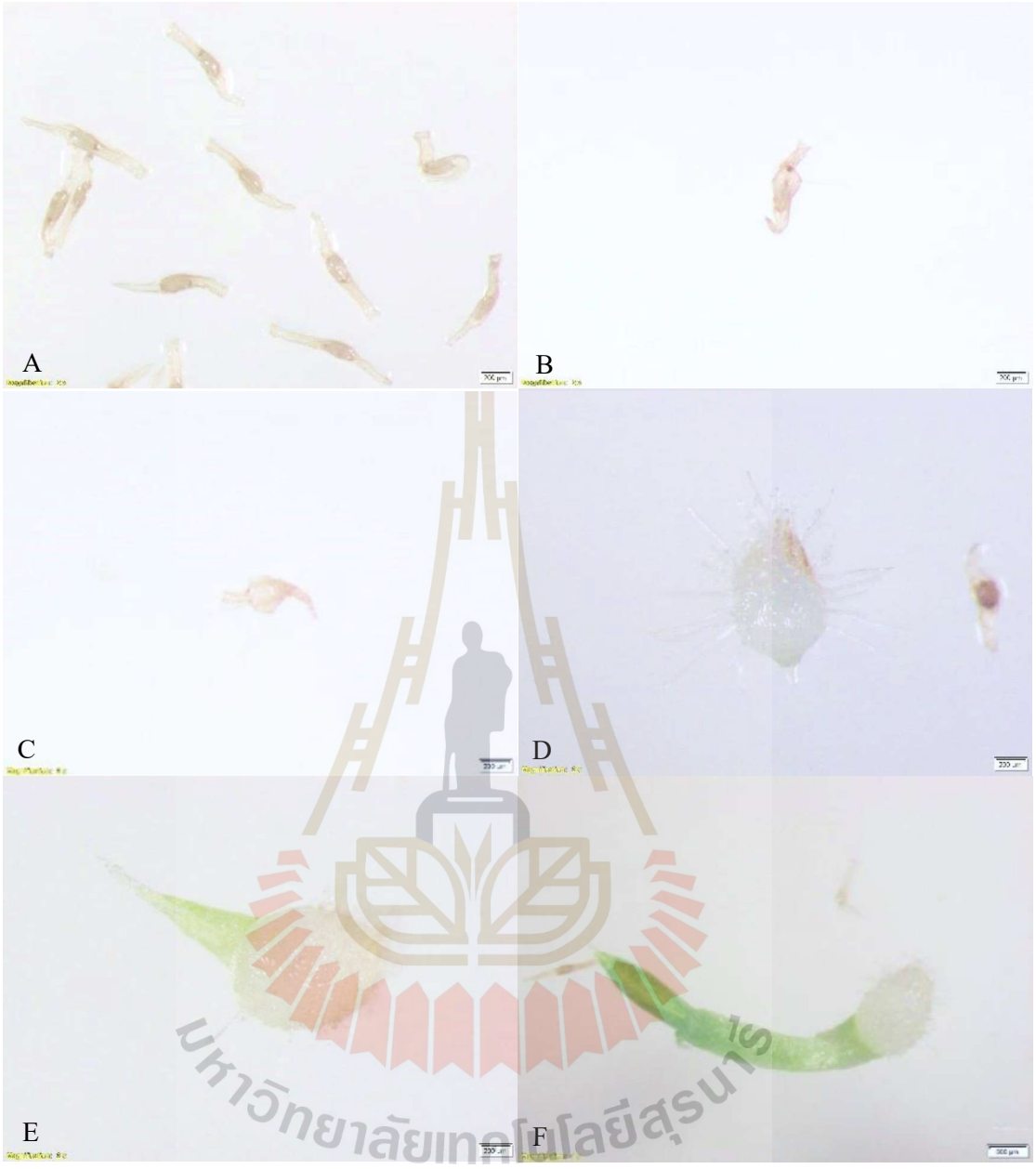


**Figure 6.10** Seed germination and protocorm developmental stages of *Habenaria rhodocheila* on ½VW media, A Stage 0, bar = 500 µm; b Stage 1, bar = 500 µm; c Stage 2, bar = 500 µm; d Stage 3, bar = 500 µm; e Stage 4, bar = 500 µm; f Stage 5, bar = 500 µm.

The results of the testing of basal media on germination rate index showed that both plant species highly germinated in stage 1 on the ½VW media and a higher germination percentage than the previous report by Kohkalang *et al*. (2014) who found 3.25 % germination of *H. rhodochila* pink form on VW formula with 15 % coconut water and 1 gram of peptone per liter for 16 weeks. Those seeds cultivated on the ½MS were at stage 2 while they did not develop stage 2 on ½VW. *Cymbidium* Sw. seeds, on the other hand, germinated effectively on Knudson medium (1922). Many studies have applied this technique asymbiotically, and a variety of asymbiotic media have been developed for the germination of orchid seeds (Knudson, 1946; Vacin and Went, 1949; Johnson *et al*., 2007). Terrestrial orchid species rely on mycorrhizal fungal association to facilitate seed germination and growth (Rasmussen, 1995; Alghamdi, 2019). Although Malmagen (1996) modified terrestrial orchid medium (MM) is widely used, the efficacy of the basal media efficiency has been reported to vary greatly amongst species (Hoque *et al*., 1994).

**6.3.2 Testing BAP, GA and TDZ capacity to promote germination and protocorm development**

The results of 10 tested media on the germination and protocorm development of *H. janellehayneiana* showed that their seeds began germinating 4 weeks after sowing in all tested media (Figure 6.11). The addition of 3 mg/L BAP resulted in the greatest seed germination (5.47 %). After 6 weeks of sowing, the 1 mg/L BAP and 1 mg/L TDZ showed protocorm development in stage 2 with the 1 mg/L BAP showing the most protocorm development (0.68 %). At 8 weeks of sowing, 1 mg/L BAP (0.34 %) and 5 mg/L BAP (0.19 %) added demonstrated stage 3 protocorm. At 10 weeks of sowing, 5 mg/L BAP (0.19 %) added indicated stage 4 protocorm. In all investigated media treatments, *H. rhodocheila* seeds began germinating 4 weeks after sowing (Figure 6.12). The addition of 1 mg/L BAP resulted in the highest seed germination (6.71 %). After 4 weeks of sowing, the addition of 1, 3 mg/L BAP, and all GA treatments showed protocorm development in stage 2 with the 1 mg/L BAP added having the maximum protocorm development (0.79 %). At 6 weeks of sowing, BAP and GA added demonstrated stage 3 protocorm, with the 1 mg/L BAP added showing the highest protocorm development (0.99 %). Stage 4 protocorms were observed in the control, BAP and 3 mg/L BAP added, with the 3 mg/L BAP added having the highest protocorm development (1.19 %). Almost all treatments developed to stage 5 at 12 weeks, with the exception of 3 and 5 mg/l TDZ added, which developed to stage 5 at 14 weeks.

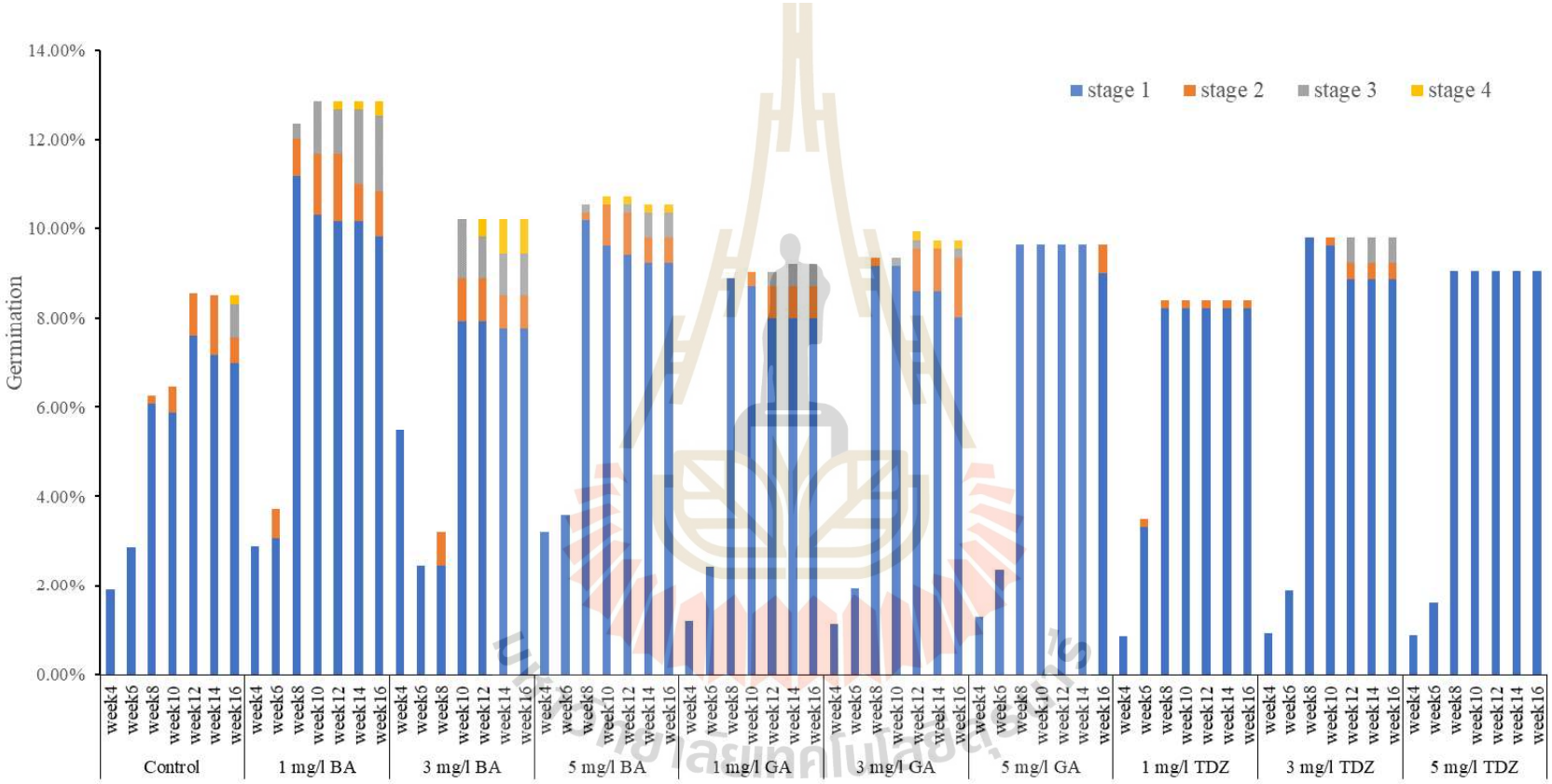


**Figure 6.11** Comparison of total seed germination and protocorm development of *Habenaria janellehayneiana* on various BAP, GA, and TDZ for 16 weeks.

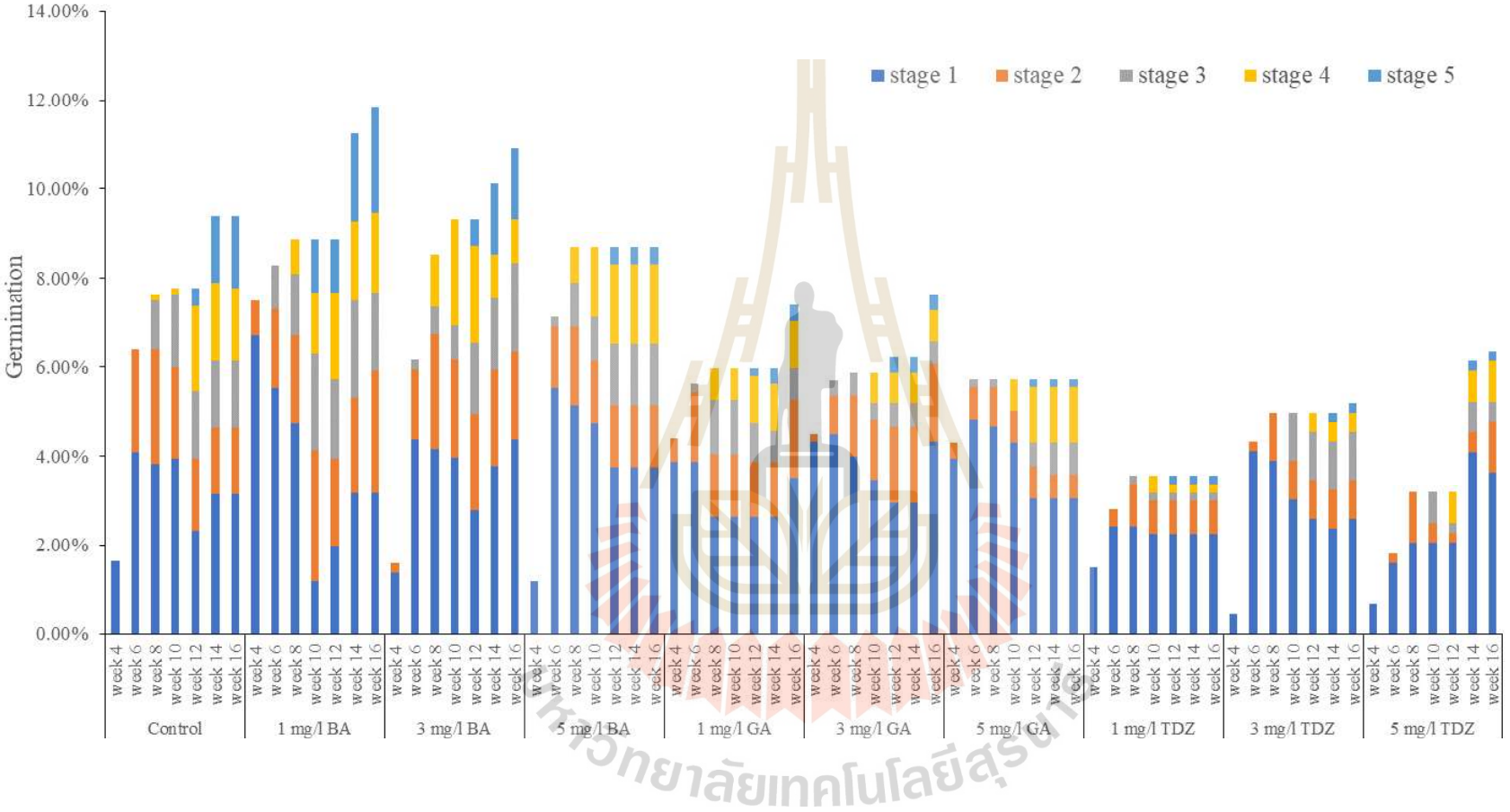


**Figure 6.12** Comparison of total seed germination and protocorm development of *Habenaria rhodocheila* on various BAP, GA, and TDZ for 16 weeks.

The GRI and DRI percentages of both orchid species on ten tested media differed after 16 weeks of culture. The GRI percentage of *H. janellehayneiana* on ½VW +1 mg/l BAP was 11.80 % per week. In stage 4, the GRI percentage was larger than that of other media (0.10 %). The results revealed that the GRI percentage on the ½VW +1 mg/l BAP was significantly higher than other media (Table 6.6). The *H. rhodocheila* had the highest GRI percentage on ½VW +1 mg/l BAP with 08.08 % per week and the DRI percentage was much higher than other media in stages 3 (2.02 %) and 5 (0.86 %). At 16 weeks, the effect of BAP, GA and TDZ on the total seed germination showed that ½VW +1 mg/l BAP had the highest germination at 11.83 % (Table 6.7; Figure 6.13).

**Table 6.6** Influence of BAP, GA, and TDZ on germination rate index (GRI) at stage 1 and developmental rate index (DRI) at stages 2-5 for *Habenaria janellehayneiana* cultured on ½ VW media for 16 weeks.

| Stages<br>Basal media | Stage 1<br>GRI (% per week) | Stage 2<br>DRI (% per week) | Stage 3<br>DRI (% per week) | Stage 4<br>DRI (% per week) | Stage 5<br>DRI (% per week) |
|---|---|---|---|---|---|
| control | 7.74±0.60 | 0.58±0.07$^{bc}$ | 0.09±0.04$^{bc}$ | 0.02±0.02 | 0.00±0.00 |
| 1 mg/l BAP | 11.80±1.06 | 1.24±0.34$^{a}$ | 1.02±0.37$^{a}$ | 0.10±0.07 | 0.00±0.00 |
| 3 mg/l BAP | 10.80±1.37 | 0.73±0.19$^{b}$ | 0.65±0.29$^{ab}$ | 0.26±0.16 | 0.00±0.00 |
| 5 mg/l BAP | 11.70±1.45 | 0.56±0.06$^{bc}$ | 0.22±0.08$^{bc}$ | 0.16±0.16 | 0.00±0.00 |
| 1 mg/l GA | 8.71±0.57 | 0.37±0.08$^{bcd}$ | 0.22±0.08$^{bc}$ | 0.00±0.00 | 0.00±0.00 |
| 3 mg/l GA | 9.19±1.49 | 0.57±0.12$^{bc}$ | 0.07±0.05$^{c}$ | 0.11±0.11 | 0.00±0.00 |
| 5 mg/l GA | 9.78±1.86 | 0.08±0.05$^{cd}$ | 0.00±0.00$^{c}$ | 0.00±0.00 | 0.00±0.00 |
| 1 mg/l TDZ | 8.77±0.60 | 0.22±0.22$^{bcd}$ | 0.00±0.00$^{c}$ | 0.00±0.00 | 0.00±0.00 |
| 3 mg/l TDZ | 9.16±1.57 | 0.19±0.11$^{cd}$ | 0.24±0.24$^{bc}$ | 0.00±0.00 | 0.00±0.00 |
| 5 mg/l TDZ | 8.88±0.88 | 0.00±0.00$^{d}$ | 0.00±0.00$^{c}$ | 0.00±0.00 | 0.00±0.00 |

Different letters in each column show significant differences at $P < 0.05$ (DMRT) for each plant.

**Table 6.7** Influence of BAP, GA, and TDZ on germination rate index (GRI) at stage 1 and developmental rate index (DRI) at stages 2-5 for *Habenaria rhodocheila* cultured on ½ VW 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) |
| 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$^{c}$ |

Different letters in each column show significant differences at P < 0.05 (DMRT) for each plant.

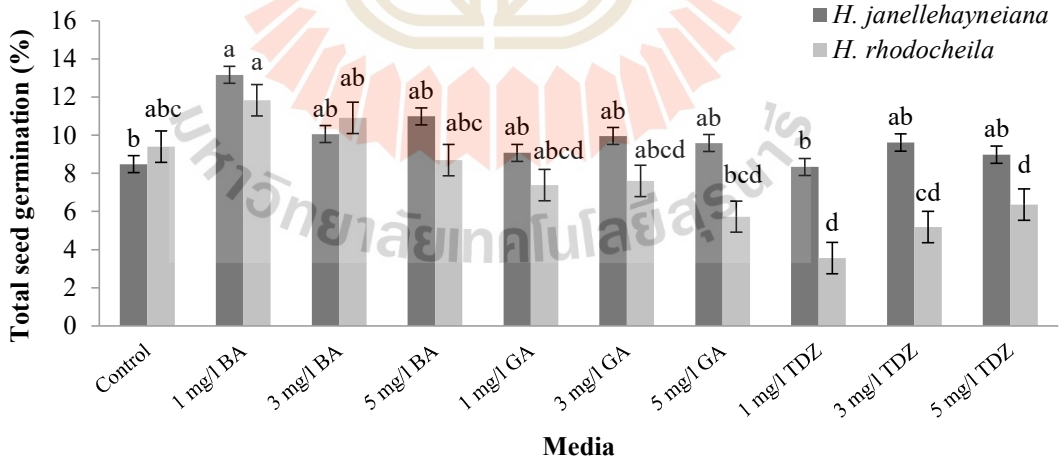


**Figure 6.13** Effect of BAP, GA, and TDZ on total seed germination of *Habenaria rhodocheila* and *H. janellehayneina* at 16 weeks after inoculation. Different letters show significant differences according to DMRT ($p < 0.05$) for each plant.

The results of this study indicate that the ½VW supplemented with 1 mg/l BAP had a significant effect on promoting stages 3 and 5 protocorm development of *H. rhodochila*. On the same media *H. janellehayneiana* had higher germination percentage than *H. rhodochila*, with an extension of the elongation of first leaf. In order to promote leaf growth in *Paphiopedilum spicerianum,* an appropriate amount of BAP was added (Chen *et al.*, 2015). This germination rate in this experiment was higher than in other studies. Piyatrakul and Apavatjrut (2004) cultured *H. rhodocheila* (pink form) on modified VW media (CMU1 + 0.1 mg/L NAA + 1 mg/L BAP) and detected 2.46 % total germination after 20 weeks of cultivation Later on, Piyatrakul (2014) found a maximum of 5.48 % germination of *H. rhodocheila* on modified VW medium (CMU1+ NAA 0.1 mg/L + BAP 1 mg/L). The addition of GA and TDZ did not improve germination and protocorm development in both species, which resulted in significantly less germination than the control. This result was inconsistent with the previous studies which GA and TDZ helped for adventitious shoot induction in *Malaxis acuminata* (Cheruvathur *et al.,* 2010) and multiple shoot induction in *Rhynchostylis retusa* (Thomas and Michael, 2007). Exogenous GA3 concentration change was regulated by endogenous hormonal change in *Dendobium officinale* seed germination, indicating that GA3 participates in the crosstalk between the hormone biosynthesis route and the common symbiotic signal system (Chen *et al*., 2020). Some reports compared basal media with or without modification for terrestrial or epiphytic orchids, undefined growth additives such as CW, compared to a standard symbiotic germination ½MS basal salt with an additional 5 % (v/v) CW. The seeds of an Australian terrestrial orchid developed an equivalent germination process (Bustam *et al*., 2014). Due to mechanical or physiological mechanisms which maintain seed dormancy, *in vitro* seed germination may fail. Certain temperature regimes, prolonged imbibition, chemical softening of the testa with $Ca(OCl)_2$ or NaOCl, or mechanical damage can break seed dormancy.

# CHAPTER VII
# CONCLUSIONS

The first objective in this study was a taxonomic revision of the *Habenaria rhodocheila* complex, consisting of different color forms viz. red, orange, yellow and pink with closely related species viz. *H. janellehayneiana* and *H. carnea*. Morphometric and molecular phylogeny were employed. ITS, *matK* and *rbcL* genes in various color forms of *H. rhodocheila*, *H. janellahayneiana* and *H. carnea* were first time sequenced.

Based on the morphometric data, the PCA and dendrogram analysis among 6 populations of various color forms of *H. rhodocheila* and one population of *H. janellehayneiana* using 26 floral characters revealed 4 discriminated groups; 1) *H. janellehayneiana*; 2) *H. rhodocheila* population 06; 3) *H. rhodocheila* population 03; and 4) *H. rhodocheila* populations 01, 02, 04, and 05. The morphological characters can be used to distinguish effectively between *H. janellehayneiana* and *H. rhodocheila*, with stigmaphore convergent character state supporting the species delimination by Choltco *et al.* (2017). For *H. rhodocheila,* the morphometric result suggested that the rostellum length, and pedicel length are discreted characters for discriminating populations, 03 (pink form) and 06 (red form), respectively. The red forms were not grouped together, indicated that the color form is a variable character. Thus, the result supports the broad variation of species concept of *H. rhodocheila.*

Phylogenetic analyses based on the combined ITS, *matK* and *rbcL* genes using maximum-parsimony showed that *H. janellehayneiana* mixed with *H. rhodocheila* (Figure 4.6), contravocy with morphological analysis. Since the molecular evident has more informative information, it is likely that the *H. janellehayneiana* should be circumscripted in *H. rhodocheila.* However, it is too hurry to conclude like this, due to weak resolution of phylogenetic analysis. Thus, more evidences such more specimens which representative all geographic ranges of *H. rhodocheila* and other genomic regions are required, due to unstable position in phylogenetic analysis.

The second objective is to compare the reproductive biology of *H. rhodocheila* and *H. janellehayneiana*. This study has focused on breeding system, flowering and fruit set, and population size structure. The breeding systems showed no fruits created by untouched (no pollination) of both species on caged individuals set fruit, however all manually self-pollinated flowers set fruit (100 %). This indicates that both species were genetically self-compatibility, non-autogamous specieswith no

apomixis. Pollinators were required for both species. Number of mature seeds form natural pollination of *H. janellehayneiana* and *H. rhodocheila* are significantly different ($p < 0.05$). The *H. janellehayneiana* had the percentage of fruit set ranging from 43.68 to 47.66 %, while *H. rhodocheila* had the percentage of fruit set ranging from 41.22 to 70.00 % in the natural pollination. The environment of the population may be the important role for reproductive success. For population size structure study, I compare the subpopulations of *H. janellehayneiana* occured on stone boulders in the evergreen forest near a waterfall and *H. rhodocheila* occured on sandstone boulders in hill evergreen forest. Each species was represented in 2 plots. Leaf length is used as a size class in this study. The apparence of low number of seedling in each population indicated that low reproductive success which led to extinct from the wild. The re-introduction is suggested to maintain the population. Likewise, the high number of large plants are needed to protect to keep genetic variation.

The symbiotic and asymbiotic seed germination of *H. rhodocheila* (orange form) and *H. janellehayneiana* were investigated. Seed viability testing revealed that *H. janellehayneiana* and *H. rhodocheila* has a viability rate of 14.89 %, and 56.71 %, respectively. Twenty fungal isolates of *H. janellehayneiana* and twelve isolates of *H. rhodochila* were obtained from the roots and tubers of both plants. Symbiotic seed germination of *H. janellehayneiana* in three fungal treatments (I03, I20, and control) showed the testa were broken and seed germination. Seeds treated with I20 had the highest GRI value (14.00 %). I08 showed stage 1 protocorms after 14 weeks of incubation. At 16 weeks of sowing, the highest total seed germination percentage was on I20 (14.08 %), followed by I03 (11.04 %) and I08 (4.22 %), respectively. On the other hand, no germination was observed in the *H. rhodocheila* seeds treated with fungal inoculation at 16 weeks. Fungal identification based on the DNA similarity of ITS to the GenBank database showed that I03 belonged to the family Glomerellaceae, *Colletotrichum* sp. (92.61% identity), I08 belonged to the family Hypoxylaceae, *Hypoxylon* sp. (84.97 % identity), and I20 belonged to the family Trichocomaceae, *Aspergillus* sp. (97.92 % identity).

For asymbiotic seed germination, the *H. janellehayneiana* at 16 weeks of sowing on four tested media had the highest total seed germination percentage on ½VW media (19.11 %), followed by ½MS (13.79 %), MS (12.52 %) and VW (11.83 %), respectively. Then, they had developed to stage 4 within 14 weeks. Stage 5 protocorm development was observed after 18 weeks on ½VW media. The overall seed germination percentages of *H. rhodocheila* at 16 weeks after sowing on the tested media were highest on 1/2VW media (15.82 %), followed by VW (9.99 %), MS (9.40 %),

and 1/2MS (7.59 %), respectively. After 16 weeks, they had progressed to stage 5. The results of testing the BAP, GA and TDZ' capacity to promote germination showed that *H. janellehayneiana* seeds began germinating 4 weeks after sowing in all tested media, with the addition of 3 mg/l BAP giving the highest seed germination (5.47 %). In all of the investigated media treatments, *H. rhodocheila* seeds germinated 4 weeks after sowing, with the addition of 1 mg/l BAP having the maximum seed germination (6.71 %). After 16 weeks of culture, seed germination of both plant species on 10 tested media had difference of GRI and DRI percentages. The GRI percentage of *H. janellehayneiana* on 1/2VW +1 mg/l BAP was 11.80 percent per week which wasy greater than the other media. Similarly, the *H. rhodocheila* had the highest GRI (8.08 % per week) on 12VW +1 mg/l BAP. At 16 weeks, the effects of BAP, GA, and TDZ on total seed germination showed that the 1/2VW + 1 mg/l BAP had the highest total germination percentage of 11.83 %. In this study, asymbiotic seed germination had higher efficiency as compared to symbiotic seed germination.

Finally, this study reorganized the taxonomic and phylogenetic relationships of *H. rhodocheila* complex in Thailand to provide a relationship between populations and classification. Study of reproductive patterns and populations size structure of *H. rhodocheila* and *H. janellehayneiana* representatives in the field suggested to maintain the population management and to propagate of *H. rhodocheila* and *H. janellehayneiana* by symbiotic and asymbiotic seed germination techniques for future conservation of there orchids and others.

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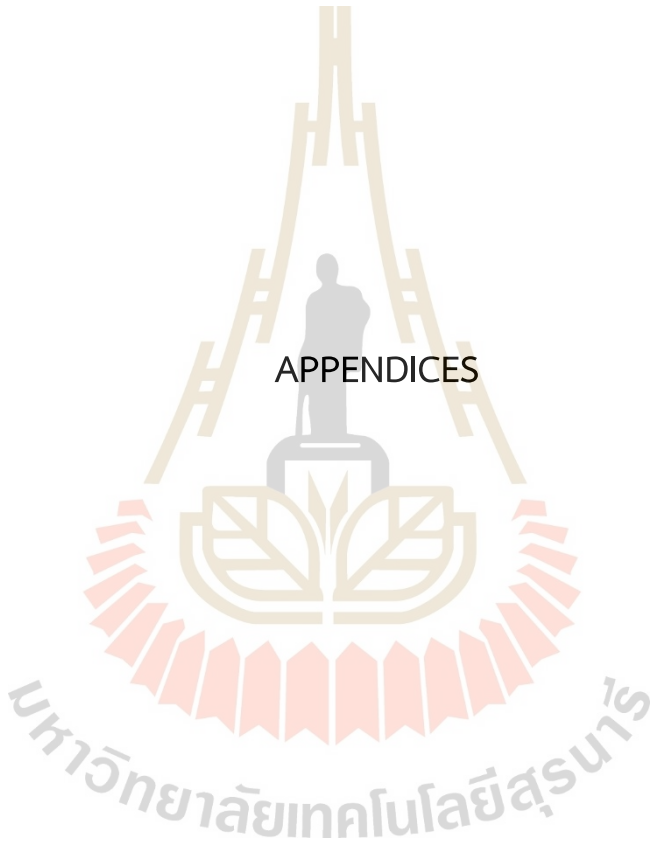
# APPENDICES



# APPENDIX A
# MEDIUM

## A.1 Medium

**Table A.1** Vacin and Went medium (VW), 1949 (Torres, 1989).

| Chemical | Concentration (mg/L) | Concentration in Stock solution (mg/L) | Fold | Value used (mL/L) |
|---|---|---|---|---|
| **Stock solution 1** | | | | 10 |
| $(NH_4)_2SO_4$ | 500 | 50,000 | 100 | |
| $MgSO_4.7H_2O$ | 250 | 25,000 | | |
| $KNO_3$ | 525 | 52,500 | | |
| $KH_2PO_4$ | 250 | 25,000 | | |
| $MnSO_4.4H_2O$ | 7.5 | 750 | | |
| **Stock solution 2** | | | | 10 |
| $Ca_3(PO_4)_2$ | 200 | 20,000 | 100 | |
| **Stock solution 3** | | | | 10 |
| $FeSO_4.7H_2O$ | 27.8 | 2,780 | 100 | |
| $Na_2EDTA$ | 37.3 | 3,730 | | |

Remark: Sugar 20 g/L, Agar 7 g/L, Adjusted pH at 4.8-5.0

**Table A.2** Murashige and Skoog medium (MS) (Murashige and Skoog, 1962).

| Chemical | Concentration (mg/L) | Concentration in Stock solution (mg/L) | Fold | Value used (mL/L) |
|---|---|---|---|---|
| **Stock solution 1** | | | | 50 |
| NH4NO3 | 1,650 | 33,000 | 20 | |
| KNO3 | 1,900 | 38,000 | | |
| CaCl2.2H2O | 440 | 8,800 | | |
| MgSO4.7H2O | 370 | 7,400 | | |
| KH2PO4 | 170 | 3,400 | | |
| **Stock solution 2** | | | | 5 |
| KI | 0.83 | 1.66 | 200 | |
| H3BO3 | 6.2 | 1,240 | | |
| MnSO4.4H2O | 22.3 | 4,460 | | |
| ZnSO4.7H2O | 8.6 | 1,720 | | |
| Na2MoO4.2H2O | 0.25 | 50 | | |
| CuSO4.5H2O | 0.025 | 5 | | |
| CoCl2.6H2O | 0.025 | 5 | | |
| **Stock solution 3** | | | | 5 |
| FeSO4.7H2O | 27.85 | 5,560 | 200 | |
| Na2.EDTA.5H2O | 37.3 | 7,460 | | |
| **Stock solution 4** | | | | 5 |
| Myo-inosital | 100.50 | 20,000 | 200 | |
| Nicotinic acid | 0.5 | 100 | | |
| Pyridoxine HCl | 0.5 | 100 | | |
| Thiamine HCl | 0.5 | 100 | | |
| Glycine | 2 | 400 | | |

Remark: Sugar 30 g/L, Agar 7 g/L, Adjusted pH at 5.7-5.8

## A.2 Plant hormones 100 mg/L of 6-benzylaminopurine (BAP)

Preparation of stock: the BAP is weighted at 100 mg and then dissolved in 1-2 mL of ethanol, mixed thoroughly until dissolved and brought volume up to 100 mL. The solution is kept in a plastic container in the dark and stored at room temperature.

## A.3 Plant hormones 100 mg/L of gibberellic acid (GA)

Preparation of stock: the GA is weighted at 100 mg and then dissolved in 1-2 mL of ethanol, mixed thoroughly until dissolved and brought volume up to 100 mL. The solution is kept in a plastic container in the dark and stored at room temperature.

## A.4 Plant hormones 100 mg/L of thidiazuron (TDZ)

Preparation of stock: the TDZ is weighted at 100 mg and then dissolved in 1-2 mL of ethanol, mixed thoroughly until dissolved and brought volume up to 100 mL. The solution is kept in a plastic container in the dark and stored at room temperature.

## A5. Potato dextrose agar (PDA) 1000 mL

| | | |
|---|---|---|
| Potato Infusion (from 200 g of potatoes) | 4.0 | g |
| Dextrose | 20.0 | g |
| Agar | 17.0 | g |

Final pH at 5.6 ± 0.2.

# APPENDIX B
# DATA OF MORPHOLOGICAL CHARACTERS

**Table B1** Data of morphological characters for morphometric analysis of *H. rhodocheila* (01).

| No | Characters | No. of plant | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 1 | Shoot length | 114.2 | 96.8 | 112.6 | 145.2 | 131.4 | 79.6 | 83.5 | 100.1 | 97.6 | 136.7 |
| 2 | Leaf length | 101.2 | 88.6 | 136.6 | 107.7 | 96.2 | 109.7 | 99.5 | 90.4 | 78.6 | 105.9 |
| 3 | Leaf width | 18.2 | 16.8 | 15.2 | 16.1 | 11.8 | 15.2 | 18.3 | 16 | 15.3 | 16.7 |
| 4 | Leaf margin | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | Leaf apex | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | Flower number | 7 | 4 | 4 | 4 | 4 | 2 | 3 | 3 | 5 | 7 |
| 7 | Dorsal sepal length | 9.2 | 9.5 | 9.6 | 9.8 | 9.4 | 9.7 | 9.2 | 9.1 | 9.6 | 9.8 |
| 8 | Dorsal sepal width | 4.7 | 4.7 | 4.3 | 4.8 | 4.1 | 4.6 | 4.7 | 4.4 | 4.9 | 4.8 |
| 9 | Lateral sepal length | 9.6 | 9.7 | 9 | 9.9 | 9.6 | 9.6 | 9 | 9.7 | 9.6 | 9.4 |
| 10 | Lateral sepal width | 5.8 | 2.3 | 5.4 | 5.5 | 5 | 5.3 | 5.2 | 5 | 5.1 | 5.3 |
| 11 | Lateral sepal apex | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 12 | Petal length | 8.1 | 8.4 | 8.5 | 8.5 | 8.3 | 8.6 | 8.6 | 9.1 | 7.8 | 8.8 |
| 13 | Petal width | 1.8 | 1.9 | 2.2 | 2.3 | 2.2 | 2.1 | 2.0 | 2.4 | 2.0 | 1.8 |
| 14 | Petal margin | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 15 | Petal color | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 16 | Lip length | 27.3 | 25.3 | 22.9 | 27.5 | 25.0 | 23.0 | 25.3 | 27.2 | 24.9 | 22.9 |
| 17 | Lip width | 24.9 | 24.3 | 19.4 | 27.5 | 21.1 | 27.4 | 19.4 | 24.4 | 21.2 | 24.9 |
| 18 | Lip claw length | 5.7 | 4.7 | 4.0 | 4.8 | 4.6 | 4.5 | 4.8 | 5.6 | 4.8 | 4.1 |
| 19 | Side-lobe of lip length | 12.3 | 12.5 | 10.3 | 14.5 | 10.7 | 10.3 | 10.7 | 12.5 | 12.4 | 12.3 |
| 20 | Side-lobe of lip width | 11 | 11.2 | 9.1 | 12.9 | 11.4 | 11.1 | 11.3 | 12.9 | 9.1 | 11.5 |
| 21 | Ratio between side-lobe of lip length and width | 1.12 | 1.12 | 1.13 | 1.12 | 0.94 | 0.93 | 0.95 | 1.00 | 1.36 | 1.07 |
| 22 | Epichile length | 15.4 | 14.2 | 12.6 | 16.3 | 14.7 | 12.7 | 14.8 | 14.3 | 15.5 | 16.2 |
| 23 | Epichile width | 13.5 | 13.2 | 13.3 | 16.9 | 12.8 | 13.7 | 12.9 | 16.6 | 13.3 | 16.8 |

**Table B1** Data of morphological characters for morphometric analysis of *H. rhodocheila* (01) (Continued).

| No | Characters | No. of plant | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 24 | Ratio between side-lobe width and epichile width | 0.81 | 0.85 | 0.68 | 0.76 | 0.89 | 0.81 | 0.88 | 0.78 | 0.68 | 0.68 |
| 25 | Sinus deep of epichile | 6.7 | 6.1 | 4.8 | 7.3 | 5.7 | 4.9 | 6.2 | 5.7 | 6.6 | 6.2 |
| 26 | Sinus width of epichile | 6.5 | 8.1 | 5.8 | 6.9 | 5.4 | 7.0 | 5.5 | 6.6 | 5.8 | 6.9 |
| 27 | Ratio between sinus deep and width of epichile | 1.03 | 0.75 | 0.83 | 1.06 | 1.06 | 0.70 | 1.13 | 0.86 | 1.14 | 0.90 |
| 28 | Spur length | 43.6 | 44.2 | 33.1 | 41.3 | 36.3 | 41.3 | 44.2 | 43.6 | 44.3 | 36.0 |
| 29 | Rostellum length | 4.1 | 4.4 | 4.1 | 4.2 | 4.0 | 4.3 | 4.4 | 4.1 | 4.0 | 4.4 |
| 30 | Rostellum width | 1.5 | 1.9 | 1.7 | 1.9 | 1.9 | 1.8 | 1.9 | 1.9 | 1.6 | 1.8 |
| 31 | Stigmatophore | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 32 | Pedicel length | 33.4 | 27.9 | 22.2 | 21.1 | 27.7 | 22.2 | 33.3 | 28.0 | 21.1 | 22.1 |

**Table B2** Data of morphological characters for morphometric analysis of *H. rhodocheila* (02).

| No | Characters | No. of plant | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 1 | Shoot length | 76.6 | 99.2 | 62.8 | 78.5 | 63.0 | 76.5 | 48.8 | 48.9 | 32.77 | 36.89 |
| 2 | Leaf length | 108.0 | 69.6 | 84.9 | 85.6 | 87.9 | 109.2 | 101.9 | 72.5 | 70.5 | 72.8 |
| 3 | Leaf width | 8.8 | 11.2 | 8.2 | 11.0 | 8.9 | 13.2 | 11.0 | 8.4 | 13.1 | 8.9 |
| 4 | Leaf margin | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 5 | Leaf apex | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | Flower number | 5 | 5 | 3 | 3 | 3 | 8 | 2 | 2 | 4 | 2 |
| 7 | Dorsal sepal length | 8.3 | 8.4 | 8.4 | 8.2 | 8.3 | 9.2 | 8.2 | 9.3 | 8.1 | 9.4 |
| 8 | Dorsal sepal width | 3.8 | 3.6 | 3.8 | 3.7 | 4.1 | 3.8 | 3.6 | 4.0 | 3.7 | 4.0 |
| 9 | Lateral sepal length | 9.2 | 9.5 | 9.1 | 9.6 | 10.6 | 9.6 | 9.4 | 10.5 | 9.2 | 10.6 |
| 10 | Lateral sepal width | 5.4 | 5.7 | 5.5 | 5.2 | 5.8 | 5.8 | 5.8 | 5.5 | 5.3 | 5.8 |
| 11 | Lateral sepal apex | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 12 | Petal length | 7.5 | 8.1 | 7.5 | 8.8 | 8.2 | 7.4 | 8.1 | 7.5 | 7.4 | 8.83 |
| 13 | Petal width | 4.6 | 4.3 | 3.8 | 4.5 | 4.4 | 3.7 | 3.9 | 3.8 | 4.3 | 4.5 |
| 14 | Petal margin | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 15 | Petal color | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| 16 | Lip length | 22.2 | 21.1 | 20.5 | 23.0 | 21.1 | 22.1 | 22.6 | 20.1 | 22.0 | 22.5 |
| 17 | Lip width | 25.1 | 21.7 | 20.6 | 21.6 | 25.1 | 20.0 | 22.0 | 20.6 | 21.7 | 25.0 |
| 18 | Lip claw length | 5.2 | 4.9 | 5.5 | 5.1 | 5.0 | 5.7 | 4.9 | 5.6 | 5.0 | 5.12 |
| 19 | Side-lobe of lip length | 12.6 | 10.6 | 10.2 | 12.0 | 10.7 | 10.14 | 10.6 | 12.1 | 10.6 | 12.51 |
| 20 | Side-lobe of lip width | 8.8 | 8.5 | 8.8 | 9.8 | 8.6 | 9.5 | 8.9 | 8.5 | 9.8 | 9.7 |
| 21 | Ratio between side-lobe of lip length and width | 1.43 | 1.25 | 1.16 | 1.22 | 1.24 | 1.07 | 1.19 | 1.42 | 1.08 | 1.29 |
| 22 | Epichile length | 14.3 | 13.0 | 12.4 | 12.5 | 13.1 | 12.4 | 12.5 | 13.1 | 12.4 | 14.2 |
| 23 | Epichile width | 12.9 | 14.8 | 12.8 | 14.9 | 14.9 | 12.7 | 14.7 | 12.6 | 12.9 | 14.9 |
| 24 | Ratio between side-lobe width and epichile width | 0.68 | 0.57 | 0.69 | 0.66 | 0.58 | 0.75 | 0.61 | 0.68 | 0.76 | 0.65 |

**Table B2** Data of morphological characters for morphometric analysis of *H. rhodocheila* (02) (Continued).

| No | Characters | No. of plant | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 25 | Sinus deep of epichile | 4.1 | 3.9 | 3.8 | 3.9 | 4.7 | 4.1 | 4.8 | 3.7 | 3.9 | 4.8 |
| 26 | Sinus width of epichile | 9.4 | 9.77 | 7.0 | 9.3 | 6.7 | 8.5 | 7.1 | 7.0 | 9.7 | 9.4 |
| 27 | Ratio between sinus deep and width of epichile | 0.44 | 0.40 | 0.54 | 0.42 | 0.70 | 0.48 | 0.68 | 0.53 | 0.40 | 0.51 |
| 28 | Spur length | 39.3 | 33.8 | 36.7 | 39.4 | 33.9 | 36.1 | 36.8 | 33.7 | 33.8 | 39.4 |
| 29 | Rostellum length | 4.1 | 4.1 | 4.3 | 4.0 | 4.1 | 4.9 | 4.2 | 4.1 | 4.8 | 4.0 |
| 30 | Rostellum width | 1.9 | 1.5 | 1.8 | 1.8 | 1.6 | 1.8 | 1.5 | 1.9 | 1.6 | 1.9 |
| 31 | Stigmatophore | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 32 | Pedicel length | 22.0 | 23.8 | 26.8 | 21.9 | 13.8 | 24.3 | 23.9 | 26.7 | 24.4 | 21.9 |

**Table B3** Data of morphological characters for morphometric analysis of *H. rhodocheila* (03).

| No | Characters | No. of plant | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 1 | Shoot length | 75.6 | 72.8 | 108.3 | 107.8 | 73.9 | 75.8 | 72.0 | 74.0 | 108.0 | 72.2 |
| 2 | Leaf length | 161.4 | 153.1 | 166.5 | 166.5 | 145.2 | 154.0 | 167.7 | 161.5 | 166.5 | 166 |
| 3 | Leaf width | 18.3 | 17.0 | 13.3 | 13.5 | 16.8 | 13.2 | 17.1 | 13.4 | 16.8 | 18.5 |
| 4 | Leaf margin | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | Leaf apex | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | Flower number | 5 | 5 | 4 | 4 | 3 | 3 | 5 | 4 | 4 | 3 |
| 7 | Dorsal sepal length | 10.9 | 11.2 | 10.9 | 11.0 | 11.1 | 11.0 | 10.9 | 11.2 | 11.1 | 10.9 |
| 8 | Dorsal sepal width | 4.0 | 4.3 | 4.6 | 4.2 | 2.7 | 4.3 | 4.5 | 4.2 | 4.0 | 2.8 |
| 9 | Lateral sepal length | 10.8 | 11.6 | 10.9 | 11.2 | 11.1 | 11.0 | 10.8 | 11.6 | 11.1 | 10.7 |
| 10 | Lateral sepal width | 4.6 | 4.5 | 4.5 | 4.3 | 4.6 | 4.6 | 4.3 | 4.2 | 4.5 | 4.7 |
| 11 | Lateral sepal apex | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 12 | Petal length | 10.2 | 10.4 | 10.2 | 10.3 | 10.8 | 10.5 | 10.3 | 10.7 | 10.2 | 10.2 |
| 13 | Petal width | 3.8 | 3.9 | 3.4 | 3.3 | 3.2 | 3.9 | 3.3 | 3.8 | 4.0 | 3.4 |
| 14 | Petal margin | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 15 | Petal color | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 16 | Lip length | 24.3 | 22.3 | 24.0 | 22.0 | 22.1 | 22.4 | 24.1 | 24.4 | 22.0 | 22.1 |
| 17 | Lip width | 16.4 | 15.6 | 11.8 | 18.1 | 17.7 | 11.9 | 18.2 | 16.5 | 15.5 | 11.8 |
| 18 | Lip claw length | 4.9 | 3.5 | 3.5 | 3.3 | 3.5 | 3.6 | 3.4 | 4.8 | 3.5 | 3.3 |
| 19 | Side-lobe of lip length | 8.2 | 7.8 | 5.9 | 9.0 | 8.8 | 6.0 | 8.9 | 8.8 | 1.9 | 8.3 |
| 20 | Side-lobe of lip width | 14 | 14.1 | 14.4 | 13.2 | 13.5 | 14.5 | 13.3 | 13.9 | 14.2 | 14.5 |
| 21 | Ratio between side-lobe of lip length and width | 0.59 | 0.55 | 0.41 | 0.68 | 0.65 | 0.41 | 0.67 | 0.63 | 0.13 | 0.57 |
| 22 | Epichile length | 14.4 | 12.9 | 15.3 | 13.3 | 13.3 | 14.5 | 13 | 15.3 | 13.4 | 15.2 |
| 23 | Epichile width | 12.0 | 11.2 | 13.9 | 12.1 | 11.6 | 11.3 | 12.2 | 12.0 | 14.0 | 11.3 |
| 24 | Ratio between side-lobe width and epichile width | 1.17 | 1.26 | 1.04 | 1.09 | 1.16 | 1.28 | 1.09 | 1.16 | 1.01 | 1.28 |

**Table B3** Data of morphological characters for morphometric analysis of *H. rhodocheila* (03) (Continued).

| No | Characters | No. of plant | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 25 | Sinus deep of epichile | 5.0 | 3.8 | 6.1 | 5.1 | 3.8 | 3.7 | 6.2 | 5.0 | 3.8 | 5.1 |
| 26 | Sinus width of epichile | 4.5 | 4.9 | 5.4 | 5.8 | 4.6 | 5.5 | 5.0 | 4.6 | 5.4 | 4.7 |
| 27 | Ratio between sinus deep and width of epichile | 1.11 | 0.78 | 1.13 | 0.88 | 0.83 | 0.68 | 1.24 | 1.09 | 0.70 | 1.09 |
| 28 | Spur length | 47.0 | 48.2 | 45.3 | 41.6 | 40.6 | 48.2 | 47.0 | 45.3 | 41.7 | 47.1 |
| 29 | Rostellum length | 3.5 | 3.2 | 3.2 | 3.2 | 3.6 | 3.3 | 3.5 | 3.7 | 3.2 | 3.3 |
| 30 | Rostellum width | 2.1 | 2.1 | 2.7 | 2.3 | 2.7 | 2.2 | 2.7 | 2.6 | 2.8 | 2.2 |
| 31 | Stigmatophore | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 32 | Pedicel length | 34.7 | 33.5 | 28.0 | 24.7 | 25.5 | 28.1 | 24.8 | 33.6 | 24.7 | 34.6 |

**Table B4** Data of morphological characters for morphometric analysis of *H. rhodocheila* (04).

| No | Characters | No. of plant | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 1 | Shoot length | 54.3 | 87.8 | 87.3 | 93.4 | 33.2 | 51.6 | 54.6 | 64.8 | 86.1 | 52.8 |
| 2 | Leaf length | 111.7 | 148.4 | 173.3 | 117.8 | 102.8 | 123.7 | 100.6 | 112.0 | 116.4 | 95.9 |
| 3 | Leaf width | 12.1 | 12.9 | 13.6 | 11.8 | 10.1 | 13.2 | 12.2 | 16.2 | 14.5 | 9.6 |
| 4 | Leaf margin | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | Leaf apex | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | Flower number | 1 | 2 | 4 | 3 | 3 | 5 | 2 | 8 | 5 | 2 |
| 7 | Dorsal sepal length | 8.7 | 8.1 | 7.8 | 7.8 | 7.6 | 8.2 | 8.7 | 7.9 | 7.9 | 8.1 |
| 8 | Dorsal sepal width | 3.8 | 3.6 | 3.1 | 3.7 | 3.3 | 3.7 | 3.4 | 3.9 | 3.6 | 3.2 |
| 9 | Lateral sepal length | 9.5 | 9.6 | 9.6 | 9.2 | 7.6 | 9.7 | 9.3 | 9.5 | 9.2 | 9.6 |
| 10 | Lateral sepal width | 4.8 | 4.9 | 4.9 | 4.7 | 4.0 | 4.8 | 4.7 | 5.0 | 5.2 | 4.8 |
| 11 | Lateral sepal apex | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 12 | Petal length | 7.4 | 7.3 | 7.4 | 7.0 | 7.4 | 8.0 | 7.5 | 7.3 | 7.7 | 8.1 |
| 13 | Petal width | 2.7 | 2.6 | 2.4 | 2.8 | 2.7 | 2.8 | 2.9 | 2.5 | 2.4 | 2.8 |
| 14 | Petal margin | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 15 | Petal color | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| 16 | Lip length | 21.6 | 21.1 | 20.6 | 18.8 | 21.2 | 21.0 | 18.9 | 20.7 | 21.1 | 18.8 |
| 17 | Lip width | 19.7 | 19.8 | 18.5 | 19.8 | 19.9 | 19.7 | 19.8 | 18.6 | 18.5 | 18.7 |
| 18 | Lip claw length | 4.7 | 4.6 | 5.4 | 4.7 | 4.7 | 5.5 | 5.4 | 4.7 | 4.6 | 5.5 |
| 19 | Side-lobe of lip length | 9.8 | 9.9 | 9.3 | 9.4 | 10.0 | 9.9 | 9.8 | 9.5 | 9.3 | 9.9 |
| 20 | Side-lobe of lip width | 8.4 | 8.8 | 9.3 | 8.8 | 8.9 | 9.4 | 8.8 | 8.5 | 8.9 | 9.3 |
| 21 | Ratio between side-lobe of lip length and width | 1.17 | 1.13 | 1.00 | 1.07 | 1.12 | 1.05 | 1.11 | 1.12 | 1.04 | 1.06 |
| 22 | Epichile length | 11.4 | 11.7 | 10.6 | 9.4 | 11.5 | 10.6 | 9.5 | 10.7 | 11.4 | 11.8 |
| 23 | Epichile width | 11.3 | 12.5 | 11.0 | 11.2 | 11.1 | 12.6 | 11.2 | 11.4 | 11.3 | 12.5 |
| 24 | Ratio between side-lobe width and epichile width | 0.74 | 0.70 | 0.85 | 0.79 | 0.80 | 0.75 | 0.79 | 0.75 | 0.79 | 0.74 |

**Table B4** Data of morphological characters for morphometric analysis of *H. rhodocheila* (04) (Continued).

| No | Characters | No. of plant | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 25 | Sinus deep of epichile | 3.6 | 5.3 | 3.7 | 3.0 | 3.1 | 5.3 | 3.7 | 5.2 | 3.8 | 3.7 |
| 26 | Sinus width of epichile | 6.1 | 7.0 | 5.1 | 5.4 | 7.0 | 6.2 | 6.1 | 5.2 | 5.5 | 5.4 |
| 27 | Ratio between sinus deep and width of epichile | 0.59 | 0.76 | 0.73 | 0.56 | 0.44 | 0.85 | 0.61 | 1.00 | 0.69 | 0.69 |
| 28 | Spur length | 37.4 | 34.4 | 37.8 | 33.5 | 37.9 | 37.5 | 34.5 | 33.6 | 37.9 | 34.5 |
| 29 | Rostellum length | 4.7 | 4.8 | 4.7 | 4.1 | 4.2 | 4.8 | 4.7 | 4.3 | 4.6 | 4.7 |
| 30 | Rostellum width | 1.8 | 1.6 | 1.7 | 1.7 | 1.6 | 1.8 | 1.7 | 1.9 | 1.8 | 1.6 |
| 31 | Stigmatophore | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 32 | Pedicel length | 18.6 | 23.9 | 16.0 | 19.4 | 20.1 | 24.0 | 19.5 | 20.0 | 23.8 | 18.7 |

**Table B5** Data of morphological characters for morphometric analysis of *H. rhodocheila* (05).

| No | Characters | No. of plant | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 1 | Shoot length | 74.5 | 118.0 | 111.2 | 116.5 | 109.5 | 16.4 | 44.8 | 84.3 | 120.5 | 130.7 |
| 2 | Leaf length | 125.5 | 195.4 | 106.4 | 59.8 | 106.7 | 112.3 | 82.4 | 90.8 | 123.4 | 79.8 |
| 3 | Leaf width | 16.6 | 22.0 | 16.0 | 22.1 | 16.7 | 21.2 | 16.0 | 15.2 | 21.1 | 21.6 |
| 4 | Leaf margin | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | Leaf apex | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | Flower number | 7 | 12 | 2 | 1 | 3 | 2 | 7 | 2 | 4 | 1 |
| 7 | Dorsal sepal length | 9.7 | 10.0 | 9.0 | 9.0 | 9.0 | 8.5 | 10.0 | 10.2 | 9.7 | 8.6 |
| 8 | Dorsal sepal width | 4.3 | 4.2 | 4.0 | 4.2 | 4.0 | 4.3 | 4.2 | 4.4 | 3.7 | 4.1 |
| 9 | Lateral sepal length | 9.2 | 9.9 | 9.1 | 9.2 | 9.0 | 8.5 | 9.9 | 9.6 | 8.9 | 9.2 |
| 10 | Lateral sepal width | 5.0 | 5.7 | 5.2 | 5.4 | 5.6 | 5.3 | 5.7 | 5.1 | 5.7 | 5.3 |
| 11 | Lateral sepal apex | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 12 | Petal length | 8.3 | 9.5 | 8.1 | 8.0 | 7.9 | 7.9 | 9.5 | 9.6 | 8.8 | 9.6 |
| 13 | Petal width | 2.1 | 2.5 | 2.2 | 2.1 | 2.1 | 2.1 | 2.5 | 2.5 | 2.3 | 2.2 |
| 14 | Petal margin | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 15 | Petal color | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 16 | Lip length | 22.9 | 22.6 | 22.1 | 21.5 | 21.4 | 20.5 | 21.5 | 21.4 | 21.2 | 21.5 |
| 17 | Lip width | 23.2 | 23.3 | 22.3 | 21.7 | 20.9 | 19.4 | 20.9 | 21.4 | 21.1 | 22.4 |
| 18 | Lip claw length | 3.2 | 3.5 | 3.2 | 2.5 | 2.4 | 2.1 | 2.7 | 3.2 | 3.1 | 2.9 |
| 19 | Side- lobe of lip length | 11.6 | 11.6 | 11.1 | 10.8 | 10.4 | 9.7 | 10.4 | 10.7 | 10.5 | 10.9 |
| 20 | Side- lobe of lip width | 11.6 | 12.0 | 12.0 | 11.3 | 11.1 | 11.3 | 9.2 | 9.4 | 8.9 | 11.4 |
| 21 | Ratio between side-lobe of lip length and width | 1.00 | 1.00 | 0.93 | 0.96 | 0.94 | 0.86 | 1.13 | 1.14 | 1.18 | 0.96 |
| 22 | Epichile length | 14.5 | 13.7 | 13.6 | 12.7 | 12.7 | 11.7 | 13.6 | 13.2 | 14.0 | 12.8 |
| 23 | Epichile width | 14.4 | 14.1 | 13.9 | 13.7 | 14.2 | 14.1 | 15.2 | 14.1 | 14.4 | 15.2 |
| 24 | Ratio between side-lobe width and epichile width | 0.81 | 0.85 | 0.86 | 0.82 | 0.78 | 0.80 | 0.61 | 0.67 | 0.62 | 0.75 |

**Table B5** Data of morphological characters for morphometric analysis of *H. rhodocheila* (05) (Continued).

| No | Characters | No. of plant | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 25 | Sinus deep of epichile | 4.2 | 3.3 | 3.7 | 3.6 | 4.0 | 4.3 | 4.2 | 3.9 | 5.0 | 3.7 |
| 26 | Sinus width of epichile | 4.9 | 5.1 | 4.3 | 4.9 | 4.6 | 4.6 | 6.2 | 5.2 | 5.7 | 4.7 |
| 27 | Ratio between sinus deep and width of epichile | 0.86 | 0.65 | 0.86 | 0.73 | 0.87 | 0.93 | 0.68 | 0.75 | 0.88 | 0.79 |
| 28 | Spur length | 34.2 | 42.0 | 34.2 | 34.4 | 34.3 | 35.0 | 42.6 | 41.3 | 41.9 | 34.3 |
| 29 | Rostellum length | 4.5 | 5.7 | 4.8 | 4.8 | 4.6 | 4.8 | 5.7 | 5.6 | 5.7 | 4.9 |
| 30 | Rostellum width | 3.1 | 3.0 | 3.1 | 3.1 | 3.0 | 3.0 | 3.0 | 3.4 | 3.0 | 3.2 |
| 31 | Stigmatophore | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 32 | Pedicel length | 31.8 | 29.7 | 31.9 | 31.0 | 30.0 | 29.8 | 29.7 | 28.0 | 26.2 | 29.9 |

**Table B6** Data of morphological characters for morphometric analysis of *H. rhodocheila* (06).

| No | Characters | No. of plant | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 1 | Shoot length | 241.8 | 153.0 | 29.7 | 59.5 | 74.9 | 76.0 | 74.3 | 63.6 | 61.6 | 61.4 |
| 2 | Leaf length | 185.6 | 177.3 | 142.6 | 111.2 | 111.2 | 110.5 | 102.2 | 124.5 | 110.4 | 103.5 |
| 3 | Leaf width | 29.7 | 27.9 | 11.2 | 11.8 | 9.2 | 9.2 | 12.2 | 15.0 | 12.0 | 9.2 |
| 4 | Leaf margin | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | Leaf apex | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | Flower number | 12 | 8 | 7 | 7 | 8 | 11 | 9 | 11 | 8 | 12 |
| 7 | Dorsal sepal length | 12.3 | 13.1 | 12.9 | 12.9 | 12.8 | 12.4 | 12.9 | 12.8 | 12.3 | 12.9 |
| 8 | Dorsal sepal width | 5.5 | 5.6 | 5.0 | 5.4 | 5.6 | 5.7 | 5.4 | 5.6 | 5.6 | 5.5 |
| 9 | Lateral sepal length | 11.4 | 11.3 | 11.3 | 11.2 | 12.0 | 11.8 | 11.3 | 12.0 | 11.9 | 11.4 |
| 10 | Lateral sepal width | 6.8 | 7.0 | 7.1 | 6.6 | 6.3 | 6.4 | 7.1 | 6.3 | 6.7 | 6.9 |
| 11 | Lateral sepal apex | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 12 | Petal length | 12.3 | 11.9 | 12.3 | 12.1 | 13.0 | 12.1 | 12.1 | 12.5 | 11.9 | 12.0 |
| 13 | Petal width | 2.9 | 2.8 | 2.9 | 2.8 | 2.5 | 2.6 | 2.9 | 2.6 | 3.0 | 2.6 |
| 14 | Petal margin | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 15 | Petal color | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 16 | Lip length | 28.9 | 30.2 | 29.3 | 30.6 | 27.8 | 28.1 | 29.3 | 30.2 | 28.1 | 27.8 |
| 17 | Lip width | 22.9 | 20.1 | 21.4 | 21.2 | 21.4 | 21.0 | 20.1 | 21.0 | 21.4 | 21.2 |
| 18 | Lip claw length | 6.2 | 8.4 | 6.1 | 7.9 | 7.3 | 7.7 | 6.1 | 8.4 | 7.3 | 7.9 |
| 19 | Side-lobe of lip length | 11.5 | 10.2 | 10.4 | 10.4 | 11.0 | 10.6 | 10.2 | 10.4 | 11.0 | 10.2 |
| 20 | Side-lobe of lip width | 16.0 | 16.5 | 17.2 | 16.2 | 15.2 | 14.9 | 16.3 | 16.2 | 15.2 | 17.2 |
| 21 | Ratio between side-lobe of lip length and width | 0.71 | 0.62 | 0.60 | 0.64 | 0.72 | 0.71 | 0.63 | 0.64 | 0.72 | 0.59 |
| 22 | Epichile length | 14.8 | 14.3 | 15.4 | 15.3 | 14.1 | 14.4 | 14.8 | 15.3 | 15.4 | 14.4 |
| 23 | Epichile width | 12.7 | 12.7 | 12.9 | 13.0 | 12.4 | 12.4 | 12.7 | 12.4 | 13.0 | 12.7 |
| 24 | Ratio between side-lobe width and epichile width | 1.26 | 1.30 | 1.34 | 1.25 | 1.23 | 1.20 | 1.28 | 1.31 | 1.17 | 1.35 |

**Table B6** Data of morphological characters for morphometric analysis of *H. rhodocheila* (06) (Continued).

| No | Characters | No. of plant | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 25 | Sinus deep of epichile | 5.1 | 5.7 | 5.7 | 5.8 | 3.4 | 3.9 | 5.7 | 3.4 | 5.1 | 5.8 |
| 26 | Sinus width of epichile | 6.1 | 6.3 | 6.2 | 6.2 | 6.3 | 5.8 | 6.3 | 6.2 | 6.1 | 6.2 |
| 27 | Ratio between sinus deep and width of epichile | 0.84 | 0.90 | 0.92 | 0.94 | 0.54 | 0.67 | 0.90 | 0.55 | 0.84 | 0.94 |
| 28 | Spur length | 41.0 | 42.2 | 37.1 | 39.2 | 36.5 | 36.4 | 42.2 | 36.5 | 39.2 | 42.2 |
| 29 | Rostellum length | 8.6 | 7.0 | 7.2 | 7.1 | 7.0 | 7.1 | 8.6 | 7.0 | 7.0 | 7.1 |
| 30 | Rostellum width | 2.6 | 2.6 | 2.4 | 2.6 | 2.3 | 2.4 | 2.6 | 2.6 | 2.4 | 2.6 |
| 31 | Stigmatophore | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 32 | Pedicel length | 34.9 | 36.5 | 36.5 | 34.8 | 38.1 | 37.9 | 34.9 | 36.5 | 37.9 | 36.5 |

**Table B7** Data of morphological characters for morphometric analysis of *H. janellehayneiana* (07).

| No | Characters | No. of plant | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 1 | Shoot length | 95.1 | 46.6 | 60.5 | 75.0 | 105.2 | 110.6 | 90.3 | 100.4 | 110.5 | 80.7 |
| 2 | Leaf length | 111.8 | 102.7 | 135.8 | 147.5 | 140.5 | 145.0 | 138.8 | 160.4 | 150.2 | 132.6 |
| 3 | Leaf width | 18.8 | 6.8 | 20.1 | 22.5 | 21.3 | 18.8 | 16.6 | 14.1 | 10.8 | 10.4 |
| 4 | Leaf margin | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 5 | Leaf apex | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | Flower number | 12 | 7 | 6 | 3 | 3 | 6 | 4 | 6 | 5 | 4 |
| 7 | Dorsal sepal length | 9.7 | 8.2 | 9.5 | 8.2 | 8.6 | 8.1 | 7.6 | 8.6 | 8.5 | 8.4 |
| 8 | Dorsal sepal width | 6.5 | 2.7 | 5.8 | 5.5 | 5.0 | 5.5 | 5.0 | 5.6 | 5.1 | 5.9 |
| 9 | Lateral sepal length | 13.0 | 11.6 | 11.3 | 10.7 | 10.6 | 11.7 | 10.5 | 10.5 | 12.0 | 10.1 |
| 10 | Lateral sepal width | 8.3 | 8.1 | 8.3 | 7.8 | 7.6 | 7.5 | 7.2 | 7.6 | 7.1 | 7.6 |
| 11 | Lateral sepal apex | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 12 | Petal length | 8.8 | 8.7 | 8.9 | 8.4 | 8.7 | 8.0 | 7.4 | 8.1 | 8.8 | 8.5 |
| 13 | Petal width | 4.1 | 4.2 | 3.6 | 4.4 | 4.3 | 4.0 | 3.8 | 4.3 | 4.8 | 4.7 |
| 14 | Petal margin | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 15 | Petal color | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 16 | Lip length | 22.8 | 21.2 | 20.6 | 21.9 | 21.6 | 21.2 | 20.8 | 20.6 | 21.5 | 20.1 |
| 17 | Lip width | 24.9 | 21.2 | 20.8 | 21.3 | 21.0 | 20.8 | 20.0 | 21.5 | 21.2 | 20.8 |
| 18 | Lip claw length | 6.1 | 5.5 | 6.4 | 7.6 | 5.5 | 5.6 | 5.2 | 6.4 | 6.4 | 5.9 |
| 19 | Side- lobe of lip length | 12.9 | 10.4 | 10.5 | 10.6 | 10.5 | 10.4 | 10.0 | 10.7 | 10.6 | 10.4 |
| 20 | Side- lobe of lip width | 10.6 | 9.1 | 9.4 | 9.3 | 9.4 | 9.1 | 9.4 | 10.4 | 11.7 | 8.9 |
| 21 | Ratio between side-lobe of lip length and width | 1.22 | 1.14 | 1.12 | 1.14 | 1.12 | 1.14 | 1.06 | 1.03 | 0.90 | 1.17 |
| 22 | Epichile length | 11.3 | 11.0 | 11.3 | 11.8 | 11.7 | 11.0 | 11.3 | 11.0 | 10.5 | 10.6 |
| 23 | Epichile width | 12.7 | 12.4 | 12.7 | 11.5 | 12.4 | 12.6 | 12.7 | 12.4 | 12.0 | 12.6 |
| 24 | Ratio between side-lobe width and epichile width | 0.83 | 0.73 | 0.74 | 0.81 | 0.76 | 0.72 | 0.74 | 0.84 | 0.98 | 0.71 |

**Table B7** Data of morphological characters for morphometric analysis of *H. janellehayneiana* (07) (Continued).

| No | Characters | No. of plant | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 25 | Sinus deep of epichile | 2.8 | 2.4 | 2.4 | 1.7 | 2.4 | 2.3 | 2.4 | 2.3 | 2.7 | 2.6 |
| 26 | Sinus width of epichile | 3.3 | 3.3 | 3.5 | 4.1 | 3.3 | 3.8 | 3.5 | 3.3 | 4.8 | 3.9 |
| 27 | Ratio between sinus deep and width of epichile | 0.85 | 0.73 | 0.69 | 0.41 | 0.73 | 0.61 | 0.69 | 0.70 | 0.56 | 0.67 |
| 28 | Spur length | 51.4 | 51.2 | 55.3 | 46.4 | 47.7 | 42.2 | 47.1 | 50.4 | 46.8 | 47.4 |
| 29 | Rostellum length | 4.9 | 4.5 | 5.1 | 5.0 | 4.3 | 5.0 | 4.0 | 5.3 | 4.7 | 4.1 |
| 30 | Rostellum width | 2.1 | 2.1 | 2.0 | 1.9 | 1.9 | 2.1 | 1.9 | 2.1 | 2.5 | 2.1 |
| 31 | Stigmatophore | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| 32 | Pedicel length | 27.1 | 26.2 | 29.5 | 26.2 | 27.4 | 22.2 | 24.4 | 28.0 | 26.3 | 25.8 |

# APPENDIX C
# DNA SEQUENCES

**Table C.1** DNA Sequences ITS, *matK* and *rbcL* of plant.

| Region/ No. | DNA Sequence |
|---|---|
| ITS | |
| 01 | GCCTGCGATGCTGCGAGAAGTCCATTGAACCTTATCATTTAGAGGAAGGAGAAGTCGTAACAAGG<br>TTTCCGTAGGTGAACCTGCGGAAGGATCATTGACGAGACACAAAAGAGATCGAGCGACTTGATAA<br>CTTGTGAATTATTTCGGCAGCTCACTTTGTTTGCTGCGCACCCACCCATCGGTGGCATGACTTTCC<br>TTATGGAGCCATGACGTTGGTGGAGGGGAGAACAATTGGGCACAGCTTCGTGCCAAGGTAAATAT<br>GACAGCACGAGCAAATCTTCAACCGCATATCCTCAAAACGTGCCATGGTTTGGGGAGTTGATGTT<br>TGCTCCTTTGGAATTGTAGGACTCTCGGCAATGGATATCTTGGCTCTTGCATCGATGAAGAGCGC<br>AGCGAAATGCGATACGTGGTGCGAATTGCAGAATCCCGTGAACCATCGAGTTTTTGAACGCAAGT<br>TGCGCCTGAGGCCACCTGGCCAAGGGCACGTCCACCTGGGCGTCAAGCATTAAATCGCTCTACTA<br>GACGCCAGACCTTCAATGCTATGGGGCGGCGTATTGGATGCGGAGAATGGCCCGTCATGCGTGAC<br>GTGTGGCGGGTTGAAGGGAAGGATGATTTTCTCTTGGCGATGATCGATTAATTGGTGGGATGAAG<br>CCCCAGTAACCTCCTAATCATCAGCTTGCTTTGAGGAATAGCATATCCTAGGCCAACCCAACCAG<br>AGTTTTGGAAGCAAATCGAAATGCGACCCCAGGATGGGCGGGGTGACCCGCTGAGTTTAAGCATA<br>TCAATAAGCGGAG |
| 02 | GCCTGCGATGCTGCGAGAAGTCCATTGAACCTTATCATTTAGAGGAAGGAGAAGTCGTAACAAGG<br>TTTCCGTAGGTGAACCTGCGGAAGGATCATTGACGAGACACAAAAGGGATCGAGCGACTTGATAA<br>CTTGTGAATTATTTCGGCAGCTCAATTTCTTTGCTGCGCACCCACCCATCGGTGGCATGGCTTTCC<br>TTGTGGAGCCATGACGCTGGTGGAGGGGAGAACAATCGGGCACAGCTTCGTGCCAAGGTAAATAT<br>GATAGCACGAGCAAATCTTCAACCGCATATCCTCAAAACGTGCCATGGTTTGGGGAGTTGATGTT<br>TGCTCCTTTGGAATTGTAGGACTCTCGGCAATGGATATCTTGGCTCTTGCATCGATGAAGAGCGC<br>AGCGAAATGCGATACGTGGTGCGAATTGCAGAATCCCGTGAACCATCGAGTTTTTGAACGCAAGT<br>TGCGCCCGAGGCCACCTGGCCAAGGGCACGTCCACCTGGGCGTCAAGCATTAAATCGCTCTACTA<br>GACGCCAGACCTTCAATGCTATGGGGCGGCGTATTAGATGCGGAGAATGGCCCGTCATGCGTGAC<br>GTGTGGCGGGTTGAAGGGAAGGATGATTTTCTCTTGGCAATGATCGATTAATTGGTGGGATGAAG<br>CCCCCAGTAACCTCCTAATCATCAGCTTGCATTGAGGAACAGCATATCCTAGGCCAACCCAACCA<br>GAGTTTTGGAAGCAAATCGAAATGCGACCCCAGGATGGGCGGGGTGACCCGCTGAGTTTAAGCAT<br>ATCAATAAGCGGA |

**Table C.1** DNA Sequences ITS, *matK* and *rbcL* of plant (Continued).

| Region /No. | DNA Sequence |
|---|---|
| 03 | GCCTGCGATGCTGCGAGAAGTCCATTGAACCTTATCATTTAGAGGAAGGAGAAGTCGTAACAAGG<br>TTTCCGTAGGTGAACCTGCGGAAGGATCATTGACGAGACACAAAAGGGATCGAGCGACTTGATAA<br>CTCGTGAATTATTTCGGCAGCTCACTTTCTTTGCTGCGCACCCACCCATCGGTGGCATGACTTCCT<br>TGTGGAGCCATGGCGTTGGTGGAGGGGAGAACAATCGGGCACAGCTTCGTGCCAAGGTAAATATG<br>ACAGCATGAGCAAATCTTCAACCGCATATCCTCAAAACGTGCCATGGTTTGGGGAGTTGATGTTT<br>GCTGCTTTGGAATTGTAGGACTCTCGGCAATGGATATCTTGGCTCTTGCATCGATGAAGAGCGCA<br>GCGAAATGCGATACGTGGTGCGAATTGCAGAATCCCGTGAACCATCGAGTTTTTGAACGCAAGTT<br>GCGCCTGAGGCCACCTGGCCAAGGGCACGTCCACCTGGGCGTCAAGCATTAAATCGCTCTACTAG<br>ACGCCAGACCTTCAATGCTATGGGGGCGGCGTATTGGATGCGGAGAGTGGCCCGTCATGCGTGAC<br>GTGTGGCGGGTTGAAGGGAAGGATGATTTTCTCTTGGCAATGATCGATTAATTGGTGGGATGAAG<br>CCCCAGTAACCTCCTAATCATCAGCTTGCTTTGAGGAACAGCATATCCTAGGCCAACCCAACCAG<br>AGTTTTGGAAGCAAATCGAAATGCGACCCCAGGATGGGCGGGGTGACCCGCTGAGTTTAAGCATA<br>TCAATAAGCGGAG |
| 04 | GCCTGCGATGCTGCGAGAAGTCCATTGAACCTTATCATTTAGAGGAAGGAGAAGTCGTAACAAGG<br>TTTCCGTAGGTGAACCTGCGGAAGGATCATTGACGAGACACAAAAGGGATCGAGCGACTTGATAA<br>CTCGTGAATTATTTCGGCAGCTCACTTTCTTTGCTGCGCACCCACCCATCGGTGGCATGACTTCCT<br>TGTGGAGCCATGGCGTTGGTGGAGGGGAGAACAATCGGGCACAGCTTCGTGCCAAGGTAAATATG<br>ACAGCATGAGCAAATCTTCAACCGCATATCCTCAAAACGTGCCATGGTTTGGGGAGTTGATGTTT<br>GCTGCTTTGGAATTGTAGGACTCTCGGCAATGGATATCTTGGCTCTTGCATCGATGAAGAGCGCA<br>GCGAAATGCGATACGTGGTGCGAATTGCAGAATCCCGTGAACCATCGAGTTTTTGAACGCAAGTT<br>GCGCCTGAGGCCACCTGGCCAAGGGCACGTCCACCTGGGCGTCAAGCATTAAATCGCTCTACTAG<br>ACGCCAGACCTTCAATGCTATGGGGGCGGCGTATTGGATGCGGAGAGTGGCCCGTCATGCGTGAC<br>GTGTGGCGGGTTGAAGGGAAGGATGATTTTCTCTTGGCAATGATCGATTAATTGGTGGGATGAAG<br>CCCCAGTAACCTCCTAATCATCAGCTTGCTTTGAGGAACAGCATATCCTAGGCCAACCCAACCAG<br>AGTTTTGGAAGCAAATCGAAATGCGACCCCAGGATGGGCGGGGTGACCCGCTGAGTTTAAGCATA<br>TCAATAAGCGGAG |
| 05 | GCCTGCGATGCTGCGAGAAGTCCATTGAACCTTATCATTTAGAGGAAGGAGAAGTCGTAACAAGG<br>TTTCCGTAGGTGAACCTGCGGAAGGATCATTGACGAGACACAAAAGGGACCGAGCGACTTGATAA<br>CTTGTGAATTATTTTGGCAGCTCAATTTCTTTGCTGCACACCCACCCATCGGTGGCATGGCTTTCC<br>TTGTGGAGCCATGACGCTGGTGGAGGGGAGAACAATTGGGCACAGCTTCGTGCCAAGGTAAATAT<br>GACAGCACGAGCAAATCTTCAACCGCATATCCTCAAAACATGCCATGGTTTGGGGAGTTGATGTT<br>TGCTCCTTTGGAATTGTAGGACTCTCGGCAATGGATATCTTGGCTCTTGCATCGATGAAGAGCGC<br>AGCGAAATGCGATACGTGGTGCGAATTGCAGAATCCCGTGAACCATCGAGTTTTTGAACGCAAGT<br>TGCGCCCGAGGCCACCTGGCCAAGGGCACGTCCACCTGGGCGTCAAGCATTAAATCGCTCTACTA<br>GACGCCAGACCTTCAATGCTATGGGGCGGCGTATTAGATGCGGAGAATGGCCCGTCATGCGTGAC<br>GTGTGGCGGGTTGAAGGGAAGGATGATTTTCTCTTGGCAATGATCGATTAATTGGTGGGATGAAG<br>CCCCAGTAACCTTCTAATCATCAGCTTGCATTGAGGAACAACATATCCTAGGCCAACCCAACCAG<br>AGTTTTGGAAGCAAATCGAAATGCGACCCCAGGATGGGCGGGGTGACCCGCTGAGTTTAAGCATA<br>TCAATAAGCGGAG |

**Table C.1** DNA Sequences ITS, *matK* and *rbcL* of plant (Continued).

| Region/No. | DNA Sequence |
|---|---|
| 06 | GCCTGCGATGCTGCGAGAAGTCCATTGAACCTTATCATTTAGAGGAAGGAGAAGTCGTAACAAGG<br>TTTCCGTAGGTGAACCTGCGGAAGGATCATTGACGAGACACAAAAGGGGATCGAGCGACTTGATA<br>ACTCGTGAATTATTTCGGCAGCTCACTTTCTTTGCTGCGCACCCACCCATCGGTGGCATGACTTC<br>CTTGTGGAGCCATGGCGTTGGTGGAGGGGAGAACAATCGGGCACAGCTTCGTGCCAAGGTAAATA<br>TGACAGCATGAGCAAATCTTCAACCGCATATCCTCAAAACGTGCCATGGTTTGGGGAGTTGATGT<br>TTGCTGCTTTGGAATTGTAGGACTCTCGGCAATGGATATCTTGGCTCTTGCATCGATGAAGAGCG<br>CAGCGAAATGCGATACGTGGTGCGAATTGCAGAATCCCGTGAACCATCGAGTTTTTGAACGCAAG<br>TTGCGCCTGAGGCCACCTGGCCAAGGGCACGTCCACCTGGGCGTCAAGCATTAAATCGCTCTACT<br>AGACGCCAGACCTTCAATGCTATGGGGCGGCGTATTGGATGCGGAGAGTGGCCCGTCATGCGTGA<br>CGTGTGGCGGGTTGAAGGGAAGGATGATTTTCTCTTGGCAATGATCGATTAATTGGTGGGATGAA<br>GCCCCAGTAACCTCCTAATCATCAGCTTGCTTTGAGGAACAGCATATCCTAGGCCAACCCAACCA<br>GAGTTTTGGAAGCAAATCGAAATGCGACCCCAGGATGGGCGGGGTGACCCGCTGAGTTTAAGCAT<br>ATCAATAAGCGGAG |
| 07 | GCCTGCGATGCTGCGAGAAGTCCATTGAACCTTATCATTTAGAGGAAGGAGAAGTCGTAACAAGG<br>TTTCCGTAGGTGAACCTGCGGAAGGATCATTGACGAGACACAAAAGGGATCGAGCGACTTGATAA<br>CTCGTGAATTATTTCGGCAGCTCACTTTCTTTGCTGCGCACCCACCCATCGGTGGCATGACTTCCT<br>TGTGGAGCCATGGCGTTGGTGGAGGGGAGAACAATCGGGCACAGCTTCGTGCCAAGGTAAATATG<br>ACAGCATGAGCAAATCTTCAACCGCATATCCTCAAAACGTGCCATGGTTTGGGGAGTTGATGTTT<br>GCTGCTTTGGAATTGTAGGACTCTCGGCAATGGATATCTTGGCTCTTGCATCGATGAAGAGCGCA<br>GCGAAATGCGATACGTGGTGCGAATTGCAGAATCCCGTGAACCATCGAGTTTTTGAACGCAAGTT<br>GCGCCTGAGGCCACCTGGCCAAGGGCACGTCCACCTGGGCGTCAAGCATTAAATCGCTCTACTAG<br>ACGCCAGACCTTCAATGTTATGGGGCGGCGTATTGGATGCGGAGAGTGGCCCGTCATGCGTGACG<br>TGTGGCGGGTTGAAGGGAAGGATGATTTTCTCTTGGCAATGATCGATTAATTGGTGGGATGAAGC<br>CCCAGTAACCTCCTAATCATCAGCTTGCTTTGAGGAACAGCATATCCTAGGCCAACCCAACCAGA<br>GTTTTGGAAGCAAATCGAAATGCGACCCCAGGATGGGCGGGGTGACCCGCTGAGTTTAAGCATAT<br>CAATAAGCGGAGG |
| ***matK*** | |
| 01 | TCAATCTTTCCTTTTTTAGAAGATAAATTCTTGCATTTAAATTCTGTGTTAGATCTACTAATACCCC<br>ATCCCATCCATCTTGAAATCTTAGTTCGAATCCTTCAATACTGGATCAAAGATGTTCCTTCTTTGC<br>ATTTGTTGCGATTGATTTTCCACGAATATCATAATTTAAATAGTTTCATTACTTCAAAGAAAGARA<br>TTTACGTCTTTTCAAAAATAAAAAAAAGATTTTTTTTATTTTTACATAATTCTTATGTATATGAATG<br>CGAATATATATTCCTGTTTCTTCGCGAACAGTCTTCTTATTTACGATCAACATCTTTTGAAGTCTT<br>TCTTGAGCGAACACATTTCTATAGAAAAATAGAATATTTTAGAGTAATATATTGTAATTCTTTTAA<br>GAGGATTCTATGGTTTATCAAAGAACCTTTCATACATTATGTTCGATATCAAGGAAAAGCAATTCT<br>GGCTTCAAAGCTAACTCTAATTCTGATGAATAAATGGAAATTTTATCTTGTTCATTTTTGGCAATT<br>TTATTTTCACTTTTGGTCTCAACCTTATAGGATCCTTATAAAGGAATTACCCAACTATTGCTTCTT<br>TTTTCTAGGGTATTTTTTAAGTGTACTAAAAAAGACTTTGGTAGTAAGAAATAAAATGCTGGAGAA<br>TTTATATTTAATAAATACTCTGGTTAATAAATTCGATACCATAGCCCCAGTTATTTTTCTTATTGGA<br>GCATTGTCAAAAACTCSATTTTGTACAGTATTCGGRCATCCCATTAGCAAACCAATTTGGACTGAT<br>TTATCGGATTCTGATATTATTGATCGATTTTGTCGTATATGTAGA |

**Table C.1** DNA Sequences ITS, *matK* and *rbcL* of plant (Continued).

| Region/No. | DNA Sequence |
|---|---|
| 02 | TCAATCTTTCCTTTTTTAGAAGATAAATTCTTGCATTTAAATTCTGTGTTAGATCTACTAATACCCC |
| | ATCCCATCCATCTTGAAATCTTAGTTCGAATCCTTCAATACTGGATCAAAGATGTTCCTTCTTTGC |
| | ATTTGTTGCGATTGATTTTCCACGAATATCATAATTTAAATAGTTTCATTACTTCAAAGAAAGACA |
| | TTTACGTCTTTTCAAAAATAAAAAAAAAGATTTTTTTTATTTTTACATAATTCTTATGTATATGAATG |
| | CGAATATATATTCCTGTTTCTTCGCGAACAGTCTTCTTATTTACGATCAACGTCTTTTGAAGTCTT |
| | TCTTGAGCGAACACATTTCTATAGAAAAATAGAATATTTTAGAGTAATATATTGTAATTCTTTTAA |
| | GAGGATTCTATGGTTTATCAAAGAACCTTTCATACATTATGTTCGATATCAAGGAAAAGCAATTCT |
| | GGCTTCAAAGCTAACTCTAATTCTGATGAATAAATGGAAATTTTATCTTGTTCATTTTTGGCAATT |
| | TTATTTTCACTTTTGGTCTCAACCTTATAGGATCCTTATAAAGGAATTACCCAACTATTGCTTCTT |
| | TTTTCTAGGGTATTTTTTAAGTGTACTAAAAAAGACTTTGGTAGTAAGAAATAAAATGCTGGAGAA |
| | TTTATATTTAATAAATACTCTGGTTAATAAATTAGATACCATAGCCCCAGTTATTTTTCTTATTGGA |
| | GCATTGTCAAAAACTCAATTTTGTACTGTATTGGGCCATCCCATTAGCAAACCAATTTGGACTGAT |
| | TTATCGGATTCTGATATTATTGATCGATTTTGTCGTATATGTAGA |
| 03 | TCAATCTTTCCTTTTTTAGAAGATAAATTCTTGCATTTAAATTATGTGTTAGATCTACTAATACCCC |
| | ATCCCATCCATCTTGAAATCTTAGTTCGAATCCTTCAATACTGGATCAAAGATGTTCCTTCTTTGC |
| | ATTTGTTGCGATTGATTTTCCACGAATATCATAATTTAAATAGTTTCATTACTTCAAAGAAAGACA |
| | TTTACGTCTTTTCAAAAATAAAAAAAAGATTTTTTTTATTTTTACATAATTCTTATGTATATGAATG |
| | CGAATATATTTTCCTGTTTCTTCGCGAACAGTCTTCTTATTTACGATCAACGTCTTTTGAAGTCTT |
| | TCTTGAGCGAACACTTTTCTATAGAAAAATAGAATATTTTAGAGTAATATATTGTAATTCTTTTAA |
| | GAGGATTCTATGGTTTATCAAAGAACCTTTCATACTTTATGTTCGATATCGAGGAAAAGCAATTCT |
| | GGCTTCAAAGCTAACTCTAATTCTGATGAATAAATGGAAATTTTATCTTGTTCATTTTTGGCAATT |
| | TTATTTTCACTTTTGGTCTCAACCTTATAGGATCCTTATAAAGGAATTACCCAACTATTACTTCTTT |
| | TTTCTAGGGTATTTTTTAAGTGTACTAAAAAAGACTTTGGTAGTAAGAAATAAAATGCTGGAGAAT |
| | TTATATTTAATAAATACTCTGGTTAATAAATTCGATACCATAGCCCCAGTTATTTTTCTTATTGGA |
| | GCATTGTCAAAAACTCAATTTTGTACKGTAATCGGSCATCCCATTAGCAAACCAATTTGGACTGAT |
| | TTATCGGATTCTGATATTATTGATCGATTTTGTCGTATATGTAGA |
| 04 | TCAATCTTTCCTTTTTTAGAAGATAAATTCTTGCATTTAAATTCTGTGTTAGATCTACTAATACCCC |
| | ATCCCATCCATCTTGAAATCTTAGTTCGAATCCTTCAATACTGGATCAAAGATGTTCCTTCTTTGC |
| | ATTTGTTGCGATTGATTTTCCACGAATATCATAATTTAAATAGTTTCATTACTTCAAAGAAAGACA |
| | TTTACGTCTTTTCAAAAATAAAAAAAAAGATTTTTTTTATTTTTACATAATTCTTATGTATATGAATG |
| | CGAATATATATTCCTGTTTCTTCGCGAACAGTCTTCTTATTTACGATCAACGTCTTTTGAAGTCTT |
| | TCTTGAGCGAACACATTTCTATAGAAAAATAGAATATTTTAGAGTAATATATTGTAATTCTTTTAA |
| | GAGGATTCTATGGTTTATCAAAGAACCTTTCATACATTATGTTCGATATCGAGGAAAAGCAATTCT |
| | GGCTTCAAAGCTAACTCTAATTCTGATGAATAAATGGAAATTTTATCTTGTTCATTTTTGGCAATT |
| | TTATTTTCACTTTTGGTCTCAACCTTATAGGATCCTTATAAAGGAATTACCCAACTATTACTTCTTT |
| | TTTCTAGGGTATTTTTTAAGTGTACTAAAAAAGACTTTGGTAGTAAGAAATAAAATGCTGGAGAAT |
| | TTATATTTAATAAATACTCTGGTTAATAAATTCGATACCATAGCCCCAGTTATTTTTCTTATTGGA |
| | GCATTGTCAAAAACTCAATTTTGTACTGTATTGGGCCATCCCATTAGCAAACCAATTTGGACTGAT |
| | TTATCGGATTCTGATATTATTGATCGATTTTGTCGTATATGTAGA |

**Table C.1** DNA Sequences ITS, *matK* and *rbcL* of plant (Continued).

| Region/No. | DNA Sequence |
|---|---|
| 05 | TCAATTTTTCCTTTTTTAGAAGATAAATTCTTACATTTAAATTATGTGTTAGATCTACTAATACCCC<br>ATCCCATCCATCTTGAAATCTTAGTTCGAATCCTTCAATGCTGGATCAAAGATGTTCCTTCTTTGC<br>ATTTGTTGCGATTGATTTTCCACGAATATCATAATTTAAATAGTTTCATTACTTCAAAGAAAGACA<br>TTTACGTCTTTTCAAAAATAAATAAAAGATTTTTTTTATTTTTACATAATTCTTATGTATATGAATG<br>CGAATATATATTCCTGTTTCTTCGCGAACAGTCTTCTTATTTACGATCAACGTCTTTTGAAGTCTT<br>TCTTGAGCGAACACATTTCTATAGAAAAATAGAATATTTTAGAGTAATGTATTGTAATTCTTTTAA<br>GAGGATTCTATGGTTTATCAAAGAACCTTTCATACATTATGTTCGATATCAAGGAAAAGCAATTCT<br>GGCTTCAAAGGTAACTCTAATTCTGATGAATAAATGGAAATTTTATCTTGTTCATTTTTGGCAATT<br>TTATTTTCACTTTTGGTCTCAACCTTATAGGATCCATATAAAGGAATTACCCAACTATTGCTTCTT<br>TTTTATAGGGTATTTTTTAAGTGTACTAAAAAAGACTTTGGTAGTAAGAAATAAAATGCTGGAGAA<br>TTTATATTTAATAAATACGCTGGTTAAGAAATTAGATACCATAGCCCCAGTTATTTTTCTTATKGG<br>AGCATTGTCAAAAACTCAATTTTGTACTGTATTGGGCCATCCCATTAGCAAACCAATTTGGACTGA<br>TTTATCGGATTCTGATATTATTGATCGATTTTGTCGTATATGTAGA |
| 06 | TCAATCTTTCCTTTTTTAGAAGATAAATTCTTGCATTTAAATTCTGTGTTAGATCTACTAATACCCC<br>ATCCCATCCATCTTGAAATCTTAGTTCGAATCCTTCAATATTGGATCAAAGATGTTCCTTCTTTGC<br>ATTTGTTGCGATTGATTTTCCACGAATATCATAATTTAAATAGTTTCATTACTTCAAAGAAAGACA<br>TTTACGTCTTTTCAAAAATAAAAAAAATCTTTTTTTTATTTTTACATAATTCTTATGTATATGAATG<br>CGAATATATATTCCTGTTTCTTCGCGAACAGTCTTCTTATTTACGATCAACGTCTTTTGAAGTCTT<br>TCTTGAGCGAACACATTTCTATAGAAAAATAGAATATTTTAGAGTAATATATTGTAATTCTTTTAA<br>GAGGATTCTATGGTTTATCAAAGAACCTTTCATACATTATGTTCGATATCGAGGAAAAGCAATTCT<br>GGCTTCAAAGCTAACTCTAATTCTGATGAATAAATGGAAATTTTATCTTGTTCATTTTTGGCAATT<br>TTATTTTCACTTTTGGTCTCAACCTTATAGGATCCTTATAAAGGAATTACCCAACTATTGCTTCTT<br>TTTTCTAGGGTATTTTTTAAGTGTACTAAAAAAGACTTTGGTAGTAAGAAATAAAATGCTGGAGAA<br>TTTATATTTAATAAATACTCTGGTTAATAAATTCGATACCATAGCCCCAGTTATTTTTCTTATTGGA<br>GCGTTGTCAAAAACTCAATTTTGTACTGTATTGGGCCATCCCATTAGCAAACCAATTTGGACTGAT<br>TTATCGGATTCTGATATTATTGATCGATTTTGTCGTATATGTAGA |
| 07 | TCAATCTTTCCTTTTTTAGAAGATAAATTCTTGCATTTAAATTCTGTGTTAGATCTACTAATACCCC<br>ATCCCATCCATCTTGAAATCTTAGTTCGAATCCTTCAATACTGGATCAAAGATGTTCCTTCTTTGC<br>ATTTGTTGCGATTGATTTTCCACGAATATCATAATTTAAATAGTTTCATTACTTCAAAGAAAGACA<br>TTTACGTCTTTTCAAAAATAAAAAAAAAGATTTTTTTTATTTTTACATAATTCTTATGTATATGAATG<br>CGAATATATATTCCTGTTTCTTCGCGAACAGTCTTCTTATTTACGATCAACGTCTTTTGAAGTCTT<br>TCTTGAGCGAACACATTTCTATAGAAAAATAGAATATTTTAGAGTAATATATTGTAATTCTTTTAA<br>GAGGATTCTATGGTTTATCAAAGAACCTTTCATACATTATGTTCGATATCGAGGAAAAGCAATTCT<br>GGCTTCAAAGCTAACTCTAATTCTGATGAATAAATGGAAATTTTATCTTGTTCATTTTTGGCAATT<br>TTATTTTCACTTTTGGTCTCAACCTTATAGGATCCTTATAAAGGAATTACCCAACTATTGCTTCTT<br>TTTTCTAGGGTATTTTTTAAGTGTACTAAAAAAGACTTTGGTAGTAAGAAATAAAATGCTGGAGAA<br>TTTATATTTAATAAATACTTTGGTTAATAAATTCGATACCATAGCCCCAGTTATTTTTCTTATTGGA<br>GCATTGTCAAAAACTCAATTTTGTACTGTATTGGGCCATCCCATTAGCAAACCAATTTGGACTGAT<br>TTATCGGATTCTGATATTATTGATCGATTTTGTCGTATATGTAGA |

**Table C.1** DNA Sequences ITS, *matK* and *rbcL* of plant (Continued).

| Region/No. | DNA Sequence |
|---|---|
| *rbcL* | |
| 01 | GAGCTATTATCTCGTCCCTCATTACGAGCTTGATCACTTGCTTCTGAAGCCACCCGATTAGCTACT<br>GCTCCGGGTGCATCTCCCCAAGGGTGCCCTAAAGTTCCTCCACCATATTGTAGTACGGAATCATC<br>CCCAAAGATATCGGTTAGGATAGGCATATGCAAAACATGAATACCCCCTGAAGCCACGGGAAGAA<br>CACCTGGCATAGAGACCCAGTCTTGAGTGAAAAAAATACCGCGACTTCGATCTTTTTCAATAAAAT<br>CATCACGTAATATATCAACAAAACCCAAAGTCATCTCACGTTCCCCCTCCAGTTTACCTACTACTG<br>TACCAGCGTGAATATGATCTCCACCAGACATACGTGATGCTTTAGATAGTACACGAAAATGCATA<br>CCAGGATTTTTCTGTCTATCAAGAACTGCATGCATTGCGCGATGGATGTCAAGAAGTAGACCGTT<br>GTCGCGGCAATAATGAGCCAAGGTAGTATTTGCGGTGAATCCCCCGGTTAAGTAGTCATGCATTG<br>ACGATAGGAACTCCCAATTCTCTGGCAAATACCGCTCTTTTGATCATTTCTTCACATGTACCCGCA<br>GTTGCATTCAAGTGATGCCCTTTGATTTCACCCGTTTCAGCTTGTGCTTTATAAAGAGATTCGGCA<br>CAAAATAAGAAACGATCTCTCCAACGCATAAATGGTTGTGAGTTCACGTTTTCATCATCACTTAGT<br>GAAATCAAGTCCACCCCGTAGACATTCATAAACTGCTCTACCACAGTTTTTTGCGGATAACCCCAA<br>TTTTGGGTTAATAGTACATCCCATTAGGGACGACCATGCTTGTTCAATTGATCTCTTTCAACTAGG<br>AAGCATGGAGGCGGACTTCGGAAGGTTTTAGGATAAGCAGGGGGAATTCGCAGATCTTCCAGACG<br>TAGAGCTCGCAGAGCTTTGAAACCGAGAACATGACCCACA |
| 02 | GAGCAAGTATCACGTCCCTCATTACGAGCTTGTACACATGCTTCTGAAGCCACCCGATTAGCTAC<br>TGCACCGGGTGCATTTCCCCAAGGGTGCCCTAAAGTTCCTCCACCAAACTGTAGTACGGAATCAT<br>CCCCAAAGATTTCGGTTAGGGCAGGCATATGCCAAACATGAATACCCCCTGAAGCCACGGGCAGA<br>ACACCTGGCATAGAGACCCAGTCTTGAGTGAAAAAAATACCGCGACTTCGATCTTTTTCAATAAA<br>ATCATCACGTAATAAATCAACAAAACCCAAAGTCATCTCACGTTCCCCCTCCAGTTTACCTACTAC<br>TGTACCAGCGTGAATATGATCTCCACCAGACATACGTAATGCTTTAGCTAGTACACGAAAATGCA<br>TACCATGATTTTTCTGTCTATCAAGAACTGCATGCATTGCGCGATGGATGTGAAGAAGTAGACCG<br>TTGTCGCGGCAATAATGAGCCAAGGTAGTATTTGCGGTGAATCCCCCGGTTAAGTAGTCATGCAT<br>TACGATAGGAACTCCCAATTCTCTGGCAAATACCGCTCTTTTGATCATTTCTTCACATGTACCCGC<br>AGTTGCATTCAAGTAATGCCCTTTGATTTCACCCGTTTCAGCTTGTGCTTTATAAAGAGATTCGGC<br>ACAAAATAAGAAACGATCTCTCCAACGCATAAATGGTTGTGAGTTCACGTTTTCATCATCCTTAGT<br>AAAATCAAGTCCACCCCGTAGACATTCATAAACTGCTCTACCATAGTTTTTTGCGGATAATCCCAA<br>TTTTGGTTTAATAGTACATCCCAATAGGGGACGACCATACTTGTTCAATTTATCTCTTTCAACTTG<br>GATGCCATGAGGCGGACCTTGGAAAGTTTTGGAATAAGCAGGGGGAATTCGCAGATCTTCCAGAC<br>GTAGAGCTCGCAGAGCTTTGAAACCAAAAACATTACCCACAAT |

**Table C.1** DNA Sequences ITS, *matK* and *rbcL* of plant (Continued).

| Region/No. | DNA Sequence |
|---|---|
| *rbcL* | |
| 03 | GAGCAAGTATCACGTCCCTCATTACGAGCTTGTACACATGCTTCTGAAGCCACCCGATTAGCTAC<br>TGCACCGGGTGCATTTCCCCAAGGGTGCCCTAAAGTTCCTCCACCAAACTGTAGTACGGAATCAT<br>CCCCAAAGATTTCGGTTAGGGCAGGCATATGCCAAACATGAATACCCCCTGAAGCCACGGGCAGA<br>ACACCTGGCATAGAGACCCAGTCTTGAGTGAAAAAAATACCGCGACTTCGATCTTTTTCAATAAA<br>ATCATCACGTAATAAATCAACAAAACCCAAAGTCATCTCACGTTCCCCCTCCAGTTTACCTACTAC<br>TGTACCAGCGTGAATATGATCTCCACCAGACATACGTTATGTTTTAGCTAGTACACGAAAATGCAT<br>ACCATGATTTTTCTGTCTATCAAGAACTGCATGCATTGCGCGATGGATGTGAAGAAGTAGACCGT<br>TGTCGCGGCAATAATGAGCCAAGGTAGTATTTGCGGTGAATCCCCCGGTTAAGTAGTCATGCATT<br>ACGATAGGAACTCCCAATTCTCTGGCAAATACCGCTCTTTTTATCATTTCTTCACATGTACCCGCA<br>GTTGCATTCAAGTAATGCCCTTTGATTTCACCCGTTTCAGCTTGTGCTTTATAAAGAGATTCGGCA<br>CATAATAAGAAACGATCTCTCCAACGCATAATTGGTTGTGAGTTCACGTTTTCATCATCCTTAGTA<br>GAAATCAAGTCCACCCCGTAGACTTTCATAAACTGCTCTACCATAGTTTTTTGCGGATAATCCCAA<br>TTTTGGGTTAAATAGTACATCCCAATAGGGGACGACCATACTTGTCCAATTTATCTCTTTCAACTT<br>GGATGCCATGAGGCGGACCTTGGAAAGTTTTGGAATAAGCGGGGGGAATTCGCAGATCTTCCAGA<br>CGTAGAGTTCGCAGAGCTTTGAAACCAAGAACATTACCCACA |
| 04 | GAGCAAGTATCACGTCCCTCATTACGAGCTTGTACACATGCTTCTGAAGCCACCCGATTAGCTAC<br>TGCACCGGGTGTATTTCCCCAAGGTCGCCCTAAAGGTCCTCCACCAAACTGTAGTACGGAATCAT<br>CCCCAAAGATTTCGGTTAGGGCAGGCATATCCGCCACATGAATACCCCCTGAAGCCACGGGCAGA<br>ACACCTGGCATAGAGACCCAGTATTGAGTGAAAAAAATACCGCGACTTCGATCTTTTTCAATAAAA<br>TCATCACGTAATAAATCAACAAAACCCAAAGTCATCTCACGTTCCCCCTCCAGTTTACCTACTACT<br>GTACCAGCGTGAATATGATCTCCACCAGACATACGTAATGCTTTAGCTAGTACACGAAAATGCAT<br>ACCATGATTTTTCTGTCTATCAAGAACTGCATGCATTGCGCGATGGATGTGAAGAAGTAGACCGT<br>TGTCGCGGCAATAATGAGCCAAGGTAGTATTTGCGGTGAATCCCCCGGTTAAGTAGTCATGCATT<br>ACGATAGGAACTCCCAATTCTCTGGCAAATACCGCTCTTTTTATCATTTCTTCACATGTACCCGCA<br>GTTGCATTCAAGTAATGCCCTTTGATTTCACCCGTTTCAGCTTGTGCTTTATAAAGAGATTCGGCA<br>CATAATAAGAAACGATCTCTCCAACGCATAAATGGTTGTGAGTTCACGTTTTTCATCATCCTTAGT<br>AGAAATCAAGTCCACCCCGTAGACATTCATAAAACTGCTCTACCACAGTTTTTTGCGGATAATCCC<br>AATTTTGGGTTTAAATAGTACATCCCCATTAGGGGGACGACCCATACCTTGTTCCAATTTAATCTT<br>TTTTCAACTTGGAATGCCATGAAGCGGGACCTTGGGAAAAGTTTTTGGAAATAAGCAGGGGGGAA<br>ATTCCGCAGAATCTTCCCAGAACGTAGGAGTTCGCAGAACTT |

**Table C.1** DNA Sequences ITS, *matK* and *rbcL* of plant (Continued).

| Region/No. | DNA Sequence |
|---|---|
| ***rbcL*** | |
| 05 | TAGTCAAGATCACGTCCCTCATTACGAGCTTGTACACATGCTTCTGATGTCACCCGATTAGCAACT<br>GCACCGGGTGATTCTCCCCAAGGTTGCCCTAAAGTTCCTCCACCAAACTGTAGTACGGAATCATC<br>CCCAAAGATTTCGGTTAGGGCAGGCATATGCCAAACATGAATACCCCCTCGAAGCCACGGGCAGA<br>ACACCTGGCATAGAGACCCAGTCTTGAGTGAAAAAAATACCGCGACTTCGATCTTTTTCAATAAA<br>ATCATCACGTAATAAATCAACAAAACCCAAAGTCATCTCACGTTCCCCCTCCAGTTTACCTACTAC<br>TGTACCAGCGTGAATATGATCTCCACCAGACATACGTAATGCTTTAGCTAGTACACGAAAATGCA<br>TACCATGATTTTTCTGTCTGTCAAGAACTGCATGCATTGCGCGATGGATGTGAAGAAGTAGACCG<br>TTGTCGCGGCAATAATGAGCCAAGGTAGTATTTGCGGTGAATCCCCCGGTTAAGTAGTCATGCAT<br>TACGATAGGAACTCCCAATTCTCTGGCAAATACCGCTCTTTTGATCATTTCTTCACATGTACCCGC<br>AGTTGCATTCAAGTCATGCCCTTTGATTTCACCCGTTTCAGCTTGTGCTTTATAAAAGAGATTCGG<br>CACATAAATAAAGAAAACGATCTCTTCCAACGCATAAATTGGTTTGTGAAGTTCCACGTTTTTCAT<br>CATCCCTTAGGGAGAAATCCAAGTCCCACCCCCGTAGAACATTTCATAAAACTGGCTCTACCCGC<br>AGTTTTTTTGCGGGATAGATCCCCAATTTTTGGGTTTAAATAAGGGACCTTCCCCATTAGGGGGA<br>CGACCCATACCTTTGTTCCAAATTGTATTCTTTTTTTCAAACTTGGGAATGCCCATGGAGGGCGGA<br>ACCTTTGGGAAAAGTTTTTGGAAAAATAAGCCAGGGGGGAAAT |
| 06 | GAGTAAGATCACTCTCCCTCATTACGAGCTTGTACACCCGCTTATTTCGCCTCCCGATTAGCTACT<br>GCACCGGGTGAATTTCCCCAAGCCTCCCCCAAAATACCTCCACCAAACTCTAGTAAGCCATCATC<br>CCCTCAGAGTTCGGTAAGGGCAATGATATCCTCGCTGTGTCTGCCCTCGTAAGACAGGTGGAGAT<br>CATCTGGCTTAGAGAACCAGTCTTGAACGAAATATATACCGCGACTTCAATCTTTTTCAATAAAAT<br>CATAGCGTAATTGCCAAAGAAACCCATAGCATCTCATGTTCCCCCTCCAGTTTACCTACTACTGTA<br>CCAGCGTGAATATGATCTCCACCAGACATACGTAATGCTTTAGCTAGTACACGAAAATGCATACC<br>ATGATTTTTCTGTCTATCAAGAACTGCATGCATTGCGCGATGGATGTGAAGAAGTAGACCGTTGT<br>CGCGGCAATAATGAGCCAAGGTAGTATTTGCGGTGAATCCCCCGGTTAAGTAGTCATGCATTGAC<br>GATAGGAACTCCCAATTCTCTGGCAAATACCGCTCTTTTTATCATTTCTTCACATGTACCCGCAGT<br>TGCATTCAAGTCATGCCCTTTGATTTCACCCGTTTCAGCTTGTGCTTTATAAAAGAGATTCGGCAC<br>AAATTAAGAAACGATCTCTCCGACGCATAAATGGTTGTGAGTTCCACGTTTTCATCATCACTTAGT<br>GAAATCAAGTCCACCCCCGTAGACATCCATAAACTGCTCTACCATCAGTTATTTGCCGGATGATC<br>CCAATTTTGGCTTAATAGTACATCCCATTAGGGGACGACCATACTTGTTCACTTATCTTCTATCAA<br>CTTGGGATGCCATGGAGCGGACCTTCGGGAAAGCTCTTCGCAATGAGCAGGGGGAATTCGCAGAT<br>CTTCCAGACGTAGAGCTCCGCAAGAGCTTTGGAACTAGA |

**Table C.1** DNA Sequences ITS, *matK* and *rbcL* of plant (Continued).

| Region/No. | DNA Sequence |
|---|---|
| 07 | GAGCAGGTATCACGTCCCTCATTACGAGCTTGTACACATGCTTCTGAAGCCACCCGATTAGCTAC |
| | TGCACCGGGTGGTTTCCCCTAAGGGTGCCCTAAAGTTCCTCCACCAAACTGTAGTACGGAATCAT |
| | CCCCAAAGATTTCGGTTAGGGCAGGCATATGCTAAACATGAATACCCCCTGAAGCCACGGGCAGA |
| | ACACCTGGCATAGAGACCCAGTCTTGAGTGAAAAAAATACCGCGACTTCGATCTTTTTCAATAAA |
| | ATCATCACGTAATAAATCAACAAAACCCAAAGTCATCTCACGTTCCCCCTCCAGTTTACCTACTAC |
| | TGTACCAGCGTGAATATGATCTCCACCAGACATACGTAATGCTTTAGCTAGTACACGAAAATGCA |
| | TACCATGATTTTTCTGTCTATCAAGAACTGCATGCATTGCGCGATGGATGTCAAGAAGTAGACCG |
| | TTGTCGCGGCAATAATGAGCCAAGGTAGAATTTGCGGTGAATCCCCCGGTTAAGTAGTCATGCAT |
| | TACGATAGGAACTCCCAATTCTCTGGCAAATACCGCTCTTTTTATCATTTCTTCACATGTACCCGC |
| | AGTTGCATTCAAGTCATGCCCTTTGATTTCACCCGTTTCAGCTTGTGCTTTATAAAGAGATTCGGC |
| | ACAGAATAAGAAACGATCTCTCCAACGCATAAATGGTTGTGAGTTCACGTTTTCATCATCCTTAGG |
| | AGAATCAAGTCCACCCCGTAGACTTTCATAAACTGCTCTACCACAGTTTTTTGCGGATAATCCCAA |
| | TTTTGGGTTAAAAAGTACATCCCATTAGGCGACGACCATACTTGTCCAATTTATCTCTTTAAACTT |
| | GAATGCCATGAGGCGGACTTGGGAAAGTTTTGGAATGAGCAGGGGGAATTCGCAGATCTTCCAAA |
| | CGTAGAGCTCGCAGACTTTTGAAAACCAAGAACTTTACCCACA |

**Table C.2** DNA Sequencing of fungal.

| Isolate | DNA Sequence |
|---|---|
| I03 | GCAAAAAAGGGAAGAACGGAGTCTGTACACTTGCTCCTGATGCGAACGCA |
| | GAGGTCATGTTATGCGCTCTTGACATTCCATACTTGCACGAGGGTTCGTG |
| | CAAGACAGTCTACCGTCATCTGTGAGAATTGCGACTCCGGCTGGTTGCAG |
| | CTTTGAGAGCGTGGTGACTAGGCTACAGGATGAGTGAGTCTGAGGATTTG |
| | AGGAAACAACCGAGAGTAGCTTTCACGTTTCCGAGGTCCAAGGGGACGTC |
| | CTGACGATTGTTCAAGCCACAGAGCGCGAATCGTGACCGATGTGCACCTA |
| | GCAGCGCTCACCATGGCATTCTTTGGCTGGGACTCGACTGTCGACGTGTC |
| | TGTGACCCTGGTTGAATGCAGAGCCTGCTTCCGCCGCCTTGGCCTATGGA |
| | TGTACAAACCAAAAGCCAACGGAGATGTCTCTGTCTACACATCCCTCGAC |
| | GCTGCTACCGAGCACATGGACTATTGTCCCTGGATTGACCGGACCGCCCA |
| | GAGTGGAACTGGCAAAGCGAATGAGAAGATAGCCAATCTACGAAGTGGAT |
| | GGGAAATTGTTGCCGAAGCCGTGAAGGTGAAGCACCGGAGACGACTACGC |
| | TCTACTGCATCGGTGGATACCCTCCGCACCGAACCAAGCACTCCCGCGGA |
| | GACACCAAGTGATGAAGATGCAGGGAACGAAGAGACGAAGAAGGCGGCTG |
| | ACCGCGAGTGGTGGGCTAAGATTCGACGCATGAGGCATGTCTTGACGCAC |
| | AAGTCGCCGCGGCGTAAGCCTGTGCTTCCCCAATGATTTCTTTGAGCGTT |
| | CTTCTTCATGATATCTGATAATCTCGTTTCTCTCATACATGTGCAAGGCG |
| | TTGCAGGTAAAGTGGCATAT |

**Table C.2** DNA Sequencing of fungal (Continued).

| Isolate | DNA Sequence |
|---|---|
| I04 | GCGAGTAGCGGAGCACCGTGTCGTTCCTCCTCCAGCACGCTTCTTCGGTG<br>TCTAGAACGGCCACGGTGGCGTTAGGTGCGCCGCCATTAATGACGCATCT<br>CTGTAATTCCGCAATTAATTTTTGCTTGGGGTGAAAGCTATGTGCCCGAC<br>CCAACCTATTTCCGTACAATTACATTAAATTTCGGTTGATTCTTGTTCAG<br>GTTGCAAATTACTGTCGGGACCGGCTCCACCCTTTGTTGGGGGAGGTGCT<br>CAAGAAGGAGGGGTCGTCCGGGGCCGCTCTGGGTTAGTATGAGTGAATTT<br>CAGAAAAAAAATGGGAAACGGCTTTTGGGTCCTCTTTTTCGAGGGGGAAGA<br>AGGAGGGGGGGGCGAATCCGAATTGGGAAAAACCTGGAGCACCCAGAACG<br>GGGGAAACAACGCCCGTTCCCCGTTCCCCCAAAAGGTGGGGGGGGGGGGG<br>GGGGCCGGGGTGGGGGGGGGGCAGAAACACAATCAGCGGCTGAAGATTTG<br>ACGCGGGTGGGGTGGTTATCAATGCTGATGGAGGGGGTTGGGCGTGTTGG<br>GTGGTGTAGGGCTATTTTGTGAGAATACCCGATGGAATAAGCATATCATA<br>CGCGGAGCCGGAGTAAGCGGCGGTGCGAGAAGTGAAATGCACCGCC |
| I20 | GTTTCAACGGACGTCAGTACATGATCGAGGCAACCTGGTAAATAGATTGA<br>TGACGCCGGCTGGCCCCGGCCGCCTTTATCGAGCGGGTGACAAAGCCCCA<br>TACGCTCGAGGACCGGACACGTGCCGCCGCTCCCTTTCGGGCCCGTCCCC<br>CGTGAGTCTGAAACTAAATCAGAAACAGTTTTTAAAGGGAATCAATAGGC<br>TTCGGCAGGATGCCCCCGGAATGCAAGGGGCGCATTGTGTGAACAAAAAC<br>TCTAAGTTTATCTGATTTTCGCAATTCACATATTTAATCGAGGTTCATTC<br>CTTTTTTCATGCATCCCGAAGCGAGAGACCGTTTTTCAAGCTTTGGATTG<br>TTTTATAATTAGAACCCAGACTGCATCATTCTAAGAATGAAGTTCAGGGG<br>CCCCGGCCGGCTCGCCCCCGGAAGGGGCTCCCCCGCCCAGCCAAATTTGT<br>TTAGAATTAAGGGGGGGGGGGTGGGCCCCCGGGGCAGCCCGGCACTCGTA<br>ATTAATCTTCGGTAGCCCCCCTCGGGAAATTTTAA |

# APPENDIX D

# DATA OF SYMBIOTIC AND ASYMBIOTIC SEED GERMINATION AND PROTOCORM DEVELOPMENT OF *H. JANELLEHAYNEIANA* AND *H. RHODOCHEILA*

**Table D1** Data of seed germination and protocorm development of *H. janellehayneiana* on various fungal for 16 weeks.

| Isolate | Weeks | Stages | | | | | Total |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | |
| Control | 4 | 2.43 | 0 | 0 | 0 | 0 | 2.43 |
| | 6 | 2.88 | 0 | 0 | 0 | 0 | 2.88 |
| | 8 | 5.31 | 0.44 | 0 | 0 | 0 | 5.75 |
| | 10 | 5.31 | 0.44 | 0 | 0 | 0 | 5.75 |
| | 12 | 5.31 | 0.44 | 0 | 0 | 0 | 5.75 |
| | 14 | 5.53 | 0.44 | 0.22 | 0 | 0 | 6.19 |
| | 16 | 5.31 | 0.66 | 0.22 | 0 | 0 | 6.19 |
| I03 | 4 | 3.53 | 0 | 0 | 0 | 0 | 3.53 |
| | 6 | 4.42 | 0 | 0 | 0 | 0 | 4.42 |
| | 8 | 6.4 | 0.44 | 0 | 0 | 0 | 6.84 |
| | 10 | 6.4 | 0.44 | 0 | 0 | 0 | 6.84 |
| | 12 | 8.83 | 0.44 | 0 | 0 | 0 | 9.27 |
| | 14 | 9.93 | 0.44 | 0 | 0 | 0 | 10.38 |
| | 16 | 10.15 | 0.88 | 0 | 0 | 0 | 11.04 |
| I08 | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 6 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 8 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 10 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 12 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 14 | 3.51 | 0.23 | 0 | 0 | 0 | 3.75 |
| | 16 | 3.75 | 0.47 | 0 | 0 | 0 | 4.22 |

**Table D1** Data of seed germination and protocorm development of *H . janellehayneiana* on various fungal for 16 weeks (Continued).

| Isolate | Weeks | Stages | | | | | Total |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | |
| I20 | 4 | 4.46 | 0.23 | 0 | 0 | 0 | 4.69 |
| | 6 | 8.45 | 0.47 | 0 | 0 | 0 | 8.92 |
| | 8 | 9.15 | 0.47 | 0 | 0 | 0 | 9.62 |
| | 10 | 9.39 | 0.47 | 0 | 0 | 0 | 9.86 |
| | 12 | 10.56 | 0.7 | 0 | 0 | 0 | 11.27 |
| | 14 | 11.97 | 1.17 | 0.47 | 0 | 0 | 13.62 |
| | 16 | 10.8 | 2.35 | 0.94 | 0 | 0 | 14.08 |

**Table D2** Data of seed germination and protocorm development of *H. janellehayneiana* on various basal media for 16 weeks.

| Basal media | Weeks | Stages | | | | | Total |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | |
| VW | 4 | 1.12 | 0 | 0 | 0 | 0 | 1.12 |
| | 6 | 8.48 | 0.89 | 0 | 0 | 0 | 9.38 |
| | 8 | 8.26 | 1.34 | 0.22 | 0 | 0 | 9.82 |
| | 10 | 6.03 | 2.46 | 1.56 | 0.89 | 0 | 10.94 |
| | 12 | 6.25 | 2.46 | 1.56 | 0.89 | 0 | 11.16 |
| | 14 | 6.7 | 2.68 | 1.56 | 0.89 | 0 | 11.83 |
| | 16 | 6.7 | 2.68 | 1.56 | 0.89 | 0 | 11.83 |
| ½VW | 4 | 3.33 | 0 | 0 | 0 | 0 | 3.33 |
| | 6 | 13.11 | 2.67 | 0 | 0 | 0 | 15.78 |
| | 8 | 11.11 | 3.56 | 2.44 | 0 | 0 | 17.11 |
| | 10 | 10.22 | 3.56 | 2 | 1.33 | 0 | 17.11 |
| | 12 | 10.22 | 3.33 | 2 | 1.56 | 0 | 17.11 |
| | 14 | 12 | 3.11 | 2 | 2 | 0 | 19.11 |
| | 16 | 12 | 2.89 | 2 | 2.22 | 0 | 19.11 |
| MS | 4 | 0.67 | 0 | 0 | 0 | 0 | 0.67 |
| | 6 | 8.48 | 0.22 | 0 | 0 | 0 | 8.71 |
| | 8 | 11.61 | 0.45 | 0.22 | 0 | 0 | 12.28 |
| | 10 | 9.6 | 1.34 | 0.67 | 0.89 | 0 | 12.5 |
| | 12 | 9.6 | 1.34 | 0.67 | 0.89 | 0 | 12.5 |
| | 14 | 9.6 | 1.34 | 0.67 | 0.89 | 0 | 12.5 |
| ½MS | 4 | 11.26 | 0.58 | 0 | 0 | 0 | 11.84 |
| | 6 | 8.16 | 1.94 | 0.58 | 0 | 0 | 10.68 |
| | 8 | 8.35 | 2.33 | 0.58 | 0.97 | 0 | 12.23 |
| | 10 | 7.57 | 2.91 | 0.97 | 1.17 | 0 | 12.62 |
| | 12 | 8.74 | 2.91 | 0.97 | 1.17 | 0 | 13.79 |
| | 14 | 8.74 | 2.52 | 1.36 | 1.17 | 0 | 13.79 |
| | 16 | 3.3 | 0 | 0 | 0 | 0 | 3.3 |

**Table D3** Data of seed germination and protocorm development of *H. rhodochila* on various basal media for 16 weeks.

| Basal media | Weeks | Stages | | | | | Total |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | |
| VW | 4 | 2.20 | 0 | 0 | 0 | 0 | 2.20 |
| | 6 | 4.99 | 0 | 0 | 0 | 0 | 4.99 |
| | 8 | 5.43 | 0.15 | 0 | 0 | 0 | 5.58 |
| | 10 | 9.25 | 0.44 | 0.15 | 0 | 0 | 9.84 |
| | 12 | 9.25 | 0.59 | 0.15 | 0 | 0 | 9.99 |
| | 14 | 9.10 | 0.59 | 0.29 | 0 | 0 | 9.99 |
| | 16 | 8.81 | 0.44 | 0.29 | 0.29 | 0.15 | 9.99 |
| ½VW | 4 | 0.41 | 0 | 0 | 0 | 0 | 0.41 |
| | 6 | 0.09 | 0.41 | 0 | 0 | 0 | 6.49 |
| | 8 | 7.71 | 0.61 | 0.41 | 0 | 0 | 8.72 |
| | 10 | 14.00 | 0.61 | 0.81 | 0 | 0 | 15.42 |
| | 12 | 14.00 | 1.01 | 0.81 | 0 | 0 | 15.82 |
| | 14 | 14.00 | 1.01 | 0.81 | 0 | 0 | 16.02 |
| | 16 | 13.79 | 0.20 | 1.22 | 0.41 | 0.20 | 15.82 |
| MS | 4 | 1.26 | 0 | 0 | 0 | 0 | 1.26 |
| | 6 | 2.10 | 0 | 0 | 0 | 0 | 2.10 |
| | 8 | 5.61 | 0 | 0 | 0 | 0 | 5.61 |
| | 10 | 8.98 | 0 | 0 | 0 | 0 | 8.98 |
| | 12 | 8.98 | 0.56 | 0 | 0 | 0 | 9.54 |
| | 14 | 8.98 | 0.56 | 0 | 0 | 0 | 9.54 |
| | 16 | 8.84 | 0.56 | 0 | 0 | 0 | 9.40 |
| ½MS | 4 | 0.50 | 0 | 0 | 0 | 0 | 0.50 |
| | 6 | 1.74 | 0.37 | 0 | 0 | 0 | 2.11 |
| | 8 | 3.11 | 0.37 | 0 | 0 | 0 | 3.48 |
| | 10 | 7.09 | 0.37 | 0.12 | 0 | 0 | 7.59 |
| | 12 | 7.09 | 0.12 | 0.50 | 0 | 0 | 7.71 |
| | 14 | 6.97 | 0.12 | 0.37 | 0.12 | 0 | 7.59 |
| | 16 | 6.72 | 0.25 | 0.37 | 0.25 | 0 | 7.59 |

**Table D4** Data of seed germination and protocorm development of *H. janellehayneiana* on various of BAP, GA, and TDZ on ½ VW media for 16 weeks.

| Media | Weeks | Stages | | | | | Total |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | |
| Control | 4 | 1.9 | 0 | 0 | 0 | 0 | 1.9 |
| | 6 | 2.85 | 0 | 0 | 0 | 0 | 2.85 |
| | 8 | 6.07 | 0.19 | 0 | 0 | 0 | 6.26 |
| | 10 | 5.88 | 0.57 | 0 | 0 | 0 | 6.45 |
| | 12 | 7.59 | 0.95 | 0 | 0 | 0 | 8.54 |
| | 14 | 7.17 | 1.32 | 0 | 0 | 0 | 8.49 |
| | 16 | 6.98 | 0.57 | 0.75 | 0.19 | 0 | 8.49 |
| 1 mg/L BAP | 4 | 2.88 | 0 | 0 | 0 | 0 | 2.88 |
| | 6 | 3.05 | 0.68 | 0 | 0 | 0 | 3.72 |
| | 8 | 11.17 | 0.85 | 0.34 | 0 | 0 | 12.35 |
| | 10 | 10.32 | 1.35 | 1.18 | 0 | 0 | 12.86 |
| | 12 | 10.15 | 1.52 | 1.02 | 0.17 | 0 | 12.86 |
| | 14 | 10.15 | 0.85 | 1.69 | 0.17 | 0 | 12.86 |
| | 16 | 9.81 | 1.02 | 1.69 | 0.34 | 0 | 12.86 |
| 3 mg/L BAP | 4 | 5.47 | 0 | 0 | 0 | 0 | 5.47 |
| | 6 | 2.45 | 0 | 0 | 0 | 0 | 2.45 |
| | 8 | 2.45 | 0.75 | 0 | 0 | 0 | 3.21 |
| | 10 | 7.92 | 0.94 | 0.94 | 0.38 | 0 | 10.19 |
| | 12 | 7.74 | 0.75 | 0.94 | 0.75 | 0 | 10.19 |
| | 14 | 7.74 | 0.75 | 0.94 | 0.75 | 0 | 10.19 |
| | 16 | 5.47 | 0 | 0 | 0 | 0 | 5.47 |
| 5 mg/L BAP | 4 | 3.2 | 0 | 0 | 0 | 0 | 3.2 |
| | 6 | 3.58 | 0 | 0 | 0 | 0 | 3.58 |
| | 8 | 10.17 | 0.19 | 0.19 | 0 | 0 | 10.36 |
| | 10 | 9.6 | 0.94 | 0 | 0.19 | 0 | 10.73 |
| | 12 | 9.42 | 0.94 | 0.19 | 0.19 | 0 | 10.73 |
| | 14 | 9.23 | 0.56 | 0.56 | 0.19 | 0 | 10.55 |
| | 16 | 9.23 | 0.56 | 0.56 | 0.19 | 0 | 10.55 |

**Table D4** Data of seed germination and protocorm development of *H. janellehayneiana* on various of BAP, GA, and TDZ on ½ VW media for 16 weeks (Continued).

| Media | Weeks | Stages | | | | | Total |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | |
| 1 mg/L GA | 4 | 1.22 | 0 | 0 | 0 | 0 | 1.22 |
| | 6 | 2.43 | 0 | 0 | 0 | 0 | 2.43 |
| | 8 | 8.87 | 0 | 0 | 0 | 0 | 8.87 |
| | 10 | 8.7 | 0.35 | 0 | 0 | 0 | 9.04 |
| | 12 | 8 | 0.7 | 0.35 | 0 | 0 | 9.04 |
| | 14 | 8 | 0.7 | 0.52 | 0 | 0 | 9.22 |
| | 16 | 8 | 0.7 | 0.52 | 0 | 0 | 9.22 |
| 3 mg/L GA | 4 | 1.15 | 0 | 0 | 0 | 0 | 1.15 |
| | 6 | 1.91 | 0 | 0 | 0 | 0 | 1.91 |
| | 8 | 9.16 | 0.19 | 0 | 0 | 0 | 9.35 |
| | 10 | 9.16 | 0 | 0.19 | 0 | 0 | 9.35 |
| | 12 | 8.59 | 0.95 | 0.19 | 0.19 | 0 | 9.92 |
| | 14 | 8.59 | 0.95 | 0 | 0.19 | 0 | 9.73 |
| | 16 | 8.02 | 16.67 | 0.19 | 0.19 | 0 | 25.06 |
| 5 mg/L GA | 4 | 1.28 | 0 | 0 | 0 | 0 | 1.28 |
| | 6 | 2.36 | 0 | 0 | 0 | 0 | 2.36 |
| | 8 | 9.64 | 0 | 0 | 0 | 0 | 9.64 |
| | 10 | 9.64 | 0 | 0 | 0 | 0 | 9.64 |
| | 12 | 9.64 | 0 | 0 | 0 | 0 | 9.64 |
| | 14 | 9.64 | 0 | 0 | 0 | 0 | 9.64 |
| | 16 | 8.99 | 0.64 | 0 | 0 | 0 | 9.64 |
| 1 mg/L TDZ | 4 | 0.87 | 0 | 0 | 0 | 0 | 0.87 |
| | 6 | 3.32 | 0.17 | 0 | 0 | 0 | 3.5 |
| | 8 | 8.22 | 0.17 | 0 | 0 | 0 | 8.39 |
| | 10 | 8.22 | 0.17 | 0 | 0 | 0 | 8.39 |
| | 12 | 8.22 | 0.17 | 0 | 0 | 0 | 8.39 |
| | 14 | 8.22 | 0.17 | 0 | 0 | 0 | 8.39 |
| | 16 | 8.22 | 0.17 | 0 | 0 | 0 | 8.39 |

**Table D4** Data of seed germination and protocorm development of *H. janellehayneiana* on various of BAP, GA, and TDZ on ½ VW media for 16 weeks (Continued).

| Media | Weeks | Stages | | | | | Total |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | |
| 3 mg/L TDZ | 4 | 0.94 | 0 | 0 | 0 | 0 | 0.94 |
| | 6 | 1.88 | 0 | 0 | 0 | 0 | 1.88 |
| | 8 | 9.79 | 0 | 0 | 0 | 0 | 9.79 |
| | 10 | 9.6 | 0.19 | 0 | 0 | 0 | 9.79 |
| | 12 | 8.85 | 0.38 | 0.56 | 0 | 0 | 9.79 |
| | 14 | 8.85 | 0.38 | 0.56 | 0 | 0 | 9.79 |
| | 16 | 8.85 | 0.38 | 0.56 | 0 | 0 | 9.79 |
| 5 mg/L TDZ | 4 | 0.89 | 0 | 0 | 0 | 0 | 0.89 |
| | 6 | 1.6 | 0 | 0 | 0 | 0 | 1.60 |
| | 8 | 9.06 | 0 | 0 | 0 | 0 | 9.06 |
| | 10 | 9.06 | 0 | 0 | 0 | 0 | 9.06 |
| | 12 | 9.06 | 0 | 0 | 0 | 0 | 9.06 |
| | 14 | 9.06 | 0 | 0 | 0 | 0 | 9.06 |
| | 16 | 9.06 | 0 | 0 | 0 | 0 | 9.06 |

**Table D5** Data of seed germination and protocorm development of *H. rhodochila* on various of BAP, GA, and TDZ on ½ VW media for 16 weeks.

| Media | Weeks | Stages | | | | | Total |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | |
| Control | 4 | 1.63 | 0 | 0 | 0 | 0 | 1.63 |
| | 6 | 4.09 | 2.32 | 0 | 0 | 0 | 6.40 |
| | 8 | 3.81 | 2.59 | 1.09 | 0.14 | 0 | 7.63 |
| | 10 | 3.95 | 2.04 | 1.63 | 0.14 | 0 | 7.77 |
| | 12 | 2.32 | 1.63 | 1.50 | 1.91 | 0.41 | 7.77 |
| | 14 | 3.13 | 1.50 | 1.50 | 1.77 | 1.50 | 9.40 |
| | 16 | 3.13 | 1.50 | 1.50 | 1.63 | 1.63 | 9.40 |
| 1 mg/L BAP | 4 | 6.71 | 0.79 | 0 | 0 | 0 | 7.50 |
| | 6 | 5.52 | 1.78 | 0.99 | 0 | 0 | 8.28 |
| | 8 | 4.73 | 1.97 | 1.38 | 0.79 | 0 | 8.88 |
| | 10 | 1.18 | 2.96 | 2.17 | 1.38 | 1.18 | 8.88 |
| | 12 | 1.97 | 1.97 | 1.78 | 1.97 | 1.18 | 8.88 |
| | 14 | 3.16 | 2.17 | 2.17 | 1.78 | 1.97 | 11.24 |
| | 16 | 3.16 | 2.76 | 1.78 | 1.78 | 2.37 | 11.83 |
| 3 mg/L BAP | 4 | 1.39 | 0.20 | 0 | 0 | 0 | 1.59 |
| | 6 | 4.37 | 1.59 | 0.20 | 0 | 0 | 6.15 |
| | 8 | 4.17 | 2.58 | 0.60 | 1.19 | 0 | 8.53 |
| | 10 | 3.97 | 2.18 | 0.79 | 2.38 | 0 | 9.33 |
| | 12 | 2.78 | 2.18 | 1.59 | 2.18 | 0.60 | 9.33 |
| | 14 | 3.77 | 2.18 | 1.59 | 0.99 | 1.59 | 10.12 |
| | 16 | 4.37 | 1.98 | 1.98 | 0.99 | 1.59 | 10.91 |
| 5 mg/L BAP | 4 | 1.19 | 0 | 0 | 0 | 0 | 1.19 |
| | 6 | 5.53 | 1.38 | 0.20 | 0 | 0 | 7.11 |
| | 8 | 5.14 | 1.78 | 0.99 | 0.79 | 0 | 8.70 |
| | 10 | 4.74 | 1.38 | 0.99 | 1.58 | 0 | 8.70 |
| | 12 | 3.75 | 1.38 | 1.38 | 1.78 | 0.40 | 8.70 |
| | 14 | 3.75 | 1.38 | 1.38 | 1.78 | 0.40 | 8.70 |
| | 16 | 3.75 | 1.38 | 1.38 | 1.78 | 0.40 | 8.70 |

**Table D5** Data of seed germination and protocorm development of *H. janellehayneiana* on various of BAP, GA, and TDZ on ½ VW media for 16 weeks (Continued).

| Media | Weeks | Stages | | | | | Total |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | |
| 1 mg/L GA | 4 | 3.87 | 0.53 | 0 | 0 | 0 | 4.39 |
| | 6 | 3.87 | 1.58 | 0.18 | 0 | 0 | 5.62 |
| | 8 | 2.64 | 1.41 | 1.23 | 0.70 | 0 | 5.98 |
| | 10 | 2.64 | 1.41 | 1.23 | 0.70 | 0 | 5.98 |
| | 12 | 2.64 | 1.23 | 0.88 | 1.05 | 0.18 | 5.98 |
| | 14 | 2.64 | 1.23 | 0.70 | 1.05 | 0.35 | 5.98 |
| | 16 | 3.51 | 1.76 | 0.70 | 1.05 | 0.35 | 7.38 |
| 3 mg/L GA | 4 | 4.33 | 0.17 | 0 | 0 | 0 | 4.50 |
| | 6 | 4.50 | 0.87 | 0.35 | 0 | 0 | 5.71 |
| | 8 | 3.98 | 1.38 | 0.52 | 0 | 0 | 5.88 |
| | 10 | 3.46 | 1.38 | 0.35 | 0.69 | 0 | 5.88 |
| | 12 | 2.94 | 1.73 | 0.52 | 0.69 | 0.35 | 6.23 |
| | 14 | 2.94 | 1.73 | 0.52 | 0.69 | 0.35 | 6.23 |
| | 16 | 4.33 | 1.73 | 0.52 | 0.69 | 0.35 | 7.61 |
| 5 mg/L GA | 4 | 3.94 | 0.36 | 0 | 0 | 0 | 4.30 |
| | 6 | 4.84 | 0.72 | 0.18 | 0 | 0 | 5.73 |
| | 8 | 4.66 | 0.90 | 0.18 | 0 | 0 | 5.73 |
| | 10 | 4.30 | 0.72 | 0.00 | 0.72 | 0 | 5.73 |
| | 12 | 3.05 | 0.72 | 0.54 | 1.25 | 0.18 | 5.73 |
| | 14 | 3.05 | 0.54 | 0.72 | 1.25 | 0.18 | 5.73 |
| | 16 | 3.05 | 0.54 | 0.72 | 1.25 | 0.18 | 5.73 |
| 1 mg/L TDZ | 4 | 1.50 | 0 | 0 | 0 | 0 | 1.50 |
| | 6 | 2.43 | 0.37 | 0 | 0 | 0 | 2.80 |
| | 8 | 2.43 | 0.93 | 0.19 | 0 | 0 | 3.55 |
| | 10 | 2.24 | 0.75 | 0.19 | 0.37 | 0 | 3.55 |
| | 12 | 2.24 | 0.75 | 0.19 | 0.19 | 0.19 | 3.55 |
| | 14 | 0.75 | 0.19 | 0.19 | 0.19 | 0.19 | 3.55 |
| | 16 | 0.75 | 0.19 | 0.19 | 0.19 | 0.19 | 3.55 |

**Table D5** Data of seed germination and protocorm development of *H. janellehayneiana* on various of BAP, GA, and TDZ on ½ VW media for 16 weeks (Continued).

| Media | Weeks | Stages | | | | | Total |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | |
| 3 mg/L TDZ | 4 | 0.43 | 0 | 0 | 0 | 0 | 0.43 |
| | 6 | 4.11 | 0.22 | 0 | 0 | 0 | 4.33 |
| | 8 | 3.90 | 1.08 | 0 | 0 | 0 | 4.98 |
| | 10 | 3.03 | 0.87 | 1.08 | 0 | 0 | 4.98 |
| | 12 | 2.60 | 0.87 | 1.08 | 0.43 | 0 | 4.98 |
| | 14 | 2.38 | 0.87 | 1.08 | 0.43 | 0.22 | 4.98 |
| | 16 | 2.60 | 0.87 | 1.08 | 0.43 | 0.22 | 5.19 |
| 5 mg/L TDZ | 4 | 0.68 | 0 | 0 | 0 | 0 | 0.68 |
| | 6 | 1.59 | 0.23 | 0 | 0 | 0 | 1.82 |
| | 8 | 2.05 | 1.14 | 0 | 0 | 0 | 3.18 |
| | 10 | 2.05 | 0.45 | 0.68 | 0 | 0 | 3.18 |
| | 12 | 2.05 | 0.23 | 0.23 | 0.68 | 0 | 3.18 |
| | 14 | 4.09 | 0.45 | 0.68 | 0.68 | 0.23 | 6.14 |
| | 16 | 3.64 | 1.14 | 0.45 | 0.91 | 0.23 | 6.36 |

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