Ecology and Ex Situ Conservation of *Vanilla siamensis* (Rolfe ex Downie) in Thailand

By

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Abstract

A loss of habitat and climate change raises concerns about change in biodiversity, in particular the sensitive species such as narrowly endemic species. *Vanilla siamensis* is one such endemic species. A preliminary cursory observation indicates that a lot of *Vanilla siamensis* vines cut when an ecosystem ismodified by human activity. Elucidating the steps of In-situ and Ex-situ conservation methods are thus required. This thesis focuses on the In-situ methods that are used for conserving biodiversity and monitoring their population under natural conditions and Ex-situ conservation methods used to preserve their diversity outside the natural habitats.

I start by presenting In-situ conservation methods. The conservation of a species in their natural habitat is done by studying the impact of ecological factors on the distribution of *Vanilla siamensis* (Orchidaceae: subfamily Vanilloideae). The tree host species in Tropical Forest at Khao Soi Dao Wildlife Sanctuary, Chantaburi, Thailand is also discussed. I then move on to focus on the natural pollinators of *Vanilla siamensis*, their floral morphology, and fruit set.

Lastly, I present the Ex-Situ Conservation Methods. This involves In vitro seed germination and plantlet regeneration of *Vanilla siamensis*, and evaluation of Cryopreservation protocols for *Vanilla siamensis*. It has been reported that the Genus Vanilla produces numerous minute seeds that do not germinate under natural conditions. Thus, using In vitro seed germination and plantlet regeneration techniques could be used to successfully germinate the seeds. Protocol for Cryopreservation of seed and embryo culture of *Vanilla siamensis* has been standardized and summarized by my co-workers.

The findings of this thesis have the potential to be applied for the conservation of *Vanilla siamensis* inside their habitat, while the Ex-situ conservation techniques can be used to grow *Vanilla siamensis* in habitats where it could not have survived unaided.

Acknowledgements

First and foremost, I would like to thank my supervisor Dr. David Roberts for his advice and support throughout this research, who encouraged me to undertake this work. This has been a big turning point of my life with personal diary, fulfillemt, and unforgettable gratitude. During these three years, my work has been full time, self financed, and as a 67 years old pensioner, I must have been probably the oldest postgraduate student in the country. This work is the result of hard work, dedication, inspiration, thoughtfulness, guidance, unlimited energy, determination, moral support, and generosity of my family, friends and colleagues. My quick three years research has come to its quest completion. I have laughed, shared, had my jaw dropped, been startled with quizzical looks many times and been to beautiful places around the world. I have been soaked with sweat and rain in the forest, stung by bees and wasps, roasted in the extreme heat of Madagascar, bitten by mosquitoes and leeches in the monsoon season and scared to death of being trampled by the wild elephants at Kao Soi Dow, being a protected wild life animal Sanctuary. In this Sanctuary, there are around twenty five wild elephants roaming around a waterfall area where the research was carried out on *V. siamensis* habitation. During the day times, *V. siamensis* are found near the waterfall and at higher elevations between 300 to 400 metre above sea level. However, during the evening around 6 to 7 pm, the herd comes down to the orchard plantations to dine on bananas, pineapples, coconuts, mangoes, rambutans, mangoestein, and any other edible plants. These disruptions cause the farmers to deter from going out until the noise minimizes. All houses within this vicinity, including mine, are boardered with a soft electric shock single wire fence. All roads have billboards with the warning “Careful driving with wild elephants crossing”. A great thank you to all these farmers for their protective advanced warning.

This research is truly my pride, passion, and not only just a job. At the same time, this research has led me to gain direct and indirect knowledge about topics related and unrelated to my research. Rejected by my own disappointment, I have been rendered speechless and struck by the unexpected generosity of some incredible people of Colombia, Madagascar, and Thailand. I have learned about the genetics of Vanilla vines and my new *V. planifolia* x *V. siamensis* hybrid may be a tetraploid as well, like the *V. tahitensis*. This process is known as polyploidization. This is quite common in orchid hybrids like the Vanda coerulea supra (Lord Rothschild’s Variety), one of the parents is *Vanda coerulea*, found in the same area as *V. siamensis*.

I got mesmerized by evolution as described by Charles Darwin's trails at his Down

house and have revisited it on a few occasions.This was the former home of the English naturalist Charles Darwin and his family. It was in this house and garden that Darwin worked on his theories of evolution by natural selection which he had conceived in London. I trailed his voyage to the Galapagos Islands, followed his disappointed vision to explore the mimic orchid (The Bee-Ophyrs) at Cudham in Kent, popularly known as Darwins'orchid Bank. Darwin's observations of orchids and their insect pollinators at Orchid Bank provided the evidence for his important book.

Fertilization of orchids, published in 1862, which inspired his now famous book, Origin of Species. Presently, Downe Bank is a nature reserve owned and managed by the Kent Wildlife trust in the North Downs, close to Downe in the London borough of Bromley. It is on Site of Special Scientific Interest (SSSI) together with the neighbouring High Elm Country Park located close to Charles Darwin's home, Down House. It became his favourite place and helped inspire his work.

The British and French plant-hunters like Dr. Arthur Francis Kerr, who discovered and collected the first *V. siamensis* specimen in Siam in 1905. His original collection can be seen at the Royal Botanic Gardens, Kew in the Herbarium. This *V. siamensis* is listed in CITES as an endangered species in Thailand, but at the same time being cut down by the unconcern, uninformed authority due to their massive growth in situ. It was named "siamensis" by Robert Allen Rolfe and Dorothy G Downie to honour, then Siam in 1925. The country changed name from Siam to Thailand, from being an Absolute Monarchy to Constitutional Elected Government on the 24th June 1946 with the Monarchy being the head of States. It is true, without the motivation and assistance, there is a good chance that I would have mysteriously vanished in the tangled maze of various rain forests in Colombia, Peru, Ecuador, Costa Rica, Sarawak and Thailand or even, lost my way in the labyrinths of a massive, complicated ANOVA data set.

My first and most deepest gratitude must go to the late Professor Rapee Sagarik, who passed away only months ago, at the age of 96. The professor boosted and supported my interest to do a PhD level research, like the Greek "Theophrastus" the father of Botany, the Professor Rapee has been bestowed with the honour of Father of Thai Orchidology. He was not only an academic, but his wisdom extended much further into horticulture, agriculture and philosophy. He inspired me, most importantly with his talks, lectures, books, and even hand painted oil drawing of *V. siamensis* presented to me as an encouragement. Not to mention treated me to an impromptu violin recital of his own compositions. He dedicated his whole life to the conservation work of orchid in Thailand. A great educator,

he shared his knowledge freely with anyone and everyone. I have enjoyed, shared and learned a lot during my three-year research, made many new friends and contacts around the world. A second person whom I am greatly indebted to is my favourite mentor and Chemistry teacher, Dr.William Warren at my early English Public Boarding School on the Isle of Wight (1966-1972). Sadly, he passed away this July 2018 at an age of 86 year old. He opened my world into Chemistry, especially the organic, aromatic compounds. His teaching of Chemistry subject still reverberates through my life to until this day.

During the three-year research I have travelled to many countries, including Mexico, Cuba, Costa Rica, Jamaica, Colombia, Peru, Ecuador, Galapagos Islands, Madagascar, Uganda, Reunion, India, Sarawak, Indonesia, New Caledonia, Tahiti and other endless countries visited. Another readjustment to the climate change was seen at Kao Soi Dow 2016-2017 where I set up my research centre for observing *V. siamensis* in their in-situ natural conservation. A big thanks to Mr.Phitsanok Ruatreo and Mr.Petch Tripetch for their expertise in Phantom 3 and Phantom 4 drones, which I used as a part of my *V. siamensis* conservation aerial study at Kao Soi Dow over two years. So far up to now, both drones have been disastrously lost in the tree canopy. The only solution to solve this was to hire the hexagonal drone experts from the Thai National Geographic Society to obtain these remarkable aerial views of my research area at Kao Soi Dow and in Chiangmai. No tears or regret to the lost drones, but fulfillment and satisfaction.

My enormous thanks and appreciation to all those universities in Thailand where I was advised, worked in various laboratories, eventually led me to an infinite and intimate friendship with the establishments concerned. All my research work was carried out in Thailand, in situ at the eastern part as well as in the northern part of Thailand, where *V. siamensis* grows. Ex situ work was carried out at Chiangmai University, under supervision of Dr.Hans Banziger at the Entomology Department to identify my potential pollinator bee from the four minute video clip which was taken at Kao Soi Daw. This was confirmed by Dr.Alain Pauly of the Royal National History Museum in Brussels to be an Asian bee of little known species. I have tremendous appreciation for Dr.Phenphichar Wanachantararak, Faculty of Dentistry, Chiang Mai University for helping me to identify the major organic chemical fragrance of *V. siamensis*. These experiments took many months and long hours to analyze with Mass Spectrometer and High Liquid Gas Chromatography. Many thank to Dr.Sasitorn Hasin from College of Innovation Management, Valaya Alongkorn Rajabhat University Under the Royal Patronage for helping identify different tree species and to work out the statistical distribution of different tree species by using Shannon Diversity Index

with. In Bangkok, at the University of Mahidol, tremendous thanks to Associate Professor Kanchit Thammasiri, Department of Plant Science where I took ten weeks lecture course and exams on various tissue cultures or micropropogation and cryopreservation. In the south of Thailand at University of Songkhla, Department of Plant Science, Faculty of Natural Resources, Hat Yai, Songkhla where I did my entire laboratory work on tissue culture and cryopreservation. Many long hours and month were spent here. It was at this university where the Vanilla seeds were first germinated and researched after so many unsuccessful attempts. Many thanks to Dr.Sureerat Yenchom, Professor Sompong Techato, for patiently assisting, advising, and helping me to obtain the result. Big thanks are due to Associate Professor Ayut Nissapa at the Department of Agricultural Development, who gave me full guidance, access to all apparatuses, equipments, and inspiration during my work. To the pollination specialist as well, Dr. Sara Bumrungsri at the Department of Biology, recommended by Dr. Matthew Struebig and also for his helpful consultation as well as recommendation. Needless to say, they are now great friends.

The highlight of my conference trip was in November 2017 to the world Orchid Conference, held in Guayaquil Convention Centre in Ecuador for one week. The majority of topics presented were on South and Central America's Vanillas, stretching from Mexico, Colombia, Venezuela, Costa Rica, Guatemala, El Salvador, Brazil, Cuba, Peru and Bolivia. I had the opportunity to meet so many professors, lecturers, PhD candidates and keen collectors, that we exchanged information and ideas. The two most memorable persons I met at this conference are Professor Kenneth M. Cameron, of the Department of Biology at the University of Wisconsin, who has written numerous books, literature, given hundreds of lectures and can be considered as the most knowledgeable person in the world about Vanilla, and Professor Michel Grissoni, who works at Unite Mixte de Recherche, CIRAD, Saint Pierre, La Reunion. He is the most knowledgeable person on Vanilla vines on all the French Polynesian Island, and has the largest, most important collection. He is an expert on pathogens and diseases of Vanilla. I would like to thank Associate Professor Nicola Flanagan of the University of Pontificia Universidad Javeriana, Cali, Colombia, for introducing me to new Vanilla vines, recently discovered in thick rain forest of the Amazon, and who has written many papers and articles on this subject. I also learned a lot from her- so much so that we became friends. Another inspiration I received was from Dr. Minoo Divakaran, Department of Botany, Provedence Women's College Calicut, Kerala, India. Dr. Minoo was the first person to have successfully accomplished interspecific hybridization between *V. planifolia* and *V. aphylla*, an Asian Vanilla and produced the F2 hybrid in Kerala,

India. I can say I am the second person to have done the interspecific hybridization between *V. planifolia* and *V. siamensis* in Thaialnd. My F1 hybrid seedlings are growing well in the tissue culture. I would also like to thank and cherish all the endless names of people outside the United Kingdom who helped and made my passion and interest for Vanilla vines possible.

For those in the United Kingdom, where I registered my three years of full time research at the University of Kent, and especially my supervisor, Associate Professor David Roberts, who mentored, supported, and inspired me for so many years prior to me starting my PhD. He played the crucially important role in advising the layout of my thesis and helped me to organize my ideas and research. Also to Dr. Matthew Struebig for being my second supervisor. To Dr.Phillip Cribbs and Marianne Cribb, for many exciting invitation dinners, where I always had something to know and learn from their endless knowledge about Vanilla vines, their advises to where "Vanilla must see places around the world", even has a vanilla vine named after him "*V. cribbiana*" discovered in Colombia by the late Miguel Angel Soto Arenas (1963- 2009), a Mexican botanist who at that time was a PhD Scholarship candidate working on the Vanilla at Kew under the supervision of Dr. Phillip Cribb. I truly believe Miguel would have been the number one expert person on the Mexican, Central and South American vanilla vines if he were alive. His organized work, catalogued books, papers, photographs, and everything that was related to Vanilla have been professionally kept, presented, and illustrated. A deep appreciation for his dear sister and closest friend, Dr. Eduardo Perez Garcia, for showing me the above collection. The one phase sentence Dr. Eduardo said to me about Miguel's love for the Vanilla is still deeply rooted in my brain and that is "Vanilla had always been in Miguel's blood" I was deeply touched, so much so that I have decided that my four minute video clip on the pollination of *Vanilla siamensis* in Thailand will be my dedication to Miguel for his inspiration. Also to the friendly Dr. Andre Schuiteman at Kew, who discovered and registered the new species of Vanilla found in Vietnam. This *V. atrophan* or "black beard" is a close cousin of *V. siamensis* and show many similarities to *V. siamensis* or "white beard" for the white flower and "red beard" for the red flower. Thankful appreciation for their advice and guidance at the Herbarium of the Royal Botanic Gardens in Kew, London, England, where I saw the very first *V. siamensis* specimen collected in 1905 by Dr. Arthur Francis George Kerr, the first scientist to be sent to then Siam by the British government to catalogue all plant species found and sent back to the Herbarium in Kew Gardens. To Dr.Terry Want, for his interesting, and endless talks on Lady Slipper orchids (Cypripedium, Mexipedium,

Paphiopedilum, Phragmipedium, and Selenipedium) and not to mention all these orchids are in his private collection at his house near to Kew. Also big thanks to Professor Hugh Pritchard at Millennium Seed Bank, the Royal Botanic Gardens, Kew, for his advice on my Vanilla seed and distribution. We met on several occasions at Wakefield and at various conferences held over the years. The last conference we met at was in Bangkok, Thailand and was held in March 2018 entitled, "The 3rd International Symposium on Plant Cryopreservation". Again, deep appreciation to all these wonderful enthusiastic people either colleagues, friends or mentors will be remembered and continue to do so, as there are still so many new knowledge and ideas to learn, share and discover within our circle of friends. To my best friend in England Francis Bauer-Czarnomski for his patience in everything I did all through these three years.

However, before I forget my thanks and appreciation to my last trip to Madagascar in April of 2018 on the invitation of Symrise Company, Holzminden, Germany. On the first day of my invitation I gave a talk on my *V. siamensis* research. This trip would have been impossible by any other means for many reasons. First of all, it is impossible to gain access to the company plantations and warehouses in Madagascar without signing a confidentiality paper. The roads were absolutely impossible to maneuver without a four-wheel drive van and a local, experienced driver. I visited various Symrise plantations in small and large local farms in Vanilla around Benavony, Ambodymanga, Antalaha Mama Alexia and Agroressourse plantations. Thanks to Mr.Olivier Tsaranarana for showing me how Vanilla plantation has gone to the next level by growing the Vanilla in large net houses as a protection against pathogens and pests. I visited "Magasin" of Antalaha, where all Vanilla "pods" were killed, cured, sorted, graded, and packed in large boxes for worldwide export by the person in charge, Dr. Clement Cabrol. All this was possible thanks to Miss Jane Devos, Research Development Manager, Mr. Alain Bourdon, the director of Symrise Company in Madagascar. As I left Sambava airport for the connecting flight to Bangkok, I silently thanked the Symrise Company for their enormous generosity and opportunity to have been invited, seen and achieved new knowledge, a remarkably successful large Vanilla business plantation efforts. I felt immensely priviledged to have been there and standing in the large warehouse with the net valve of over a million Euros in the world market, being amongst the second most expensive herb and species in the world in Madagascar. Only with luck and by chance this simply would not have been possible if I did not research in the *V. siamensis*.

Finally, my fulfillment and satisfaction in this research project is not completed yet

without it being continued on by another research student who has an interest in this field. The ultimate goal is for me to offer a full three-year PhD candidate scholarship to carry on with my research in Thailand, especially into the new Vanilla hybrid between *V. siamensis* and *V. planifolia*, which I have managed to obtain and the seeds (F1 generation) are growing well in a tissue culture at the University of Songkla, Thailand, and, secondly, the extraction of phytoestrogen from the green seed 'pods' for the treatment of osteoporosis patients by a pharmaceutical company. Last, but by no means least, I have learned and gained a lot of knowledge on Vanilla vines from my research abroad and around the world. From the enjoyment of my research, I have opened a Vanilla nursery or a vallinery in my new home in the cool climate of Chiang Mai. It is here where I am growing and cultivating *Vanilla siamensis* and other vines as well. I want to conserve and to continue on my new hybrid as a hobby and passion between *V. siamensis* and *V. tahitensis* with vanillin fragrance from the gene-pool of Vanilla planifolia, only second to *V. tahitensis* (*Vanilla planifolia* x *Vanilla odorata*), which grows on the island of Tahiti, in the South Pacific and which is the first naturally growing hybrid Vanilla in the world.

Before I conclude my final acknowledgement, I would like to express our enormous congratulations and gratitude to the Royal Botanic Gardens, Kew, the committees and the organizers Dr. Michael Fay, for recently hosting one of the best conferences in June 2019. It was a very well organized conference set in the perfect "mecca" botanical gardens ambience. I met so many interesting people from every corner of the world, some were familier faces and some were new comers. To mention that all Thai representatives enjoyed this conference very much, especially with the extra trip to Charles Darwin"s home at Downes. We all hope that the next conference after Perth in 2021, Thailand will have the opportunity to be the next participating host. Nevertheless and needless to say, my final fulfillment on my Vanilla Orchiddelirium has finally been laid to rest in this presentation of my thesis.

Table of Contents

Table of Contents (continued)

Table of Contents (continued)

List of Table

List of Figure

List of Figure (continued)

List of Figure (continued)

List of Figure (continued)

List of abbreviations

Abbreviation / symbol	Meaning
ANOVA	Analysis of variance
ABA	Abscisic acid
AFLP	Amplified fragment length polymorphisms
BA	6-benzylaminoprine
°C	Degree Celsius
°C/min	Degree Celsius per minute
cm	Centimeter
CRD	Completely Randomized Design
d	Day
DAPI	4'6'-diamidino-2-phenylindole
DVR	Digital video recording
DEF	Dry evergreen forest
DMSO	Dimethylsulphoxide
DBH	Diameter at breast height
EG	Ethylene glycol
e.g.	example gratia (Latin), for example
et al.	et alli (Latin), and others
FCM	Flow cytometry
°F	Degree fahrenheit
g	gram
g/l	gram per liter
h	hour
H_3	Hyonex
IAA	ind11.5ole acetic acid
IBA	Indole butaric acid
IV	Importance Value
KC	Knudson cell reactor
KN	Kinetin
LN	Liquid nitrogen
LS	Loading solution
LSD	Lest significant difference
M	Molar

List of abbreviations (continued)

Abbreviation / symbol	Meaning
m	meter
mg	milligram
mg/l	milligram per liter
min	minute
ml	milliliter
ml/l	milliliter per liter
mm	millimeter
mM	millimolar
MS	Murashige and Skoog
NAA	α-naphthalene acetic acid
ND	New dogashima
PEG	polyethylene glycol
PGR	plant growth regulator
PI	propidium iodide
PLBs	protocorm-like bodies
PVS1	plant vitrification solution formula 1
PVS2	plant vitrification solution formula 2
PVS3	plant vitrification solution formula 3
PVS4	plant vitrification solution formula 4
rpm	round per minute
Sec.	second
TDZ	Thidiazuron
Tm	melting temperature
µl	microliter
µm	micromolar
V	voltage
v/v	volume per volume
VW	Vacine and Went
w/v	weight per volume
w/w	weight per weight
ZA	zeatin riboside
%	percentage

Chapter 1: Introduction

1. Introduction to orchids

Orchids, the largest flowering plants (Angiosperms) contain over 800 genera and 25,000 species, belong to the Orchidaceae family. They can grow in many different environmental habitats with various nutrition, light, and temperature demand. They have a complex life cycle, mycorrhizal association (seed germination) and unique pollination processes. With distinct vegetative and floral characteristics, including forms, structures, and colours, the Orchidaceae family is of considerable economic importance, especially in horticulture and floristry. Apart from being in demand for its floral displays, orchids are used in traditional herbal medicine. The orchids demand for horticultural purpose makes them a threatened species globally as a result of over collection from the natural habitats. Moreover, climate shift over the world may cause them to become endangered in-situ in their natural habitats as well. Therefore, plant tissue culture, micropropagation, and cryopreservation techniques are required in in-situ conservation and management of botanical collection to preserve orchid biodiversity (Mahendran and Bai, 2009).Orchids are classified as follows:

Kingdom: Plantae

Division: Magnoliophyta

Class: Asparagales

Family: Orchidaceae

The Orchideceae family has been divided into 5 subfamilies including: Apostasioideae, Cypripedioideae, Orchidoideae, Epidendroideae and Vanilloideae.

Orchids are also divided into two basic growth types according to the growing direction of their stems which can be either monopodials or sympodials.The stem of Monopodial orchids grows in a vertical line (single stem), as in *Vanilla* vines. They have central stems which grow continuously from the trip and flowers are produced from stem between the leaves.The stem of sympodial orchids grows in the lateral line that possesses a rhizome sending out the shoot and developing into stems and leaves (multi-stem). The mid-section of stem is often expanded into water-storage organ called pseudobulb.

In addition, orchid is one of a epiphytes plant species that grows on another plant but is not a parasite and produces its own food by photosynthesis. Orchids can also be grouped according to the way they retrieve nutrients or the way they live in the natural habitat, such

as terrestrial (growing in soil), epiphytes (growing on trees), lithophytes (growing on rocks), and saprophytes (growing on decaying organic matter). Terrestrial orchids, such as *Paphiopedilum* spp., And *Spathoglottis* spp, normally establish in the soil for nutrients and moisture. Epiphytic orchids grow on other living plants for mechanical support, but not for nutrients. They get the nutrients and moisture from the air. Therefore, they are not a parasite but live together with other plants in symbiosis mutaulism, such as *Cattleya* spp., *Vanda* spp., *Phalaenopsis* spp, and *Dendrobium* spp.

2. The unique morphology of orchid flowers

The complex structures of orchid flowers have always attracted biologists. With unique morphology, orchids can be distinguished from other flowering plants by these basic features (Arditti, 1992). Orchid flowers are typically zygomorphic (bilaterally symmetric) that can divided by only a single plane into two mirror-image halves. Most flowers are actinomorphic (radially symmetric), meaning they can be divided into symmetrical halves by more than one longitudinal plane passing through the axis, much as a pie can be cut into several equal and identical pieces. Actinomorphic flowers are a basic feature of the angiosperms while zygomorphic flowers are an evolved feature of the angiosperms derived from long periods of developing higher flowering plants (Losos et al. 2008). They are composed of an outer whorl of three petal-like sepals and an inner whorl of three petals. Sepals are often differntiated in a 'dorsal' or 'medial' sepal, which is large and showy, and two 'lateral' sepals. They usually have one greatly enlarged petal that forms a lip or labellum which corresponds to the top petal between the two lateral petals that make the flower bilaterally symmetric. Only before the flower opens doesthe flower stem rotate by 180° during development, makingthe lip point down. This unique process is called 'resupination' and is necessary to position the labellum on the bottom of the flower, where it often serves as a landing platform for pollinators.

There are no stamens with free filaments as in other plants. The male and female reproductive organs are fused into a single structure known as a columm or gynostemium ,which is positioned at the centre of the flower. The anther and sticky stigma are located at the top and bottom of the column, respectively. The rostellum, a beak-like structure, separates male and female parts and acts as a barrier to prevent self-pollination. Unlike most other plants,orchid pollen is typically derived from a single stamen and pollen grains stick together to from pollinia (singular: pollinium). Pollinia are connected directly or through a short stalk (stipe or caudicle) to the sticky foot-like viscidium. This structure is termed the 'pollinarium' (plural pollinia) and becomes attached to the pollinator.

Orchids always have numerous seed count up to a million per pod or fruit of the orchids and the seeds are very tiny (0.05-6 mm in length).Vanilla orchids have a waxy pollinia as well as rounded black , shiny ,waxy seeds, only one layer thick, which becomes the thick testa.

3. Endangered orchid species and conservation

Many orchid species in the world are facing extinction because of climate change leading to uncontrollable temperature, flood, and drought stress. In nature, the frequency with which plants occur within a population can also affect their reproductive success. Low density and fragmented populations of outcrossing, seed-producing plants may suffer from limited seed recruitment due to a number of factors including: (i) a low number of potential mates,(ii) inbreeding depression caused by mating with closely related individuals and (iii) reduced attractiveness to pollinators and pollination limitation (Duffy and Scout 2008). In addition, human behavior contributes to the deforestation and wild orchid trade, which are important fators in accelerating their extinction rate. Thai orchids are famous for their beauty and unique features; therefore, they are in high demand worldwide. Some orchid species become endangered because they have been taken out from their natural habitat.

To date, many orchid species in the world have become endangered or endemic, which has resulted in a growing interest of biologists to study and establish the natural habitat profiles and conservation strategies of rare orchids for preserving their genetic diversity. Such species include *Paphiopedilum armeniacum* in China (Zhongjian et al. 2006), *Aspasia principissa* in Panama (Zotza and Schmidt 2006), *Pterostylis* aff. *Picta* in Australia (sharma et al. 2003). *Spiranthes romanzoffiana* in Ireland (Duffy and Scout 2008), *Cleisostoma arietinum limitation* (Maneerattanarungroj et al. 2007), and *Vanda coerulea* in Thailand (Thammasiri and Soamkul 2007).

The conservation of wild orchids is such a complex issue that it depends not only on developing the education and economy but also on the biological characeristics of orchids. In recent years, there have been studies on the biological embranchment of certain species in some genera, but the conditions of the plants themselves, especially the biological factors that lead to the extinction of wild orchids, remain poorly understood (Chen and Luo 2003).

All wild orchids in the world are protected by Convention on International Trade in Endangered Species of Wild Fauna and flora (CITES) and about 90% of the entire plant species are under conservation. The study and conservation of wild orchids in Thailand plays an important part in the study and conservation of orchids worldwide. Plant genetic resources can be conserved by two basic techniques, in situ and ex situ conservation. In both in situ

and ex situ conservation, the integrated application of novel techniques and methods, such as habitat resumption, plant reintroduction, and rejuvenation establish the core of conservation strategies. These undertakings involve integrated studies of the biological characteristics, community biology, ecology, and reproduction biology of orchids, and provide a scientific basic knowledge for devising conservation strategies of wild orchids (Luo et al. 2003).

3.1 In situ conservation

In situ conservation includes the conservation of ecosystems and natural habitat, maintenance and recovery of viable populations of species in their natural surroundings including forests and national parks. Orchids can be conserved in their natural habitat (in situ conservation), which is the best way to maintain the genetic biodiversity of orchid plant. It is however, very difficult to preserve them for a long period and an appropriate management is required. Conserving by growing orchids only in field is risky as of insects, pests, plant diseases, and environmental stresses. In addition, the cost is usually high due to labour and land use (Engelmann and Takagi 2000).

3.2 Ex situ conservation

Ex situ conservation means the conservation of components of biological diversity outside their natural habitat. It includes moving the plant genetic resources from their natural environment and placing them in artificial storage conditions, such as seed storage, in vitro storage, DNA storage, pollen storage, field gene banks, green house, and botanical gardens. The orchid seeds are orthodox in nature, which can be dried to sufficiently lower the moisture content and store at room temperature or in refrigerator. However, the rate of seed viability also tends to reduce. To overcome this problem, cryopreservation of seeds can be done to improve their viability rate. Such In vitro storage techniques are time-consuming, expensive, and laborious to grow and develop orchids in a medium. Furthermore, this method can lead to somaclonal variation and loss of material through by microbial contamination (Dressler 1981).

4. Orchid propagation

Orchid propagation relies on two pathways to maintain and increase its number, sexual and asexual reproduction.

4.1 Sexual propagation

Sexual reproduction, possessed by nearly all eukaryotes, is a reproductive strategy involving the formation of ovules and their fertilization by pollen tubes after the pollination of a flower. It is considered as a primary characteristic leading to the reproduction and long-term survival of the species due to evolution potential: when the habitat changes, the sexual biology can adapt to new environments, while asexual reproduction generally has difficulty in responding to changing environments (Zhang 2004).

Out-crossing is considered to be the most effective breeding system in maintaining genetic diversity; whereas self-pollination can cause inbreeding depression, considered as one of the selective pressures during the evolution of plant breeding system. If self-pollination is easier to fertilize ovules compared to crossed pollen, then inbreeding can enhance the gene numbers contributed by the offspring through the advantage of automatic selective. Avoiding selfing was one of the reasons used to explain most of the reproductive structures of plants (Zhongjian et al. 2008). Nevertheless, the fact is that selfing is a reproductive strategy chosen and adopted by the most angiosperms (Barrett 2002). Many advantages of selfing can be described such as living in a new habitat, avoiding unreliable pollinators, maintaining adaptability in local plant populations, automatically passing on genes to the next generation and bringing about reproductive assurance (Holsinge 1996). Therefore, evolution has favoured a variety of mechanisms to promote selfing in angiosperms, such as rotating its anther for self-pollination (Liu et al. 2006) and geitonogamy (the fertilization of a flower by pollen from another flower on the same or a genetically identical plant).

The most efficient method of native terrestrial orchid propagation for conservation purposes is seed germination. Terrestrial species have more stringent requirements for germination but little is known about the specific requirements (Fast 1982). Hartmann et al. (1997) suggested that their specific endogenous growth promoting and inhibiting compounds are involved directly in regulation processes of seed development, dormancy, and germination. Initiation of germination requires three factors. Firstly, the seed must be viable which means the embryo must be alive and capable of germination. Secondly, the seed must be subjected to conducive environment. Thirdly, any primary dormancy present within the seed must be overcome (Mahendran and Bai 2009).

Among the seed-bearing plants, orchid produces the smallest seeds by size (0.05-6 mm) or weight (0.31-24 ug). A single fruit capsule can produce seeds in large numbers from 20-50 up to 4 million seeds (Roberts and Dixon 2008). With a tiny size and light weight, the orchid seeds are able to float in the air for a long period, and are thought to be important in enabling orchids to become widespread and supreme epiphytic colonizers. Unlike most of

the flowering plants, orchid pollens travel, on average, a shorter distance than an orchid seed. However, most orchid seeds drop near by the mother plant, with only a tiny fraction germinating and eventually becoming an adult plant (Roberts and Dixon 2008).

The seeds contain a small embryo but lack endosperm to enable the seed to germinate. Furthermore, they lack enzymes to metabolize polysaccharides but they utilize lipid directly as a major nutrient source. Due to the absence of enzymes, it is necessary for the orchid seeds employ a symbiotic relationship with a mycorrhizal fungus for germination. This fungus utilizes soil organic matters such as cellulose and converts it into simple sugars providing nutrients, minerals,and water to support orchid seed germination and embryo growth (Rasmussen 1992; Smith 1996). Seed germination in association with mycorrhyza is known as a symbiotic germination and widely used by terrestrial orchids.

4.2 Asexual propagation.

Vegetative or asexual reproduction does not need energy in producing male offspring (or pollen) and they could transfer all genetic material to the next generation (only half of the genes in sexual individuals). In a competition for survival, population statistics of asexual individuals have advantage obviously over sexual individuals (Zhang 2004).

Under uncertain or harsh environmental conditions, no mating modes or fruited plants have been found as the flowering and pollinating processes cease under bad climate. In order to avoid the risk of a breeding failure caused by such circumstances, Trias verrucosa (newly discovered in China, accepted name of a species in the genus , Trias family Orchidaceae) used another mechanism to safeguard its multiplication via asexual reproduction, in which the pseudobulb could produce gemmules (internal buds, that are capable of producing new cells) to enlarge the colony and ensure its continuing life for a successful reproduction even if it flowers or not during its long evolutionary process (Zhongjian et al. 2007).

In South Africa, terestial orchids like Satyrium odorum, *S. ligulatum*, *Disa sagittalis*, *D. harveiana*, *Disperis purpurata*, *Pterygodium catholicum* and *Corycium deflexum* have developed additional tubers on long stolonoid roots (aerial roots, adventitious roots arising on the stems or pseudobulbs and growing in the air, such roots are often called stolonoids found in some terrestrial orchids that form clonal colonies) several centimeters away from the old plant, which germinates new plants in the following season. *Disa uniflora*, *D. tripetaloides*, and *D. cardinalis* have underground stems called stolons which form a large number of leafy plants at their ends, resulting in these Disa species to often grow in clumps with many individuals (Kurzweil 2000).

The most widely used method to propagate orchids via asexual reproduction is the

tissue culture technique that can facilitate orchid propagation. This method can produce large number of disease-free and desirable plants in a short time, and is cost effective with very little land usage.

5. The genus Vanilla

The genus Vanilla, which belongs to the Orchid family includes 120 species with new Vanilla vines being discovered all the time. Most of these species are wild and only three of the Vanilla vines and their subspecies including 1.*Vanilla planifolia*, 2.*Vanilla pompona* subsp. *pompon* (native to Mexico), *Vanilla pompona* subsp. *grandiflora* (widespread in South America), Vanilla pompona subsp. pittieri (widespread in Costa Rica, Honduras and other parts of Central America), 3. *Vanilla tahitensis* subsp *tahitensis*, *Vanilla tahitensis* subsp. *haapape*, *Vanilla tahitensis* subsp. *rea*, *Vanilla tahitensis* subsp. *parahurahu*, *Vanilla tahitensis* subsp. *tahiti* long are grown to produce vanilla commercially with *Vanilla planifolia* providing 95% of the supply to world markets. The natural habitat of species of genus Vanilla are mainly found in tropical and subtropical regions of the American, African, and Asian continents. Most are threatened by the destruction of their original habitat, which is further accentuated by climate change. The species of *V. planifolia* is particularly endangered, as the primary gene pool in its region of origin (southern Mexico) has been subjected to considerable anthropogenic stresses linked to deforestation and overexploitation of natural resources (Soto-Arenas 1999). The vegetative reproduction process, which is predominant for the vanilla species grown, makes it impossible to maintain and extend the gene pool. The secondary gene pool of vanilla includes over 100 species that have diversified in America, Africa, and Asia. These species have individual properties that may be of particular interest for the genetic improvement of cultivated vanilla, such as autofertility, resistance to disease (fusariosis, viruses), the capacity to bear a large amount of fruits, a lower dependence on the photoperiod for the induction of flowering, a high vanillin content, the presence of other aromatic or medicinal metabolites and resistance to draught.

5.1 Pollen, Fruit, and Seeds

Vanilla flowers are pollinated when pollen is transferred from the anther to stigma of the same or different flowers. Unlike Vanilla and most other vanilloid orchids, a vast majority of pollen produced by orchids is shed as loose monads (single grains) or tetrads (units of four united pollen grains). Similar to other orchids, Vanilla flowers have a technique whereby should their flowers not become pollinated, the microscopic ovules that will develop into

seeds within the flower's ovary are not formed until after the pollen grains are deposited on the stigma. The pollen grains germinate long tubes that travel down the length of a column to deliver sperm cells that will ultimately fertilize the egg cell within the ovule. The delay in ovule development ensures that no energy is wasted in the event that no insect visits the flower during the short time that it is open and receptive. Unlike most orchids, following pollination of the flowers and fertilization of the ovules, the sepals and petals of a vanilla flower whithers and eventually falls away from the ovary as it developes into a seed-bearing fruit (vanilla bean). This separation occurs along an abscission layer of cells between the ovary and floral perianth. As the fruits developed, so do the many seeds within it. The seed coat, composed of several cell layers becomes hardened, black, and lustrous. This is in contrast with all the nearby orchid seeds, which are microscopic and dispersed by wind and in which the embryo is surrounded by only a thin, single layer of seed coat. The aromatic compound, Vanillin, which gives the fruits of some Vanilla species their characteristic fragrance, is produced not by the seeds or in the wall of a fruit, but within microscopic hairs (papillae) that line the interior wall of the fruit. The importance of the hairs as a site for vanillin production, however was only recognized in 2003. A team of researchers at Rutgers University showed that these unicellular hairs contain beta-glucosidases enzymes that are involved in vanillin biosynthesis and seem to be responsible for its secretion.

5.2 Taxonomy and phylogeny of Vanilla.

Vanilla and its relatives are members of what is likely an ancient lineage of flowering plants. Many are restricted to remote regions and some are threatened with extinction. A lot of information about *Vanilla planifolia* such as methods of cultivation, diseases that affect the domesticated vines, techniques for fruit processing is available, but the fundamental natural history of the entire genus Vanilla and its closest relatives is still poorly understood. A systematic study of these plants has been and continues to be surrounded by controversies. In recent years, their taxonomy and phylogeny studies have been done and has come about primarily through an increased use of DNA-based data in systematic studies (Cameron 2003; Cameron 2004; Cameron 2006). Botanists now consider a single fertile anther at the apex of a Vanilla flower's column to have risen by way of a different evolutionary process than that of nearly all other orchids (i.e., those classified within the Epidendroideae and Orchidoideae sub-families). For such reasons, Vanilla and related orchids are now classified with their own unique subfamily, Vanilloideae. The sub-family Vanilloideae is positioned near the base of the orchid family tree and Orchidaceae is the basal family within the large monocot order Asparagales (including onions, agaves, hyacinths, and the iris family). Molecular time clock

estimates about the evolutionary age of these plants have estimated that the origin of Orchidaceae can be traced back to at least 76-119 million years (Janssen and Bremer 2004; Ramirez et al. 2007). Vanilloid orchids, in turn, are at least 62 million years old. Molecular clocks can only provide minimum ages, so these plants are probably even older.

In the evolution of Vanilloid Orchids, an unsubstantiated hypothesis has persisted among botanists that the Orchid family has only recently evolved relative to other flowering plants. To support this opinion, botanists cite the relatively low levels of genetic diversity among orchid genera and species, many of which can be hybridized easily with one another. However, molecular phylogenetic studies of Vanilloideae challenge the notion that the entire Orchid family is recently evolved.

5.3 Distribution of *Vanilla siamensis* and beyond.

Five different vanilla vines have been found namely *Vanilla pilifera*, *Vanilla aphylla*, *Vanilla albida*, and *V. siamensis*. All these Asian vines do not produce any vanillin for commercial purposes. The Asian vanilla vines are not well known or documented and not much research has been done about them. *Vanilla siamensis*, comes in two colours, namely white and red.The white colour is nicknamed “white bread” and the red colour is nicknamed “red bread” because of the presence of long finger-like hairs at the apex of the labellum, and is the only one with a slight aroma of vanillin. These five vines can be found growing in Southeast Asia, including Thailand, Cambodia, Laos, Myanmar, and in the Yunnan province in southern China. Many close relatives of *V. siamensis* are also found within these countries like *Vanilla atrophan*, in Cambodia, discovered by Andre Schuiteman, and he nicknamed it “black bread” at Kew Gardens. In Thailand, two locations were chosen because they grow very well within suitable conditions namely moisture, sunlight, shadding, rainful, humidity, and also due to the presence of the two highest mountain ranges in the north of Thailand, namely Chiangmai and the east of Thailand, Kho Soi Dow. I started my research in 2016 on the ecology of in situ and ex situ conservation including pollination and germination of endangered *V. siamensis* (Orchidaceae:Vanilla) in the tropical forest at Khao Soi Dow Wildlife Sanctuary, Chantaburi province located in the eastern region of Thailand. This research involved studying the impact of ecological factors affecting the distribution of in situ *V. siamensis* mainly in Chantaburi above 400 metre above sea level (masl). For a successful establishment, several ecological factors and practices were observed for the wild Vanilla siamensis. The most important ecological factor is the irrigation water from the waterfalls high above 1200 masl. In the mountainous region, the soil moisture, soil temperature, host trees temperature, humidity, wind are all dependent on this important

factor. Water is the main factor influencing the growth and development of this wild vanilla as observed by their tremendous size, attaching onto their preferred host trees (Hydnocarpus illicifolia and Pterocymbium tintorium) growing near or on the edge of waterfalls at 400 masl. Also, sufficient availability of water is most critical during flowering/pollination. The three observed benefits found were 1) encouraging growth and development of the plant 2) increasing fruit yield and quality 3) prolonging the production life of the plant. Another equally important ecological factor in this tropical moist forest is the presence of mulch near the edge of waterfalls which contributes to the successful growth. The primary source of nutrition for *V. siamensis* in the wild is organic material that results from the natural decomposition of vegetable/animal residues via microorganisms or vermiculture, worm-mediated breakdown of organic material. The third important ecological factor is the elevation with regulation of light and shade, which this wild *V. siamensis* requires and thrive well in. This wild vanilla does not like direct sunlight as it brings heat which can burn the thick succulent leaves resulting in burnt black spot marks. In the dry and hot April and May months, which coincide with flowering/pollination and fruit development, the host trees Hydnocarpus illicifolia and Pterocymbium tintorium are preferred supporting trees that grow on the edge of waterfalls and have a dense canopy providing 70-80% shade. This conserves humidity and prevents burning from intense sunlight while decreasing the incidence of young fruit drop. The flowering and fruiting periods start in the months of April, May, and June. Thailand is located in the northern hemisphere, above the equator line like Mexico, India, and China, where vanilla vines flower only once a year. On the other hand, Tahiti, Madagascar, Uganda, Ecuador and Indonesia have a flowering season from September to December and flower twice a year. The forth important ecological factor observed in promoting flowering at the edge of waterfalls at high elevation of 400 masl is the climate or mechanistic stress. Water stress induces reproductivity of mature individuals of vanilla and many other plants to flower. The principle stress at Khao Soi Dow is the cool and low temperature in December and January that induces flowering. The cool temperature' burns' the apical tip, killing it, and breaks the apical dominance of the plant while stimulating lateral floral buds to develop. This practice is seen in many vanilla vine plantations.

The mountain range stretches into Cambodia, where many close *V. siamensis* relatives are found. The elevation in which the vanilla vines grow well is from 300 to 700 masl. The true pollinator, Thrinchostoma bee species was discovered for *V. siamensis* and fruit sets were formed. The most important characteristic related to *V. siamensis* is that it can be successfully cross bred by hand with the more distantly found *V. planifolia*. These two Vanilla vine species could have shared a common ancestor tens of thousands or perhaps even millions of years

ago. The success of this experiment demonstrated that *V. siamensis*, even distantly related ones, can be crossed with one another. This opened the opportunity for the further improvement of Vanilla crops such as resistance to various fungal diseases, viral diseases, cold tolerance, or even new, more aromatic, novel Vanilla fragrance present in Vanilla fruits.

5.4 Vanilla's closest orchid relatives

The hierarchical classification of genus Vanilla is as follows: kingdom Plantae, phylum Anthophyta (flowering plants), class Monocotyledones, order Asparagales, family Orchidaceae, subfamily Vanilloideae (the vanilloid orchids), tribe Vanillae, and the genus Vanilla. Within Vanilloideae, there are no few than 15 genera, including Vanilla, Pseudovanilla, Erythorchis, Cyrtosia, Galecia, Lecanorchis, Eriaxis, Clematepistephium, Epistephium, Pogonia, Isotria, Cleistes divaricata, Cleistes, and Duckeella but Vanilla is the most diverse of all. Two closely related tribes of vanilloid orchids are Vanilleae and its sister tribe, Pogonieae. Except for Vanilla, which is pantropical in distribution (i.e. occurring or distributed throughout the tropical regions of the earth), the tribe Vanilleae contains one genus from tropical South America, five genera that grow in tropical and subtropical Asiaand Australia, and two genera from New Caledonia, namely Eriaxis and Clematepistphium, which are endemic to the isolated island, both of which were observed during a visit three years ago. Both genera are monotypic (only one species each). An unusual aspect of one of these two species is that Clematepistphium smilacifolium grows in dense shade of the island rainforests as a climbing vine. Unlike other species of Vanilla, Clematepistphium vines produce no aerial roots. Instead, they climb by twisting around the trunks of small trees. Its large leathery leaves exhibit prominent venation pattern that are reticulate (net-like) rather than exclusively parallel, as in most orchids and other monocotyledons (Cameron and Dickison 1998). The fruits of these orchids are capsules that dehisce to release distinctive seeds with circular wings, a feature of Orchidaceae (Cameron and Chase 1998). Winged seeds are also found in three other genera of vanilloid orchids: Pseudovanilla, Erythrorchis, and Galeola. These are closely related and native to Southeast Asia in regions including Thailand, Cambidia and Laos, northeast Australia, and a few Pacific islands. All three genera are leafless, climbing vines, two of which (Erythrochis and Galeola) completely lack chlorophyll. These nonphotosynthetic genera are exclusively mycoheterotropic (parasitic on fungi). The leafless genus Pseudovanilla is similar to the other two in most aspects, but does eventually develop green pigment within its stems after the juvenile stages of its life cycle.

Recent studies have shown that these orchids are the closest living cousins of vanilla (Cameron and Molina 2006) in terms of hierarchical classification. They climb by means of

aerial roots produced at each node of the stem just like vanilla, and their flowers are remarkably similar to those of Vanilla species. Their fruits however are designed to accommodate winged seeds and so are dry, dehiscent, and nonaromatic at maturity.

The two vanilloid orchids are a tremendously diverse group of flowering plants. The greatest amount of research has been focused on *V. planifolia*, this is only one species of a linage that has adapted to a variety of habitats, relies on different pollinators and has developed flowers of diverse forms. In other words, *V. planifolia* may be the only orchid species of significant agricultural value, second only to saffron, as the most expensive herb and species, out of more than 25,000-30,000 naturally occurring species, but it is not entirely unique in the family. Rather it is just one of approximately 110-139 species in the genus Vanilla, all of which are similar to and yet different from one other. Many of the genera and species are rare and in great danger of extinction primarily due to habitat destruction.

5.5 Thick-Leaved Species of the Old world

These are *Vanilla tahitensis*, growing on the island of Tahiti, Vanilla kinobaluensis, just outside Mountain Kinabalu National Park in Sabah, Borneo, Vanilla grandifolia growing in Gabon, Congo, and on the island of Principe, Vanilla imperialis and Vanilla polylepis. *Vanilla imperialis* and *Vanilla polylepis* are two of the most widespread and common of Africa's twenty-two native species of Vanilla. They can grow to become massive plants with thick stems and large fleshy leaves. Their flowers are also among the largest and have the fanciest display in the genus, being primarily white with a reddish-purple labellum, similar to *V. siamensis*. Both arefound in Uganda, Angola, and Tanzania.

5.6 Closest relatives of *Vanilla siamensis* and their distribution

The closest relatives of *V. siamensis* and their distribution are the thick-leaved species of the Old world (Africa and Asia) in terms of their morohology, including *V. tahitensis* the genuine black gold, the first natural hybrid, from the island of Tahiti and the French Colonies of Reunion, Raiatea, Huahine, Moorea, Marquesas Islands, and Madagasger. This is the second most important Vanilline producing vine after Vanilla planifolia. *V. tahitensis* is a primarily a hybrid between two Neotropical species *V. planifolia* (the maternal parent) and *V. odorata* (parental parent). *Vanilla tahitensis* are similar to *V. planifolia*, except that the leaves are narrower. The hybrid's flowers are green and yellow. These have frilly margins around the labellum, much frillier than that seen in *V. planifolia*, but not unlike the flowers of *V. odorata*. Flowering is induced by a decrease in temperature (18-19 °C) and in drier and sunnier weather during the fresh season. Two flowering periods often occur in a year: the

main period between July and September while the other is possible in December-January. The ends of the hanging stems then dry up and 1-3 inflorescences (a flower or group of flowers on the stem, or the way they are arranged) from 10 to 15 flowers appear. The flower opens at dawn and fades during the evening. In a single inflorescence only 1-2 flowers open each day.

There is no pollinating insect in French Polynesia and therefore pollination is done manually early in the morning to prevent the pollen from becoming too dry to adhere to the stigmata. The bean is ripe after nine months. Green until then, the bean starts to become yellow and then natural browning occuers and allows the complete conversion of glycolated aroma compounds into corresponding aglycones that are responsible for its fragrance. The ripening process starts from the bottom of the fruit and indicates that the bean can be collected and cured. The fruits of *V. tahitensis* are often shorter than those of Bourbon vanilla, are highly fragrant, and entirely different from the more common varieties. Tahitian vanilla is best described as being floral and fruity. Indeed, the fragrance of hybrid Tahitian vanilla fruits is unlike any other, primarily due to the presence of heliotropin (piperonal, C8H6O3), a molecule with a light floral aroma that is popular in the perfume industry and as a flavour in the confectionary industry. The five subspecies of *V. tahitensis* found growing on the Island of Tahiti are *V. tahitensis* issubsp. *tahitens*, *V. tahitensis* subsp. *haapape*, *V. tahitensis* subsp. *rea rea*, *V. tahitensis* subsp. *parahurahu*, and *V. tahitensis* subsp. *tahiti long*.

For most vanillas cultivated worldwide, notably *V. planifolia* , ripeness involves dehiscence of the fruits. To avoid this stage, clusters of fruits are harvested before the have fully matured; and then ripeness is stopped through treatment at high temperature. *Vanilla tahitensis* beans rarely develop split ends and they are harvested when fully ripe, with all the aroma still intact.

Others close relatives of *V. siamensis* are *V. kinabaluensis*, found just outside of Mountain Kinabalu, National Park in Sabah, Borneo. Vanilla grandifolia is found at elevations of approximately 300 metres in the tropical forests of Gabon and Congo. *Vanilla polylepis* is found in Uganda, Tanzania, Kenya, Angola, Tanzania, and on the island of Principe. All these Vanilla vines do not have the same aroma but have a great potential for future interspecific hybridization breeding programme as a secondary gene pool. The future for Vanilla industry looks promising.

5.7 Commercial viability and security of global production of Vanilla

Vanilla is a flavouring, derived from orchids of the genus Vanilla, primarily from the Mexican species, *V. planifolia* is the most important of all the vanilla vines, only second to

saffron as the most expensive herb and species in the world, because growing the vanilla seed fruits and harvesting are both labour-intensive. The word vanilla, is derived from vainilla, a diminutive of the Spanish word vaina (vaina itself meaning a sheath or a pod), is translated simply as “little pod“. Pre-Columbian Mesoamerican people cultivated the vine of Vanilla Orchid, called tlilxochitl by the Aztecs. Spanish conquistador Hernan Cortes is credited with introducing both vanilla and chocolate to Europe in the 1520s. Pollination is required to get the vanilla fruit from which the favouring is derived. Hand-pollination allows global cultivation of the vine. Three major species of vanilla are currently grown globally, all of which are derived from a species native to Mesoamerica, including parts of modern-day Mexico. They are *V. planifolia*, (syn. *Vanilla fragran*), grown in Madagascar, Reunion and Vanilla pompona and its subspecies found in the West Indies, Central America and South America. A majority of the world’s vanilla comes from the species Vanilla planifolia, more commonly known as Bourbon vanilla (after the former name of Reunion, lle Bourbon), or Madagascar vanilla, which is produced in Madagascar and neighbouring islands in the southwestern Indian Ocean and Indonesia. Madagascar and Indonesian together produce two-third of the World’s vanilla. Despite the expense, vanilla is highly valued for its flavour. As a result, Vanilla is widely used in both commercial and domestic baking, perfume manufacturing, and aromatherapy.

Until the mid-19th century, Mexico was the chief producer of vanilla. In 1819, French entrepreneurs shipped vanilla fruits to the islands of Reunion and Mauritius in hopes of producing vanilla there. After Edmond Albius, as the black slaves discovered how to pollinate the flowers quickly by hand, the fruits began to thrive. Soon, the tropical orchids were sent from the Reunion to the Comoros Islands, Seychelles, and Madagascar along with the instruction for pollinating them. By 1898, Madagascar, Reunion, and the Comoros Islands produced 200 metric tons of vanilla fruits, about 80% of the world production. The market price of vanilla rose dramatically in the late 1970s after a tropical cyclone storm ravaged key croplands. Prices remained high through the early 1980s despite the introduction of Indonesian vanilla. In the mid-1980s, the cartel that had controlled vanilla prices and distribution since its creation in 1030, disbanded. Prices dropped by 70% over the next few years, to nearly US$20 per kilogram.

A good crop, coupled with decreased demand caused by the production of imitation vanilla, pushed the market price down to the US$40/ kg in the middle of 2005. Another cyclone Enawo caused a similar spike to US$500/kg in 2017. Madagascar, especially in the fertile Sava region, as well as in Sambawa located in the northeast region of Madagascar, accounts for a majority of the global production of vanilla. I spent many days here to observe

the cultivation and vanillin production, as well as extraction of various essential oils. Mexico, once the leading producer of natural vanilla with an annual yield of 500 tons of cured fruits, produced only 10 tons in 2006. An estimated 95% of "vanilla" products are artificially flavoured with vanillin derived lignin instead of vanilla fruits. Today, the worldwide demand for vanilla is still high and Thailand is in the process of reviving of vanilla plantations again in the north of Thailand. I myself have started the vanilla plantation in Chiangmai, located in the north of Thailand, where conditions are perfect to cultivate.

5.8 Using wild relatives in horticultural crop breeding

The diversity of orchid species is staggering. So far, as many as 25.000 naturally occurring species have been found that are distributed across the globe from the Arctic Circle to the tip of Tierra del Fuego. However, this number is small compared to the vast array of hybrids in the orchid family. The Royal Horticultural recognizes more than 100,000 registered orchid hybrids. In fact, artificial crosses not only between species, but even between differing genera, are common. A majority of the orchids can be easily crossed in the greenhouse rather than in the wild. Hybridization may result in a spectacular blending of colours, intermediate flower forms, dwarf plants or any other multitude of variations. It is not unusual for hybrids to double the number of chromosomes within each of their cells (a process known as polyploidization), often resulting in larger plants. Hybrid vigor is also a well known phenomenon. Hybrid plants may be easier to grow, more disease resistant, and/or be able to tolerate a wide range of environmental conditions. In some cases, hybrid progeny is stronger than either of the parents.

Orchids do not hybridize frequently in nature. Nearly all of the known hybridized orchids were artificially produced under greenhouse conditions. As long as the species are closely related, hybridizers find it relatively easy to bypass the various barriers that flowers have evolved to insure that they pollimate only with individuals of their own kind. In the words of Charles Darwin, they have evolved "various contrivances" to avoid self-fertilization within the same flower and hybridization between flowers of different species. Such barrier includes synchronized time and place of flowering, attraction of specific animal pollinators that remain faithful to only one orchid species and chemical or molecular mechanisms of incompatibility recognition. Therefore, one finds relatively few orchid hybrids in nature, but they do exist. The best documented are from temperate Europe and North America and include hybrid individuals from the genera Ophrys, Dactylorbiza, and Spiranthes. Confirmed natural hybrids from tropical areas are much less common. Vanilla, however, presents a scenario that is somewhat contrary: natural hybrids have been

documented among Neotropical Vanilla species and yet artificial hybrids are essentially unknown for the genus.

Three Vanilla hybrids have been documented. Several hybrid Vanilla vines are suspected, but only three Vanilla hybrids have been confirmed definitively. One of them is an undisputed natural hybrid between a pair of species that grows side by side on the island of Puerto Rico. In several localities in southwestern Puerto Rico, there exist populations of *Vanilla clariculata* and *Vanilla barbellata* growing together, and these vines frequently flower at the same time. Both species are leafless and it is difficult to distinguish one species from the other when flowers are absent, but the two can be readily identified based on flower morphology and using DNA fingerprinting methods. Within these sympatric populations can be found Vanilla vines that produce flowers of a form "intermediate" between the two common species or F1 generation. Using a combination of genetic, morphological, and ecological evidence, researchers demonstrated that these vines are, indeed natural hybrids between the two closely related sister species *V. claviculata* and *V. barbellata*.

5.9 An Artificial Vanilla Hybrid

Armed with the knowledge that Vanilla species can hybridize, Manoo Divakaran and colleagues at Department of Botany, Providence women's college, Calicut, Kerala, India, successfully crossed, by hand, another more distantly Asian vanilla vine species under artificial conditions to test whether fertile seedlings could be generated. The two species of these Vanilla vines could have shared a common ancestor tens of thousands or perhaps even millions of years ago. The success of this experiment demonstrated that Vanilla species, even distaintly related ones, can be crossed with one another. This opens the opportunity for improving future vanilla crops such as resistance to various fungal diseases, viral disease, cold tolerance, or even novel vanillin fragrance present in vanilla fruits.

Myself, the second person to have accomplished the interspecific hybridization of *V. planifolia* and *V. siamensis* as part of my research thesis. The successful pollination percentage was 26% and I have had the fruits germinated in vitro. Presently, the plantlets are growing as F1 generation and hopefully these will flower and form fruits and seeds with back-crossing to obtain F2. This will take another ten years in order to discover new hybrid species from these two Vanilla vines (*Vanilla thailandtensis*).

5.10 Vanilla improvement-opportunity and challenges

The likely candidate for wild species for Vanilla improvement-opportunity and challenges must be the *V. siamensis*. In Thailand, *V. siamensis* is considered as one of the

"Giant" and robust vanilla vines. This lends an opportunity and a good challenge in the future interspecific hybridizations resulting in promising hybrids. *V. siamensis* is resistant to both pathogenic fungal and viral diseases. Fungals include Fusarium oxysporium, Rhytophthora, Sclerotium, and Colletotrichum. However, the disease caused by Fusarium in *V. planifolia* is very serious and has resulted in crop destruction of epidemic proportions in farms or plantations in many vanilla producing countries like Uganda, Reunion Island, Madagascar, India, and even in Thailand. Fusarium rot occurs on the roots, stems, fruits and leaves, and shoots of the plant. The stem and root infections cause the greatest level of damage, leading to a significant loss of yield and hence the disease is often referred to as a stem rot, or foot rot but the attack on the stems is most harmful due to tahe complete breakdown of the vascular phloem-xylem system.

The second pathogenic disease are the virus diseases. Virus diseases became a major concern for vanilla production over the last few decades, probably as a consequence of the expansion in the cultivation areas and intensification of vanilla growing. Viruses affect *V. planifolia*, *V. pompon*, and *V. tahitensis* plantations throughout the world, particularly Cymbidium mosaic virus (CMV). Viruses are obligatory cellular parasites that can infect bacteria, fungi, plants, animals, and humans. They have probably emerged and evolved in their hosts at the beginning of the tree of life (Forterre, 2006). Viruses are generally made of one or few genomic ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) molecules encapsulated in a protein shell called the capsid.

The second opportunity and challenge in *V. siamensis* is in the extra high level of phytoestrogen in the green fruits. This is equivalent to the female hormone oestrogen produced in the ovaries. This can be extracted from the green fruits and use to substitute the chemically produced pills for the treatment of osteoporosis in women. This research was carried out in Chiangmai, Thailand by Phenphichor Wanachantararak at the Department of Chemistry, Faculty of Dentistry, Department of Biotechnology, Chiang Mai University. The conclusive effects of phytoestrogen activity on the oestroblast cells in the bone were examined on the proliferation of hFOB 119 cells (hFOB is the conditionally immortalized or frozen human faetal osteoblastic cell line) and the bone mineralization process. This study indicated that the phytoestrogen extract from *V. siamensis* exhibited the characteristic effects of a natural bone promoting compound.

The future to improve vanilla looks bright. The protoplast isolation and fusion technology developed can be used in transfer of useful traits through the production of somatic hybrids, making way for genetic manipulation in vanilla. The characterization of vanilla species, accessions, seedlings, somaclones, and interspecifuc hybrids proved the

existence and extent of genetic variations that are available and brought about by biotechnological tools. The in vitro conservation methods, through synthetic seeds, slow growth, and cryopreservation will form an integral and important part of overall conservation strategy in genetic resources management of vanilla germplasm. Furthermore, the synthetic seed technology forms an ideal means for exchanging disease-free planting material.

5.11 Conservation of crop wild relatives: in-situ and ex-situ approaches

The most effective means of protecting the diversity of vanilla species ought to be in-situ protection in their areas of origin. However, the natural areas where the primary gene pools are found are often subjected to strong demographic pressure that endangers different species. This type of conservation can therefore only be envisaged if it is associated with a global conservative strategy at the level of a territory (Soto-Arenas 1999). However, the diversity of vanilla, which includes more than 1200 species distributed over three continents and living in varied biotopes, makes it difficult to set up in-situ conservation systems. The establishment of ex-situ collection, therefore, appears to be a necessary and obvious strategy for protecting vanilla genetic resources.

Today, the most important collections are found in France: Reunion, Internationale en Cooperation International en Recherche Agronomique pour le Developpement (CIRAD), French Polynesia, Etablissement Vanille de Tahiti (EVT), and Cherbourg (council/ Museum National d'Histoire Naturelle (MNHN) and in India, Indian Cardamom research Institute (ICRI) and in the United States. Several botanical gardens and research institutions also have varying quantities of vanilla specimens in their greenhouses (Royal Botanic Gardens, Kew, Rutgers University, Rio De Janeiro Botanical Garden in Brazil).

In total, around 50% of global diversity in terms of the number of species is conserved in these collections, essentially in the form of whole plants, in- vivo or sometimes in-vitro. However, similar to numerous other plant species, vanilla genetic resources are threatened with extinction of genetic erosion in many areas of origin and diversification. Any in-situ initiatives for conserving species must therefore be encouraged but the creation of ex-situ collection is essential ti protect the diversity. This conservation method means resources are more secure and bright with a positive future to in-vitro or cryopreservation mechanisms, and also makes it easier to promote resources and to acquire scientific data. Indeed, despite many research studies that have been made possible particularly by the widespread use of molecular biology tools, knowledge of vanilla genetics, in terms of the taxonomy of genus and the properties of different species is still incomplete.

The creation of a global network of in-situ and ex-situ collections of genetic resources,

based on branches in the three continents America, Africa, and Asia, that hold most of these resources, could result in a considerable progress in terms of the conservation and scientific and economic improvement of vanilla. The development of increasing affective genomics, associated with biotechnology techniques, means that plant breeding programs can be set up. Exploiting the specific characteristics of wild species, such as resistance to drought in aphyllous, could provide a means of diversifying vanilla production areas and anticipating future climate change. The creation and development of vanilla vines that are more disease resistant, more productive, and richer in vanillin and other aromatic compounds could improve the living conditions of small-scale growers in vanilla producing areas. To ensure that these research studies and conservation network initiatives are more effective, plant material exchanges between collections and research programs should be facilitated, especially by relaxing the rules of the Convention on Biological Diversity (CBD) in line with the existing exemption in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES).

5.12 Ecology, biology, and morphology of epiphytic orchids

There exists a bit of controversy as to whether Vanilla vines should be called terrestrial or epiphytic orchids, so the term hemiepiphytes is sometimes used for them. Initially, Vanilla seeds germinate in the soil with their roots covered in fine root hairs and penetrate deep into the rich organic humus. The roots live in association with mycorrhizal fungi that help them to obtain essential nutrients. These are the feeding roots of the Vanilla vines. After germination, the young vines begin climbing, using roots with a different form and function. These aerial roots are produced singly at each node along the stem and vary in length depending on the species. Aerial roots are weak and short, no more than 2 to 3 cm (0.8 to 1.2 cm) in length, whereas those of *V. planifolia* and many other species can grow to more than 50 cm (20 inches) in length. In some species, they can grow to more than 50 cm (20 inches) in length. It can be as long as 2 to 3 metres in length. In some species, aerial roots are straight so as to absorb moisture from the air and some are flattened to grip onto the tree trunk as seen in *Vanilla siamensis*.

In others, they coil tightly around small branches to establish a particularly strong hold. As in most orchids, the aerial roots of Vanilla develop an outer layer of cells or velamen that acts to absorb and hold water and are meant to die at maturity. Vanilla vines are thirsty plants. Some, such as *V. martinezii*, thrive in areas of nearly standing water or can be found almost exclusively in tropical palm swamps, as is the case with V. grandifloria. Living cells of the aerial roots may contain chlorophyll and assist with photosynthesis. After many years

of climbing upward, it is common for the lower portion of a vanilla vine to become damaged or die. But it is still able to derive all of the water that it needs from the aerial roots and much of its nutrition from minerals dissolved in the rainwater that trickles down its host's trunk. At this point, the vines can truly be called epiphytes. Being climbers, Vanilla vines need to have flexible stems, and many do. However, their stem morphology varies from species to species, *V. bicolour*, *V. aphylla* produce thin wiry stems, no thicker than a pencil, whereas those of *V. siamensis*, *V. dilloniana*, may be as thick as a baby's arm. In most species, the stem is smooth and green. In other Vanilla vines, it may have a rough warty texture, and the stems of some of the leafless Caribbean species of Vanilla commonly exhibit a bronze or reddish orange colour when growing in full sunlight, as seen in *V. barbellata* and *V. claviculata*. In these leafless species, the stems and/or roots have become the primary photosynthetic structures. Such leafless species also exhibit a groove that runs along the length of a stem, giving the vine a C-shaped outline in cross section. With this morphology, the vines are able to shrink by curling inward during extreme periods of drought without causing permanent anatomical damage as might occur if they were entirely round.

Vanilla dietschiana stands out from other species in the genus on account of its growth habit, whose stems have lost their climbing ability. This species from eastern Brazil is a short and erect member of the primitive group of species related to *V. mexicana*, *V. edwallii* and *V. inodora*. *Vanilla planifolia* and *V. siamensis* grow as a vine, climbing up an existing tree (also called a tutor), pole or other support. It can be grown in wood (on trees), in a plantation (on trees or poles), or in a "shader", with increasing order of productivity. Its growth environment is referred to as its terroir, and includes not only the adjacent plants, but also the climate, geography, and local geology. Left alone, it grows as high as possible on the support, very much like the vines of Asian wild vanilla, *V. kinabaluensis*, *V. atrophan*, and *V. grandifloria* in Uganda, resulting in a few flowers. Every year, growers in Madagascar and other *V. planifolia* vines producing countries, fold the higher parts of the vine downward so that the vine stays at height accessible to a standing human and also greatly stimulates flowering.

5.13 Cultivation

In general, quality vanilla only comes from good vines and through careful production methods. Commercial vanilla production can be performed under either open field or "greenhouse" operation. The two production systems share these similarities: 1. Plant height and number of years before it starts producing the first seeds and fruits. 2. Shade necessities. 3. Amount of organic matter needed. 4. A tree or frame to grow around (bamboo,

coconut, mango trees, or even cement poles). Vanilla grows best in a hot, humid climate from sea level to an elevation of 1500 metres above sea level. The ideal climate should have moderate rainfall, 1500-3000 mm, evenly distributed through 10 months of the year. Optimum temperatures for cultivation are 15-30 °C (59-80 °F) during the day and 15-20 °C (59-68) °F) during the night. Ideal humidity is around 80% and under normal greenhouse conditions, it can be achieved by an evaporative cooler. However, since greenhouse vanilla is grown near the equator and under polymer netting (shading of 50%), such levels of humidity are generally available naturally. Most successful vanilla growing and processing is done in the region within a band of 10 to 200 latitude of the equator.

Soils for vanilla cultivation should be loose, have high levels of organic matter, and loamy texture. They must be well drained, and so areas with a slight slope are conducive. Soil pH level has not been well documented, but some researchers have indicated an optimum soil pH of around 5.3. Mulch is very important for proper growth of the vine, and a considerable portion of mulch should be placed in the base of a vine. During the propagation, preparation, and type of stock, the dissemination of vanilla can be achieved by stem cutting or by tissue culture. For stem cutting, a progeny garden needs to be established. All vines need to grow under 50% shade, as well as the rest of the crop. In a Vanilla tissue culture, the approach is inspired by tissue culture of other flowering plants. Several methods have been proposed for vanilla tissue culture but all of them begin from the axillary buds of a vanilla vine. In vitro multiplication has also been achieved through culture of callus masses, protocorms (a tuber-like body provided with rhizoids produced in the place of a hypocotyl with roots), root-tips, and stem nodes. Orchid pollination biology includes pollinia, male and female reproductive success, how infrequent pollination is in orchid, and how often the pollination of orchids goes unnoticed.

In general, the first flowering happen three years following the planting. When trees are used as supports, or when vanilla is cultivated in shade houses, flowering initiates during the late second year, even when smaller cuttings (80-100 cm long) are used, since the plants tend to grow more vigorously as a result of more consistent shade and management. Vanilla normally flowers once a year in the northern hemisphere and twice a year in the southern hemisphere, due to a higher availability of natural sunlight. There are several main factors promoting flowering. The physiological cue to flower is promoted by climatic or mechanical stress, for example: a. Drought: Water stress induces reproductively mature individuals of vanilla and many other plants to flower.

In Uganda, vanilla flowers twice a year, as a result of the country having two distinct dry seasons. b. Cool temperatures: The principle stress in Mexico, Central and South

Americas that induces flowering are the lower temperatures of autumn-winter, when cool air masses known as “nortes” blow down more or less unimpeded from the Artic Circle, dropping temperatures to below 10 °C; the lower the temperature, the greater is the expected flowering durig that year. Cooler temperatures “burn” the apical tip, killing it and break the apical dominance of the plant while stimulating lateral floral buds to develop. c. Plant management:

In India, growers are recommended to perform a series of tasks two months before flowering: (1) pruning the apical tip by at least 10-15 cm, (2) temporary suspension of irrigation, (3) abundant use of irrigation once 10% of the blooms have opened, (4) pruning of the support tree canopy to increase luminosity by up to 75%, but only when the intensity of sunlight is not so high that it causes burning (light is an important factor in activating floral buds: Hernandez Apolinar 1997) and (5) allowing the shoots to reach a height of 1.5 m and placing them on a horizontal support so as to increase flower quantity and the number of serial roots.

There are two methods of pollination in orchid. One is by natural pollination and the second is by artificial hand-pollination. Bees in the genus Eulaema are members of the tribe Euglossi, which are usually characterized by solitary behavior, iridescent colouration, and a fondness among the males for collecting fragrant oils and resins from orchid flowers (Odoux and Grisoni 2010).

In some cases, the male bees appear to use their collected orchid fragrances in courtship with females, Eulaema is one of the few orchid bees whose body is not metallic green. Instead, the twenty-five or so species are brown or black bees, with robust, hairy bodies that are often have yellow or orange stripes, giving them an appearance more typical of a bumble bee. After years of speculation, it is now well established that Euglossine bees are the natural pollinators of *Vanilla planifolia* and other Neotropical species. The tribe Euglossini, in the subfamily Apinae, commonly known as orchid bees or euglossine bees, are the only group of corbiculate bees whose non-parasitic members do not possess an eusocial behavior. Most of the tribe’s species are solitary though a few are communal, or exhibit simple forms of eusociality. There are about 200 described species, distributed in five genera: Euglossa, Eulaema, Eufriesea, Exaerete, and the monotypic Aglae. All these species are exclusively found in South or Central America. The genera Exaerete and Aglae are kleptoparasites, and reside in the nests of other orchid bees. All species, except Eulaema, are characterized by brilliant metallic colouration, primarily green, gold, and blue. Females gather pollen and nectar as food from a variety of plants and resins, mud, and other minerals for nest building.

Some of the same food plants are also used by the males, which leave the nest upon hatching and do not return. Fragrance collectign male orchid bees have uniquely modified legs which are used to collect and store different volatile compounds, often as esters throughout their lives, primarily from orchids in the subtribes Stanhopeinae and Catasetinae, where all species are exclusively pollinated by euglossine males. These orchids do not produce nectar and hide the pollen on a single anther under an anther cap and they are not visited by females. The whole pollinarium becomes attached to the male as it leaves the flower.

The chemicals are picked up using special brushes on the forelegs, and its transferred from there by rubbing the brushes against combs on the middle legs and finally these combs are pressed into groves on the dorsal edge of the hind legs, squeezing the chemicals past the waxy hairs which block the opening of the grove and into a sponge-like cavity inside the hind tibia. The accumulated fragrances are evidently released by the males at their display pites in the forest understory, where matings are known to take place (Odoux and Grisoni 2010). The accumulated volatiles were long believed to be used by males as a pheromone to attract females, however, female attraction to male odours or to orchid fragrances has never been demonstrated in behavioral experiments. Instead, it is now thought that the function of male odours is to signal male “genetic quality” to females (Odoux and Grisoni 2010), as collecting orchid fragrances needs great effort by males to and than only the fittest males can gather complex odour mixes.

5.14 Artificial Hand-pollination

To produce Vanilla fruits without the aid of insect pollination, humans must transfer the pollen from the anther to a stigma. The first person to have achieved hand pollination was a black slave named Edmond Albius in 1841. He discovered that with a small piece of bamboo inserted sideways into a flower just under the rostellum can be used to lift it upward and forward. In this way, the anther can be flipped forward and pressed into the stigma to deposit a smear of pollen. To avoid self-pollination in nature, Vanilla flowers have evolved flip of tissue, rostellum, that separates the anther from the stigma, the sticky receptive portion of a female pistil. Once fertilized, the ovules become seeds and the ovary become fruits. If it has failed, then the blossom drops within two to three days. The full length of *Vanilla planifolia* fruits takes up to nine months to reach maturity.

As for *Vanilla siamensis*, this takes eighteen months. The hand pollinator workers do not pollinate all the flowers that they can. The flowers near the base of an inflorescence produce the longest and straightest fruits, whereas those produced near the top do not hang

down as easily. In addition, pollinating too many flowers on a given vine can weaken the plant, resulting in inferior vanilla fruits at best or vines that fail to flower the next year at worst.

5.15 Self-pollination in Vanilla vines

In Brazil, *V. palmarum* vines also inhabit the top of palm trees; they are said to be in buds throughout the year, but only flower when they get wet after rain. This can be induced artificially with a sprinkling of water. Pollinators have never been observed near the open flowers, and yet there is an unusually high rate of fruit set (greater than 76 percent) in *V. palmarum*. This pattern is best explained by self-pollination, in which the flower's stigma secretes an excess of fluid which floods the apex of the column and allows some of the pollen grains to leak across the restrictive rostellum barrier. The mature fruits of this species are not fragrant and the flowers are small and not particularly striking. These aspects, coupled with the unusual habitat and flowering requirements of *V. palmarum*, make this a poor candidate for cultivation (Odoux and Grisoni 2010; Havkin-Frenkel and Belanger 2011).

5.16 Vanilla seed germination

Nearly all vanilla produces minute black seeds that do not germinate under natural conditions without a close relationship with mycorrhizal fungi. Tissue culture techniques can be used to successfully germinate the seeds. Protocols for seed and embryo culture of vanilla have been standarlized (Gu et al. 1987; Knudson 1950; Minoo et al. 1997; Withner 1955). Seed culture in different basal media indicate that vanilla seeds have no stringent nutritional requirements for the initiation of germination, unlike some terrestrial orchids growing in temperate climates (Minoo et al. 1997). The germination of seeds begins within four weeks of culture and the initial stages of germination are common in most orchids, such as swelling of the embryo followed by rupturing of a seed testa and the subsequent emergence of protocorms. Seeds germinate directly into plantlets in a medium supplemented with benzyladenine (BA) alone, without any intervening callus phase. In treatments with BA, most of the protocorms remain the same with a scale-like leaf primodial and developing into shoots. Murashige and Skoog's (MS) medium gives a better response than Knudson's medium, for in-vitro cultures of vanilla. However, in-vitro culture can be used for the germination of seeds and selection of useful genotypes by segregating progenies that might be mass propagated for obtaining disease-free planting material.

5.17 Vanilla Cryopreservation

Protocols for conservation of gene pools have been developed for slow growth as well as cryopreservation of accessions as encapsulated short tips, pollen, and DNA (Bahadur et al. 2015). Combining the available gene pool in the genus will help in broadening the genetic base and in combining the useful genes into cultivated vanilla from wild species. Interspecific hybridization requires synchronized flowering between the species and availability of viable pollen. Pollen from two asynchronously flowering *V. planifolia* and its wild relative *V. siamensis* were cryopreserved after desiccation, pretreated with cryoprotectant dimethyl sulfoxide and cryopreserved at -1960C in liquid nitrogen . The cryopreserved pollens of *V. planifolia* were successfully used to pollinate *V. siamensis* flowers resulting in a fruit set (Gonzalez-Arnao et al. 2009). The seeds obtained from the F1 generation were successfully cultured to develop hybrid plantlets. Viability and fertility assessment of cryopreserved pollen from Vanilla species showed that it is possible to use cryogenic methods for conservation and management of the haploid gene pool of this species. This is of great importance for facilitating crosses during the breeding programme, for distribution and exchange of germplasm and for preserving nuclear genes of the germplasm (Odoux and Grisoni 2010).

6. The Climate of Thailand

6.1 General Climatic Conditions

The climate of Thailand is influenced by monsoon winds of seasonal character, i.e., southwest and northeast monsoon. The southwest monsoon, which starts in May, brings a stream of warm moist air from the Indian Ocean towards Thailand causing abundant rain over the country, especially in the windward side of the mountains. Rainfall during this period is not only caused by the southwest monsoon but also by the Inter Tropical Convergence Zone (ITCZ) and tropical cyclones which produce a large amount of rainfall. May is the period of first arrival of the ITCZ in the Southern part. It moves northwards rapidly and prevails across southern China around June to early July, which is the reason of a dry spell over upper Thailand. ITCZ then moves in a southerly direction over the Northern and Northeastern Parts of Thailand in August and later over the Central and Southern parts in September and October, respectively. The northeast monsoon which starts in October brings cold and dry air from the anticyclones in China mainland over major parts of Thailand, especially the Northern and Northeastern parts, which are at higher latitudes. In the Southern part, this monsoon causes mild weather and abundant rain along the eastern coast. The onset

of monsoons varies to some extentwith the Southwestern monsoon usually starting in mid-May and ending in mid-October, while northeastern monsoon normally starting in mid-October and ending in mid-February.

6.2 Season

From a meteorological point of view, the climate of Thailand may be divided into three seasons as follows:

6.2.1 Rainy or southwestern monsoon season (mid-May to mid-October). The southwest monsoon prevails over Thailand and the country experiences abundant rain. The wettest period of the year is from August to September. The exception is in the East coast of Southern Thailand, where it rains abundantly until the end of the year, which is the beginning of the northeastern monsoons and November is the wettest month.

6.2.2 Winter or northeastern monsoon season (mid-October to mid-February) is the mildest period of the year with cold in December and January in upper Thailand. However, there is a great amount of rainfall in the East coast of Southern Thailand, especially during October and November.

6.2.3 Summer or pre-monsoon season during mid-February to mid-May is the transitional period from the northeast to southwest monsoons. The weather becomes warmer, especially in upper Thailand with April being the hottest month.

6.2.4 Surface Temperature

Upper Thailand, i.e., the Northern, Northeastern, Central, and Eastern parts usually experience a long period of warm weather because of they are situated inland and fall under the tropical latitude zone. March to May is the hottest period of the year, when maximum temperatures usually reach near 40ºC or more, except along coastal areas where sea breeze moderates afternoon temperatures (Table 1.1, 1.2, 1.3). The onset of rainy season also significantly a reduction in the temperatures from mid-May and are usually lower than 40ºC. In winter, the incoming cold air from China occasionally reduces temperatures to fairly low values, especially in the Northern and Northeastern parts, where temperatures may decrease to near or below zero. In the Southern part, the temperatures are generally mild throughout the year because of a maritime characteristic of this region. The high temperatures common to upper Thailand occur seldomly. The diurnal and seasonal variations in temperatures are significantly less than those in upper Thailand.6.2.5 Rainfall

Upper Thailand usually experiences dry weather in winter because of the northeast monsoon, which is a main factor controlling the climate of this region. The

summers are characterized by a gradually increasing rainfall accompanied by thunderstorms. The onset of southwestern monsoon leads to intense rainfall from mid-May until early October. Rainfall peaks around August or September, during which time some areas are flooded (Table 1.4). However, dry spells commonly occur from June to early July. Rainy season in the Southern part is different from that in upper Thailand. It rains abundantly during both the southwest and northeast monsoon periods. During the southwest monsoon, the West coast of Southern Thailand receives high rainfall and reaches its peak in September. On the contrary, the rainfall in the East coast of Southern Thailand peaks in November until January of the following year, which is also the beginning of northeast monsoon.

According to the general annual rainfall pattern, most areas of the country receive 1,200 - 1,600 mm per year (Table 1.4). Some areas on the windward side, particularly Trat province in the Eastern part and Ranong province in the West coast of Southern Thailand, receive more than 4,500 mm a year. Annual rainfall less than 1,200 mm occurs in the leeward side which lie in the central valley and the uppermost portion of Southern parts of the country.

Table 1.1 Seasonal temperatures (ºC) in various parts of Thailand. Based on the period from 1981-2010.

Temperature	Region	Winter	Summer	Rainy
Mean	North	23.4	28.1	27.3
	Northeast	24.2	28.6	27.6
	Central	26.2	29.7	28.2
	East	26.7	29.1	28.3
	South	26.3	28.2	27.8
Mean maximum	North	31.1	36.1	32.4
	Northeast	30.6	35.2	32.6
	Central	32.3	36.2	33.4
	East	32.0	34.1	32.3
	South	30.4	33.0	32.7
Mean minimum	North	17.5	21.8	23.8
	Northeast	18.7	23.2	24.4
	Central	21.2	24.6	24.8
	East	22.3	25.2	25.2
	South	22.8	24.1	24.4

Table 1.2 Extreme maximum temperatures (ºC) in summer season. Based on a period from 1951-2015.

Region	Maximum temperature	Date/Month/Year	Province
North	44.5	27 Apr 1960	Uttaradit
Northeast	43.9	28 Apr 1960	Udon Thani
Central	43.5	29 Apr 1958	Kanchanaburi
		14 Apr 1983	
		14,20 Apr 1992	
East	42.9	23 Apr 1990	Prachin Buri
South			
- East Coast	41.2	15 Apr 1998	Prachuap Khiri Khan
- West Coast	40.5	29 Mar 1992	Trang

Table 1.3 Extreme maximum temperatures (ºC) in Winter

Region	Maximum temperature	Date/Month/Year	Province
North	0.8	27 Dec 1999	Tak
Northeast	-1.4	2 Jan 1974	Sakon Nakhon
Central	5.2	27 Dec 1993	Kanchanaburi
East	7.6	16 Jan 1963	Sra Kaeo
South			
- East Coast	6.4	26 Dec 1999	Prachuap Khiri Khan
- West Coast	13.7	21 Jan 1956	Ranong

Based on 1951-2015 period

Table 1.4 Seasonal rainfall (mm) in various parts of Thailand. Based on a period between1981-2010.

Region	Winter	Summer	Rainy	Annual rainy days
North	100.4	187.3	943.2	122
Northeast	76.3	224.4	1,103.8	116
Central	127.3	205.4	942.5	116
East	178.4	277.3	1,433.2	130
South				
Coast -East	827.9	229.0	680.0	145
Coast - West	464.6	411.3	1,841.3	178

Based on 1981-2010 period

6.2.6 Relative Humidity

Thailand is covered by warm and moist air during most times of the year except areas farther inland, where the relative humidity may significantly reduce in winter and summer. For example, the extreme minimum relative humidity values are measured around only 9 % at Loei and Chiang Rai on 23 March 1983 and 23 April 1990, respectively (Table 1.5). In the Southern part with a maritime characteristic climate, the humidity is relatively higher.

Table 1.5 Relative humidity (%) in various parts of Thailand

Region	**Winter**	**Summer**	**Rainy**	**Annual**
North	74	63	81	74
Northeast	69	66	80	73
Central	70	68	78	73
East	71	75	81	76
South				
Coast - East	81	78	79	79
Coast - West	78	77	84	80

Based on 1981-2010 period

6.2.7 Cloudiness

Cloud cover is normally less from November to March. Perfectly clear skies are generally found that is a reason why extreme temperatures usually occur. Most clouds in this period are high clouds but cumulus and cumulonimbus may be seen on some occasions. During the southwest monsoon, most clouds in the sky are convective clouds. Clear skies are seldom in this period except during June which has a few days. Cloud cover is normally less from November to March. Perfectly clear skies are generally found which result in the occurrence of extreme temperatures. Most clouds during this period are high clouds but cumulus and cumulonimbus may be seen on some occasions. During the southwest monsoon, most clouds in the sky are convective clouds. Clear skies are seldom observed during this period except in June, in which the occurrence is low.

6.2.8 Thunderstorms

Thunderstorms in upper Thailand often occur during the period from April to October, while those in the southern part experience themduring March to November. The maximum frequency of thunderstorms in upper Thailand is in May. Convection and the confluence of cold and warm air streams, is the main factor for thunderstorms. The afternoon and evening thunderstorms occur due to convection while the others occur due to a confluence of winds from different airstreams.

6.2.9 Surface Wind

The pattern of surface wind direction is characterized by the monsoon system. The prevailing winds during the northeast monsoon season are mostly north and northeast in upper Thailand and east or northeast in the Southern part while they are in the south, southwest, and west direction over the country during the southwest monsoon. In summer, the prevailing wind direction is mostly south, especially in upper Thailand.

Thailand normally is affected by tropical depressions because of its location farther inland and some mountain ranges which obstruct and decrease the wind speed before they move into Thailand, except in the Southern part, which has a relatively high risk of tropical storms and typhoons (Table 1.6). For instance, the tropical storm “HARRIET” hit Nakhon Si Thammarat province in October 1962 and the typhoon “GAY” hit Chumphon province in November 1989 and the latest one was the typhoon “LINDA” which hit Prachuap Khiri Khan province in November 1997, as it was tropical storm. By considering the annual mean, tropical cyclones usually move across Thailand about 3 - 4 times a year. From January to March, Thailand has never experienced such cyclones. According to historical data, it can be seen that April

is the first month in which tropical cyclones move across Thailand. A relatively higher frequencies are found from May onwards, particularly September and October. They usually pass through the Northern and Northeastern parts during the early southwest monsoon season and will move across the southern Thailand from October to December.

Table 1.6 The frequency of tropical cyclones moving through Thailand during 65 years (1951 - 2015)

Region	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total
North	-	-	-	-	5	2	10	17	25	11	1	-	71
Northeast	-	-	-	-	1	6	4	18	33	25	4	-	91
Central	-	-	-	-	2	1	1	-	7	9	2	-	22
East	-	-	-	-	1	1	1	-	3	13	2	-	21
South	-	-	-	1	1	-	-	-	3	15	24	9	53

7. *Vanilla siamensis* (Rolfe ex Downie) in Thailand

In Thailand, five different vanilla vines are to be found, namely *Vanilla graffithii*, *V. pilifera*, *V. aphylla*, *V. albida* and *V. siamensis*. All these Asian vanilla vines do not produce any vanillin for commercial purposes. This is unlike *V. planifolia*, *V. pompona*, and *V. tahitiensis*, which all produce the vanillin aroma and fragrance. So much so that these Vanilla vines are the second most important herbs and spices in the world after saffron. The Asian vanilla vines are not well known or documented and not much research has been done till date about them. Only one of these Asian vanilla vines that has a slight, distinct vanilla aroma during maturation is the *V. siamensis*, but the amount is not enough to grow and extract the vanilla commercially.

For the distribution of *V. siamensis* on a lager scale, it is found in 11 Province in Thailand (Fig. 1.2). In this study, *V. siamensis* is found in the hill evergreen forest, Dry evergreen forest, and Tropical rain forest, which are situated at elevations of approximately 500 to 1,000 msal.

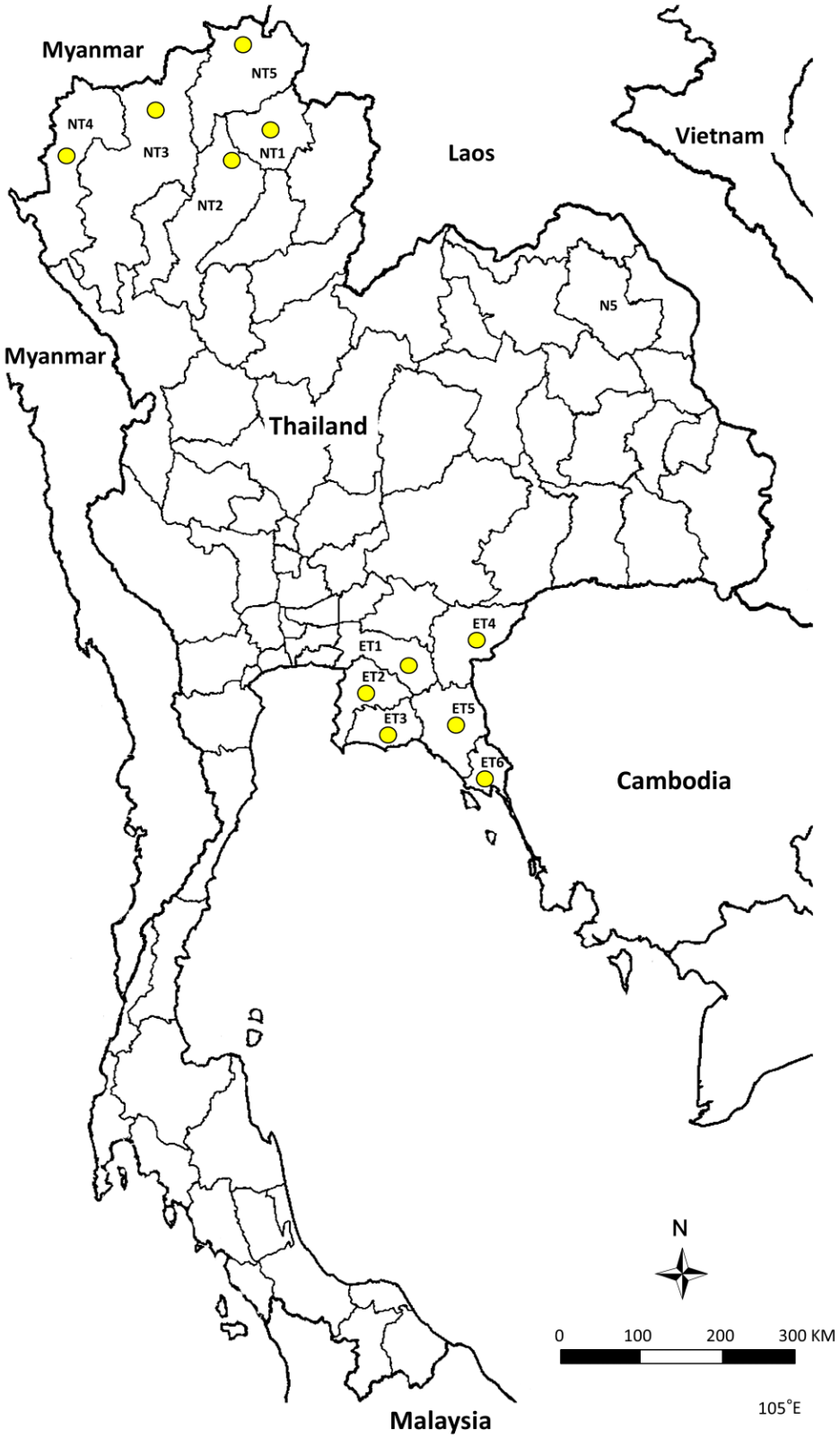

Figure 1.1 Map show the distribution of *Vanilla siamensis for* in 11 province in Thailand. Abbreviation is; NT1 = Pha Yao Prov., NT2 = Lampang, NT3 = Chiang Mai, NT4 = Mae Hong Son, NT5 = Chiang Rai, ET1= Chachoengsao, ET2= Chon Buri, ET3= Rayong, ET4= Sa Kaeo, ET5= Chanthaburi and ET6= Trat

8. Thai Orchids in general: Yesterday, Today, and Tomorrow

Thailand is situated in the heart of tropical Asia, is naturally blessed with many indigenously cultivated and wild orchid species. However, Northern Thailand, which is an extension of the Himalayan mountain range, has been endowed with lush forests and orchids of many species. For generations, local inhabitants have cultivated forest orchids in their villages but grew them in conditions similar to that observed in wild by attaching them to trunks of large shade trees surrounding their homes. They grow orchids such as Dendrobium, Vanda, and Paphiopedilium species, while the wild orchids were left unnoticed, unattended, and totally ignored. In 1875, when Thailand was still an absolute monarchy named Siam, most of the development in terms of material wealth was still confined to Bangkok, when the first assignment of European missionaries came to work in Siam and brought many new western ideas, one of them being the raising of orchids. This included the construction of an orchid house and planting them in man made containers. Some other trees introduced were rubber plants from Brazil, coffee from Africa, cocoa from Mexico, and wild orchids native to Central and South American countries, which became very popular due to their fragrances. However, most orchids introduced either died and only few have survived to this day. Likewise, in various colonies of Britain and France, like India, Indonesia, and even as far as Tahiti, the *Vanilla* vines were introduced and still thrive to this very day. In 2015, prior to my research registration, I was given permission to survey the wild *Vanilla siamensis* in Chiangmai (Pongkrai) located in the north of Thailand. This private property area of well over 100 acres is a primary forest and was found to be a perfect ecosystem with all possible flora and fauna, especially the wild trailing *V. siamensis*. All favorable external factors were perfect, with high elevation at 900 meters, high moisture content, high humility, and cool winter months needed to boost the flowering with frequent mist.

In the monsoon season, plenty of rain soaks the forest well and is well drained with waterfalls and streams. The forest itself was full of tall trees, animals, and birds. The *Vanilla* vines were clinging for strong support and shade, showing a perfect habitation. The most fascinating thing about this vine is during the flowering season in May and June. The flowers are about the size of a coffee cup and it is from these flowers that fruits or pods develope. The attractive flowers are characterized by a tuft of fingerlike projections at the apex of the labellum. The flowers come in two colours, namely white and red. However, the white flowers are most frequently found than the red flowers. Over the next few years, the owner of this private property decided to clear the forest for a coffee plantation, which is becoming a lucrative business in South East Asia.

Nowadays, more land in the north of Thailand is being cleared for such coffee plantations on a large scale. Thailand hopes to be the second major export of coffee beans both in Arabica and Robusta after Vietnam in few years time. Encouragement from the Ministry of Agriculture and the private sector for more coffee plantations has increased the area to around 50%. Strange to say, coffee beans from Thailand and Vietnam are being blended with Vanilla flavor derived from from Madagascar and Indonesia to manufacture the popular three in one instant Nestle coffee mix. In general, a lot of land is being cleared in the north of Thailand for agriculture which has resulted in the natural surviving habitation gradually diminishing and consequently the wild *V. siamensis*. At the moment *V. siamensis* is considered as a weed as it does not produce any Vanillin or is of no other usage to the farmers, but for a botanist, ecologist, conservationist, and a scientist, it has a potential future.

9. Research Aims

This thesis purposes to:

(1) To foster as much understanding as possible about the wild *V. siamensis* in Thailand. This species of Vanilla is little known and not many papers or researches have been reported on this subject in great detail, like the *V. planifolia*.

(2) To encourage their conservation in natural habitats as a part of future scientific pharmaceutical property in phytoestrogen.

(3) To discourage collection and destruction of wild Vanilla specimens by encouraging the artificial propagation of endangered species in ex situ. I have set up my own "Conservation Project", a Vanilla vines nursery in Chiangmai, in the north of Thailand. I have collected unwanted wild Vanilla vines from a private property and those that were removed from the forest by the forest rangers due to their overgrown mass collection and have planted them in my very own nursery.

(4) To promote the future commercial horticultural studies of *V. siamensis* orchids and their seed germination, plantlet regeneration, and Cryopreservation.

10. Thesis outline

I have divided the specific aims in the following parts of the Thesis and are outlined as under:

Chapter 2 The current impact of environmental factors, including the specific host on the distribution and destruction of *V. siamensis* in a forest ecosystem. The most effective

means of protecting the diversity of Vanilla species ought to be in situ protection in their areas of origin. However, the natural areas where the primary gene pools are found are often subject to strong demographic pressure that endangers the different species. The establishment of ex situ distribution therefore appears to be a necessary strategy for protecting the genetic resourses.

Chapter 3 Further investigation into the relationship and understanding of floral morphology, fruit-set, and potential pollinator of *V. siamensis.*

Chapter 4 *V. siamensis* produces numerous minute black, shiny seeds, with thick testa in the outer skin. They do not germinate under natural conditions. Tissue culture techniques can be used to successfully germinate the seeds. Protocols for seed and embryo culture of *V. siamensis* have been standardized.

Chapter 5 To develop a new protocol strategy for cryopreservation of *V. siamensis* in terms of survival rate and regeneration frequency of new multiple shoots.

Chapter 6 Conclusion and future Prospects. This is the first research ever done on wild *V. siamensis* in Thailand. Needless to say the genetic resources of *V. siamensis,* like many other wild Vanilla species, are threatened with extinction or genetic erosion in many areas of Thailand. Any in situ initiatives for conserving the species must therefore be encouraged, but the creation of ex situ collection is essential for protecting the diversity. This conservation method means resources are more secure due to in vitro or cryopreservation techniques. The collection of global network of in situ and ex situ collections of genetic resources, morphology, pollination, fruitset, and germination could result in a considerable progress in terms of the future conservation, and scientific and economic improvement of *V. siamensis* in Thailand. The development of increasing effective genomics, associated with biotechnology techniques, means Vanilla breeding programs can be set up. To exploit this, the interspecific hybridization characteristics of wild *V. siamensis*, like resistance to drought and to disease. To produce a new F1 generation richer in vanillin and other aromatic compounds, and bigger fruits. To produce and obtain a future second commercially hybridized *V. thailandtensis* after *V. tahitensis* in Tahiti, in Thailand.

Chapter 2: Impact of Ecological Factors on the Distribution of *Vanilla siamensis* Rolfe ex Downie (Orchidaceae: Vanilloideae) in Tropical Forest at Khao Soi Dao Wildlife Sanctuary, Chantaburi, Thailand

1. Abstract

Some species of the pantropical orchid genus Vanilla are widely used in a variety of industries, including food, pharmaceuticals, cosmetics, tobacco and traditional crafts. *Vanilla siamensis* is a leafy vine endemic to Thailand. Ecological factors affecting the distribution of *V. siamensis* are still unknown.

This study aims to examine the distribution of *V. siamensis* in natural conditions as well as to investigate the impact of environmental factors on its distribution in forest ecosystems. In order to achieve the proposed objectives, a study was conducted in a tropical moist forest at Khao Soi Dao Wildlife Sanctuary. The plant community and environmental factors where *V. siamensis* occurs were assessed in two study sites. At each site, two plots (10 m × 50 m) were set up near waterfalls (NWA) and in adjacent forest areas (AFA).

A total of 66 species of woody plants were recorded in the study plots. Higher values for importance value index (IV) were found for *Pterygota alata*, *Diospyros defectrix*, *Strombosia ceylanica*, *Alchornea rugosa* and *Diospyros transitoria*.

The highest number of tree species, number of individuals, Shannon diversity index and evenness were found at the NWA compared to the AFA. A total of 19 woody plant species act as support trees of *V. siamensis* in the plant families Malvaceae, Annonaceae and Achariaceae.

The results showed that *V. siamensis* might possible well adapted for survival in the NWA study zone which contained a higher level of soil moisture content compared to the AFA. All results revealed that the distribution of *V. siamensis* in the study areas could be impacted by tree hosts. These species have a larger diameter at breast height and thicker and softer bark than non- tree hosts.

2. Introduction

The genus *Vanilla* has a pantropical distribution (Medina et al. 2009; Hernandez-Ruiz et al. 2016), containing the notable species *V. planifolia* and *V. pompona* from the tropical Americas and the hybrid *V. tahitiensis*, that are used in a variety of industries, including food, pharmaceuticals, cosmetics, tobacco and traditional crafts (Medina et al. 2009). Extracts of

V. siamensis, a leafy vine endemic to Thailand (Cameron 2011a; Pedersen *et al*., 2011), exhibit the characteristic effects of a natural bone-promoting compounds such as phytoestrogen (Wanachantararak 2012). In spite of being a distinctive species of pharmacological interest, detailed information on the distribution, ecology and biology of *V. siamensis* is sparse. An understanding of the role of the various ecosystem components in terms of its survival and colonisation would be beneficial for its conservation and potential utilisation.

Vanilla is unusual amongst other orchids in that it germinates in the soil and then climb up the stems of tree hosts by use of adherent roots, eventually becoming epiphytic after losing contact with the soil (Cameron 2011b). As such, the species appear to possess the ability to colonise a range of tree species (Zimmerman and Olmsted 1992; Muñoz et al. 2003; Bergstrom and Carter 2008), although in the case of *V. siamensi*s isolated large trees seem to be favoured (Patel 1996; Harrison et al. 2003; Muñoz et al. 2003; Bergstrom and Carter 2008). Information on the distribution, identity and size of host trees for *V. siamensis* and other species of *Vanilla* remains fragmentary.

The aim of this study is to investigate the impact of environmental factors on the distribution of *V. siamensis* in forest ecosystems. Specifically, we aim to examine the degree to which *V. siamensis* is host specific and/or size class dependant under natural condition; in addition, the diversity and composition of the vegetation at various elevational levels were analysed.

3. Materials and Methods

The study was conducted in the Khao Soi Dao Wildlife Sanctuary (KSD-WS), Chanthaburi, Thailand (Fig. 2.1a), chosen for harbouring easily accessible populations of *V. siamensis*. The forest at the study site of the Khao Soi Dao waterfall is classified as a tropical moist forest. We divided the sample plots into two areas, namely, upper areas (UP) located at 439 m above sea level (asl) and lower areas (LW) located at 372 m asl (Fig. 2.1b; Fig. 2.2). At each study area, two permanent plots (10 m × 50 m) were constructed near waterfall areas (NWA) and in adjacent forest areas (AFA) at a distance of at least 20 m from each other (Fig. 2.3; Fig.2.4). In every sample plot, five quadrats of 10 m × 10 m were constructed for plant sampling and measurement of environmental factors (Fig. 2.1c). Field work was conducted over a 1-year period from November 2016 to July 2017.

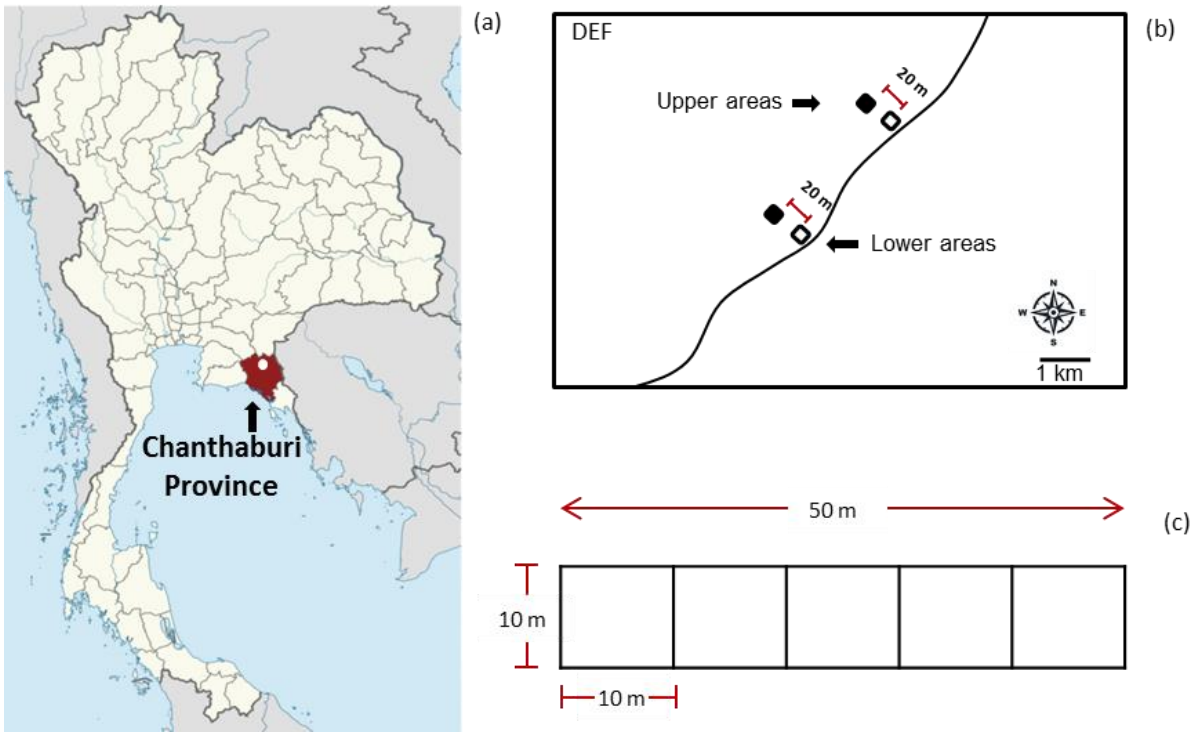

Figure 2.1 (a-b) The study site in the Khao Soi Dao Waterfall, which is located in a Dry evergreen forest (DEF) Khao Soi Dao Wildlife Sanctuary, Chanthaburi, Thailand (Fig). (b) The sample plots (50 m × 10 m) were set up near waterfall areas (□; white square) and adjacent to forest areas (■; black square), and (c) 5 quadrats of 10 m × 10 m size were set up in every sample plot.

3.1 Distribution of *V. siamensis* at Kao Soi Dow waterfalls

Surveys of distribution of *V. siamensis* in larger scale, which were conducted in Thailand where a tropical rain forest have. For small scale, here, this research was describing the distribution of *V. siamensis* within one natural forest areas, where is present of larger number of *V. siamensis* vine. This research was conducted at Kao Soi Dow waterfalls, which are located at Kao Soi Dow Plant and Animal Sanctuary, Chantaburi, in the eastern part of Thailand. Here, distribution of *V. siamensis* was described by using unmanned aerial vehicle (Drone; Fig. 2.2)

Figure 2.2 Survey on distribution of *V. siamensis* by using unmanned aerial vehicle

Phantoms 3 drone was used to take aerial pictures of Kao Soi Dow waterfalls mountainous location, from the highest level to the lowest level. The mission is to find the origin of the waterfalls from the highest point, inaccessible by foot. To study the topography of this region, especially the host trees growing on the edge (inner upper and inner lower of the waterfalls) from the highest point to the lowest point. To locate as many trees as possible with Vanilla vines growing and attaching onto the host trees. To map the "danger zone" and the movement of the wild elephants from the research area. Using rechargeable lithium polymer batteries, each drone can make flights of about 10 to 15 minutes. The computers in the drones are similar to those used in smart phone or mobile phone. When the Phantom is turned on, its computer starts the GPS and the flight control systems runs through a one to two minute process of locating and locking on to GPS satellites to establish the drone's home

position. When launched properly by allowing the flight control system to orient itself with the satellites, the Phantom drone will return one metre of its home position when the operator turns the transmitter off.

However, the new Phantom 4 Pro is now sixty per cent quieter than its predecessor, allowing for better resolution and lower latency. Both Phantom 3 and Phantom 4 drones were used at KSD. The three main purposes of Phantom 3 and 4 usages are: 1) To view land use and map covering, 2) To observe Human activity detection and 3) To evaluate Biodiversity surveys.

3.2 Plant sampling

All trees possessing a stem diameter of 1.3 m at breast height were examined for each study plot; data recorded included the species name, diameter and tree height by using the Haga hypsometer (Fig. 2.3). The presence or absence of *V. siamensis* and its host tree species were also recorded (Fig. 2.6; 2.7). Herbarium specimens were collected and identified using various regional floras and were deposited in the Forest Herbarium, Bangkok (BKF).

3.3 Measurement of Environmental factors

Soil temperature and moisture content were randomly measured in the study plots at four sampling areas in each study site. Soil temperature was measured at a depth of 10 cm in the soil with a Drip-Proof Type Digital Thermometer (MODEL PC-9215; SATO, Tokyo, Japan). Soil moisture content was also measured at a depth of 10 cm in the soil with a moisture sensor (Theta Probe type ML2x; Delta-T Devices Ltd., Cambridge, UK; Fig. 2.9). Surface temperature of the host tree was measured, comprising the trees that supported the Vanilla vines at each study site. All environmental factors were measured in daytime between 09:00 AM to 5:00 PM

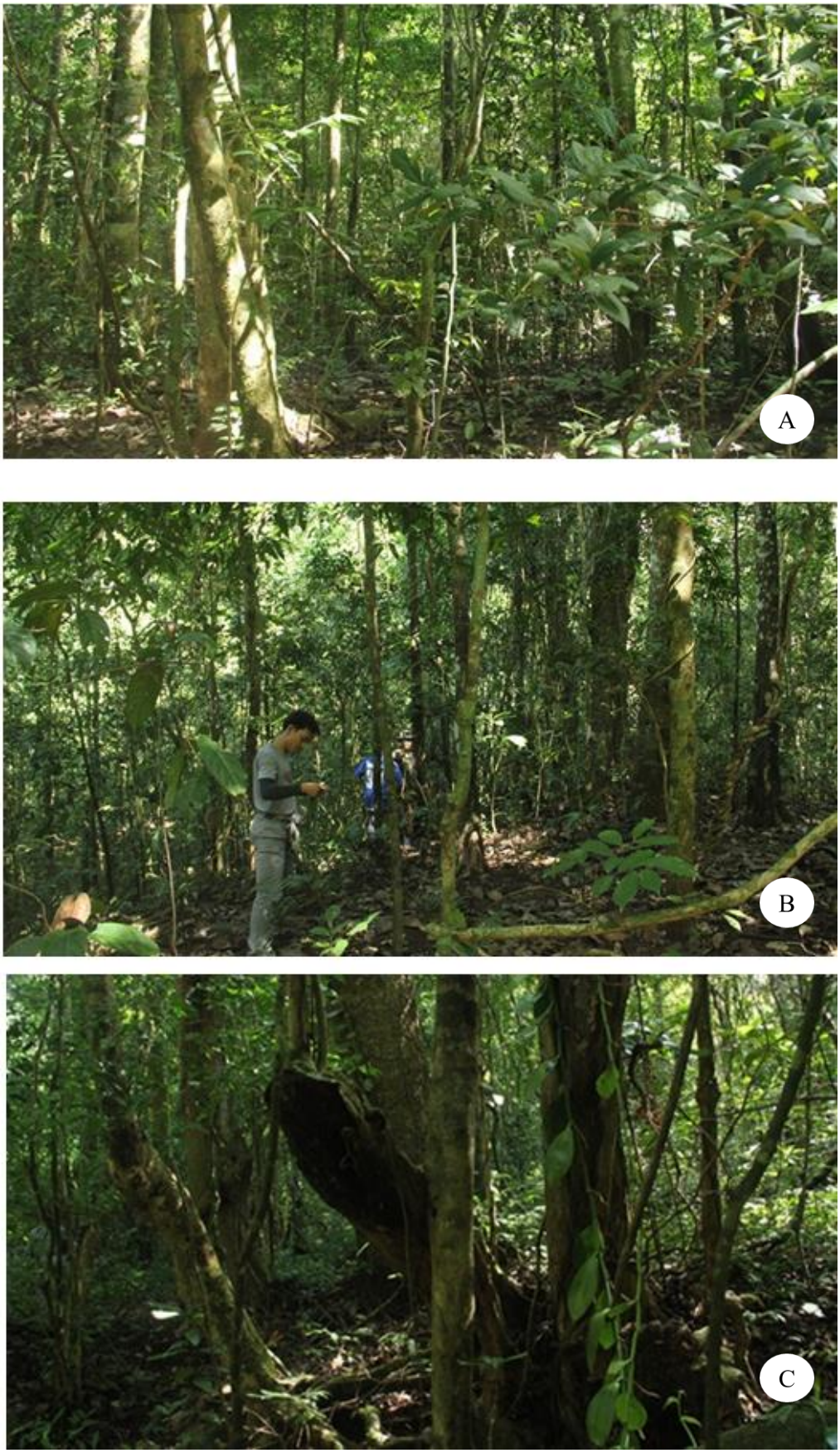

Figure 2.3 (A- C) Adjacent forest areas (AFA) were located at lower level.

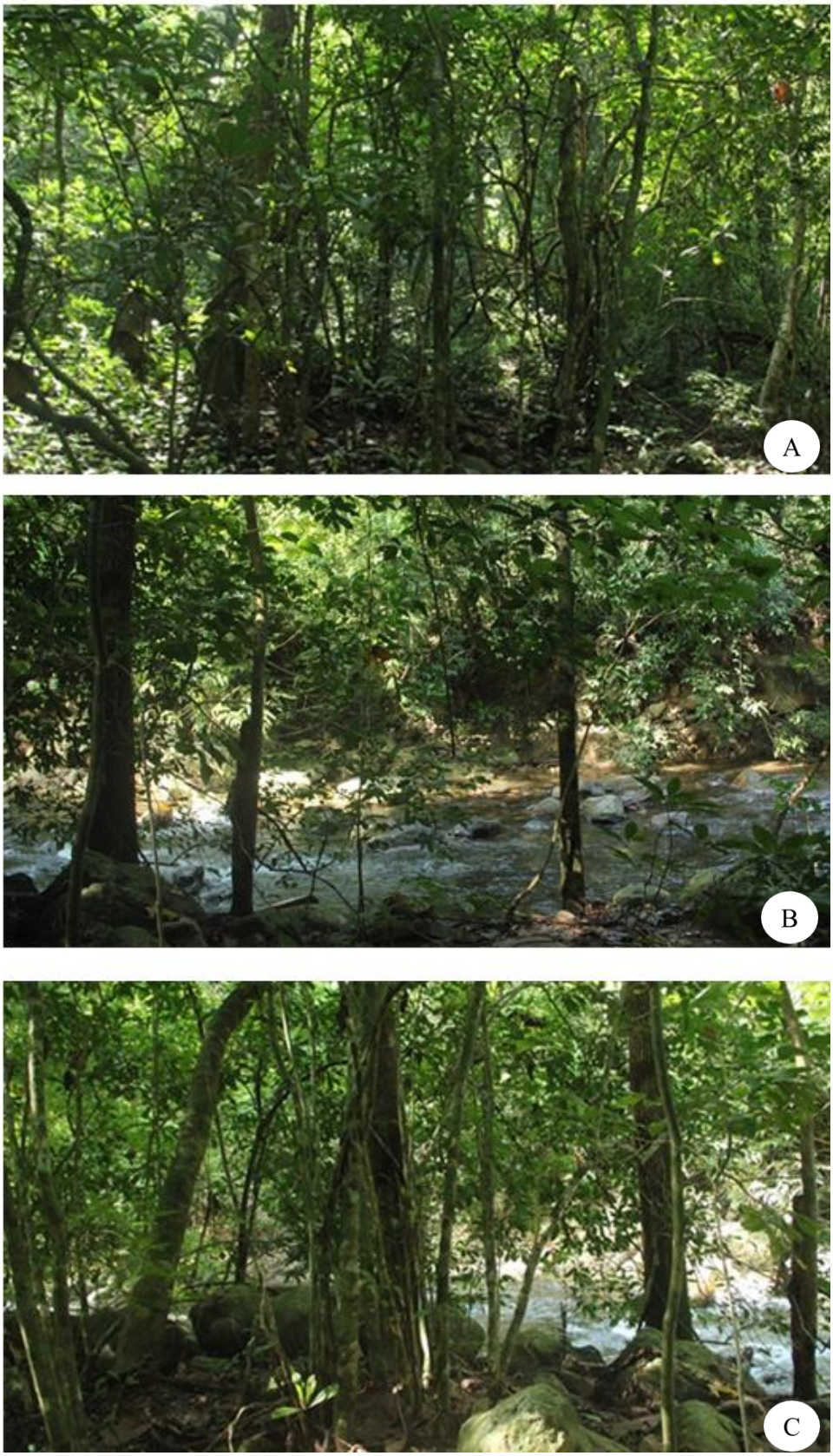

Figure 2.4 (A-C) Near waterfall areas (NWA) were located at lower level.

Figure 2.5 Adjacent forest areas (AFA) were located at upper araes.

Figure 2.6 Tree species at 5 cm in DBH were recorded at each plot (a). A stem diameter at breast height (1.3 m) and tree heights were measured (b,c)

Figure 2.7 (a-b) Flower of *V. siamensis* and (c-d) its host trees (*Pterocybium tentorium*).

Figure 2.8 Supporting tree of *V. siamensis* were recorded (A), and its stem diameter at breast height (1.3 m) were recorded (B) *V. siamensis* heights were measured

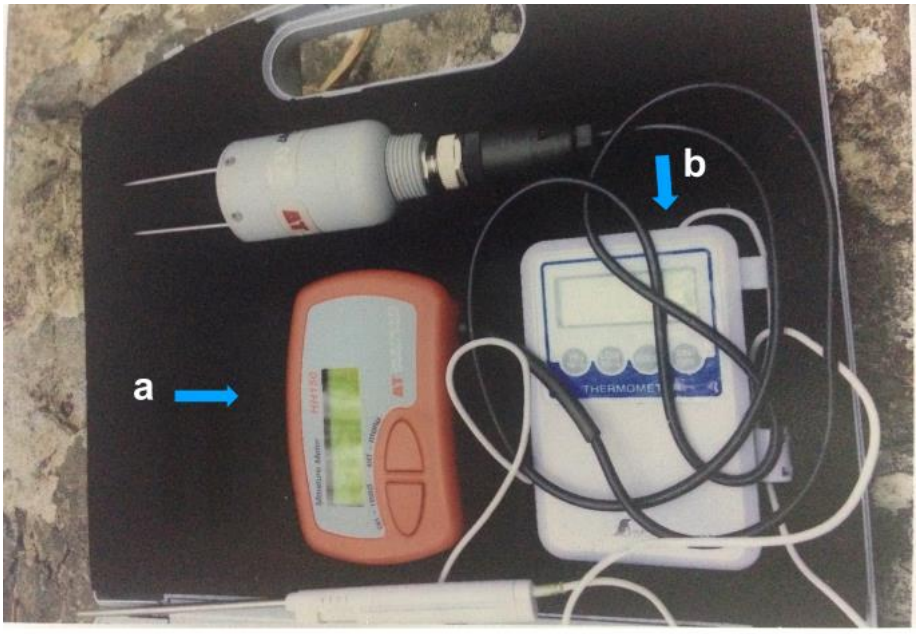

Figure 2.9 Machine for measurement of (a) soil temperature and (b) moisture content

3.4 Data analysis

The tree community was characterised using (1) richness (i.e. number of species), (2) Shannon diversity index (H′), (3) Evenness index (E), and (4) Importance Value (IV) index of tree species. T-tests were conducted to assess the differences in mean values of richness, number of tree individuals, Hand E at lower sites (LW) and upper sites (UP).

The Shannon diversity index (*H*) is commonly used to characterize species diversity in plant community (Magurran, 1988). It is estimated by:

$$H' = -\sum_{i=0}^{n}(PilnPi) \quad \text{..... (1)}$$

Where proportion of species i relative to the total number of species (pi) is calculated, and then multiplied by the natural logarithm of this proportion (lnpi). The resulting product is summed across species, and multiplied by -1:

The *E* was calculated as:

$$E = H'/\ln S \quad \text{..... (2)}$$

Evenness value ranges from 0 to 1, with the value of one indicating that all arthropod order or ant species are equally abundant (Magurran, 1988). The calculation of the *H*' and *E* were performed with the PAST software package (version 2.16, Hammer et al. 2001).

An Importance Values (IV) referred to as the importance percentage. The importance value, or the importance percentage, gives an overall estimate of the influence of importance of a plant species in the community. It is calculated by:

$$IV_i = RD_i + RF_i + RC_i \quad \text{.....(3)}$$

which is;

(1) Relative density for each species (RDi)

$$RDi = ni/\Sigma ni \quad \text{.....(4)}$$

Which *ni* is the number of individuals of a given species counted and RDi is the relative density of the given species.

(2) Relative frequency (Rf)

$$Rfi = fi/\Sigma f \qquad \text{.....(5)}$$

Which is the frequency of a given species (fi) divided by the sum of the frequencies for all species (Σf).

(3) Relative coverage for a species: RC_i

$$RC_i = Ci/\Sigma C \qquad \text{.....(6)}$$

Where the coverage (C) is the proportion of the ground occupied by a vertical projection to the ground from the aerial parts of the plant, and is given by Ci = (ai)(Di)/ni (ai is the sum of the basal areas (computed from DBH) for a species, Di is the density of the species, and ni is the total number of individuals sampled for that species., and ΣC is the total coverage or basal area for all species.

T-tests were used to compare the means of richness, diameter (DBH), number, and height of host trees for *V. siamensis* between the LW and the UP. Difference of soil temperature and moisture content between the LW and the UP were also assessed through T-tests. T-test was used to compare differences in means of soil temperature and moisture content among study areas (i.e. the UP and the LW), and study plots (i.e. the NWA and the AFA). T-test were performed with SPSS Ver. 20.0.0 for Windows (SPSS Inc., Chicago, IL, USA), and data set were checked normality and homogeneity of variance before analyses. Shannon diversity index (H′) and Evenness index (E) were evaluated with PAST: Paleontological statistics software package version 3.0

4. Results

4.1 Distribution of *Vanilla siamensis* at Kao Soi Dow waterfalls

For the distribution of *V. siamensis* in small scale at Kao Soi Dow waterfalls. Drone can be able to fly pre-programmed missions autonomously for a total flight time of approximately 30 minutes and over a distance of approximately 1-2 km, which depend on condition of climate and topography. Drone were defined waypoints along a flight path using an open-source software, and it recorded videos at up to 800 pixel resolution and acquire aerial photographs of <10 cm pixel resolution.

Result of aerial photographs can be stitched together to produce real-time on

vegetation cover maps of surveyed areas. There are separated into two forest stratums. First is along the waterfall route, which 18 meters at high above from ground (Fig. 2.10). Second is over a distance of 18 meter at high above from ground (Fig. 2.11). Result was shown that *V. siamensis* vine can be took photo by using drone along the waterfall route, while it not found at over a distance of 18 meter.

To observe Human activity detection by Drone, at Kao Soi Dow, no Human activity was noticed only tracts and trails were observed from the drone. This is a good guideline for daily enthusiastic walker in order not to get lost, as I often did on few occasions in this forest especially in heavy monsoon rain. Only 4 living quarters for the forest rangers found. In 2016 and 2017. There was an extreme drought which resulted in forest fire and mass destruction of flora and fauna. This could be seen using drone above 150 metres above the ground. This information could facilitate more targeted deployment of local rangers to patrol the problem areas.

Figure 2.10 (A-B) Aerial photographs over a distance of 18 meter at high above from ground

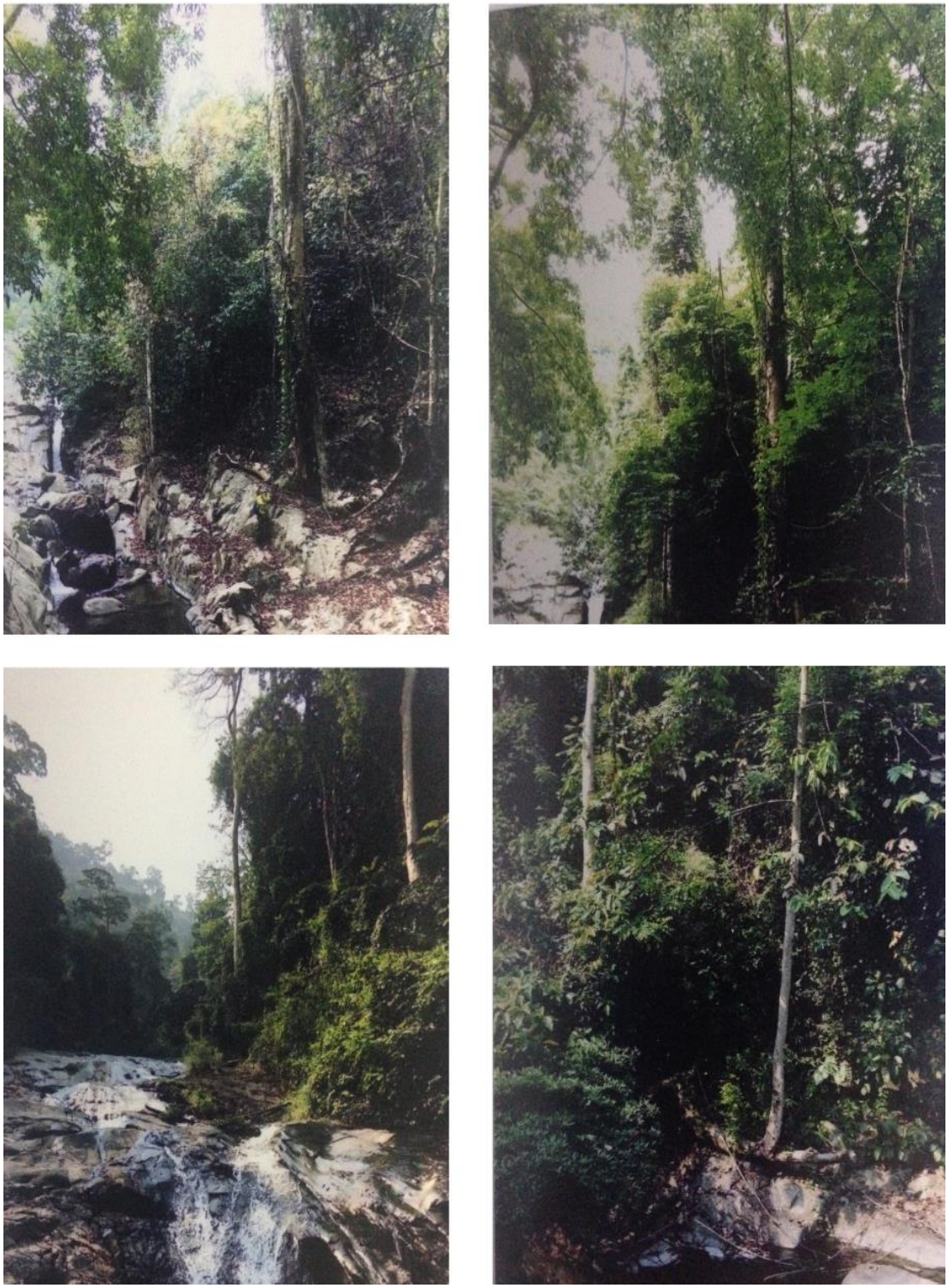

Figure 2.11 Aerial photographs along the waterfall route

4.2 Plant community

Of a total of 134 individual trees recorded in the plots, 66 species of woody plants belonging to 60 genera in 34 families were identified from the forest (Appendix Table 1). At the upper site (UP), the most dominant tree species was *Hydnocarpus ilicifolia* (320), followed by *Pterocymbium tinctorium* (190), *Orophea polycarpa* (160), *Murraya paniculata* (140), and *Diospyros variegata* (120) (Appendix Table 2). At the lower site (LW) the most dominant tree species was *Alchornea rugosa* (180 individuals/ha), followed by *Strombosia ceylanica* (150) *Diospyros defectrix* (130), *D. transitoria* (120) and *Pterygota alata* (60) (Appendix Table 2).

Importance Value index was calculated for each study site separately. At UP, higher values of IV were found for *Hydnocarpus ilicifolia* (62.0), *Pterocymbium tinctorium* (27.3), *Dalbergia oliveri* (25.3), *Diospyros variegata* (22.6) and *Murraya paniculate* (20.9) (Appendix 2). At LW higher values of IV were found for *Pterygota alata* (31.03), *Diospyros defectrix* (23.83), *Strombosia ceylanica* (22.44), *Alchornea rugose* (21.33) and *Diospyros transitoria* (20.65) (Appendix Table 3). While the greatest richness of tree species, number of individuals, Shannon diversity index and evenness were found near waterfall areas (NWA) compared with adjacent to forest areas (AFA), these differences were not statistically significant (T-test; $P > 0.05$; Fig.2.12a-2.12d).

4.3 Host trees for *Vanilla siamensis*

Of the 287 individual trees surveyed, those that hosted *V. siamensis* represented 10.5 % (n=30) of woody plants (Table 2.1). Tree species from the family Malvaceae accounted for 28.1% of all host trees, followed by Annonaceae (15.3%) and Achariaceae (10.3%). The highest number of tree host species was found in the upper sites (3.4 ±1.6 SE hosting trees per quadrat) as compared with the lower sites (1.6 ±1.0 SE), however the difference was not statistically significant (T-test; $t_9 = 2.0$, $P > 0.05$.Table 1; Fig.2.13a). The greatest number of individual tree hosts was recorded at the upper sites (6 ±3.70 SE), compared with the lower sites (1.6 ±1.03 SE), however, again difference was not significant (T-test; $t_9 = 2.1$, $P > 0.05$ Table 1; Fig.2.13b). The results suggest that neither species richness nor abundance of host trees is a key factor in terms of the abundance of *V. siamensis* in the study area.

The diameter at breast height (DBH) of host trees was significantly higher at the lower sites (36.6 ±10.79 SE) than at the upper sites (8.4 ±1.04 SE; T-test; $t_{36} = 24.3$, $P < 0.001$; Fig.2.13c). The height of the tree hosts was also significantly higher at the lower sites (23.8 ±4.51 SE) than at the upper sites (7.7 ±0.80 SE; T-test; $t_{36} = 24.3$, $P < 0.001$; Fig.2.13d)

Table 2.1 List of host trees for *V. siamensis*, and number of individuals found near waterfall areas (NWA) at lower and upper sites.

Family/ species	number of individual	
	lower site	upper sites
Achariaceae		
Hydnocarpus ilicifolia King	0	4
Annonaceae		
Miliusa mollis Pierre var. mollis	0	2
Orophea polycarpa A. DC.	0	4
Bignoniaceae		
Markhamia stipulata (Wall.)	1	1
Mayodendron igneum (Kurz) Kurz	0	1
Combretaceae		
Terminalia nigrovenulosa Pierre	0	1
Dipterocarpaceae		
Dipterocarpus alatus Roxb. ex G. Don	1	0
Ebenaceae		
Diospyros defectrix H. R. Fletcher	0	1
Diospyros transitoria Bakh.	1	0
Diospyros variegata Kurz	0	1
Euphorbiaceae		
Alchornea rugosa (Lour.) Müll. Arg.	1	0
Fabaceae		
Millettia leucantha Kurz var. leucantha	0	1
Malvaceae		
Pterocymbium tinctorium (Blanco) Merr.	0	11
Meliaceae		
Aglaia edulis (Roxb.) Wall.	1	0
Myrtaceae		
Syzygium syzygioides (Miq.) Merr. & L. M. Perry	1	0
Phyllanthaceae		
Bischofia javanica Blume	1	0
Rhizophoraceae		
Carallia brachiata (Lour.) Merr.	1	0
Rutaceae		
Murraya paniculata (L.) Jack	0	1
Vitaceae		
Tetrastigma leucostaphylum (Dennst.) Alston	0	2
Total species of host tree	8	12
Total number of host tree	8	30

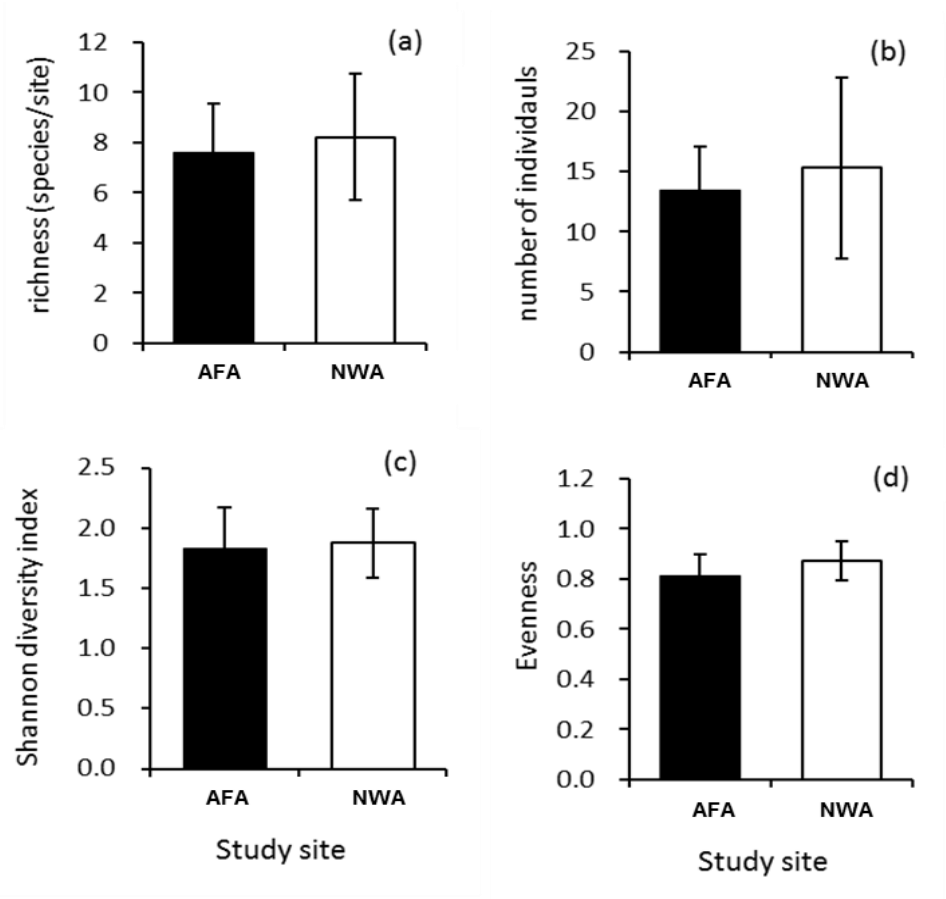

Figure 2.12 Mean (±SE) of (a) species richness, (b) number of individuals, (c) Shannon diversity index and (d) evenness of plant community at adjacent forest areas (AFA; black bar) and near waterfall areas (NWA; white bar).

4.4 Environmental factors

Soil temperature was significantly higher in the upper sites (27.7 Celsius ±0.6 SD) than at the lower sites (25.2 ±0.3 SD; $t_{36} = 14.3$, $P = 0.09$; Fig. 2.14). Soil temperature was also significantly higher at the AFA than at the NWA (T-test; $t_{36} = 19.3$, $P < 0.001$; Fig.2.14a).

Soil moisture was significantly higher in the upper sites (5.3% ±0.7 SD) than the lower sites (3.4 ±0.5 SD; $P < 0.001$). The difference was found for sampling areas, in which soil moisture was significantly higher at the NWA than the AFA (T-test; $t_{36} = 24.1$, $P < 0.001$; Fig.2.14b).

Surface temperature for host trees was higher in the upper sites (28.1 ± 0.4 SD) compared to the lower sites (27.4±0.6 SD). Surface temperature for host trees was also significantly higher at the AFA than at the NWA (T-test; $t_{36} = 18.9$, $P < 0.001$; Fig.2.14c).

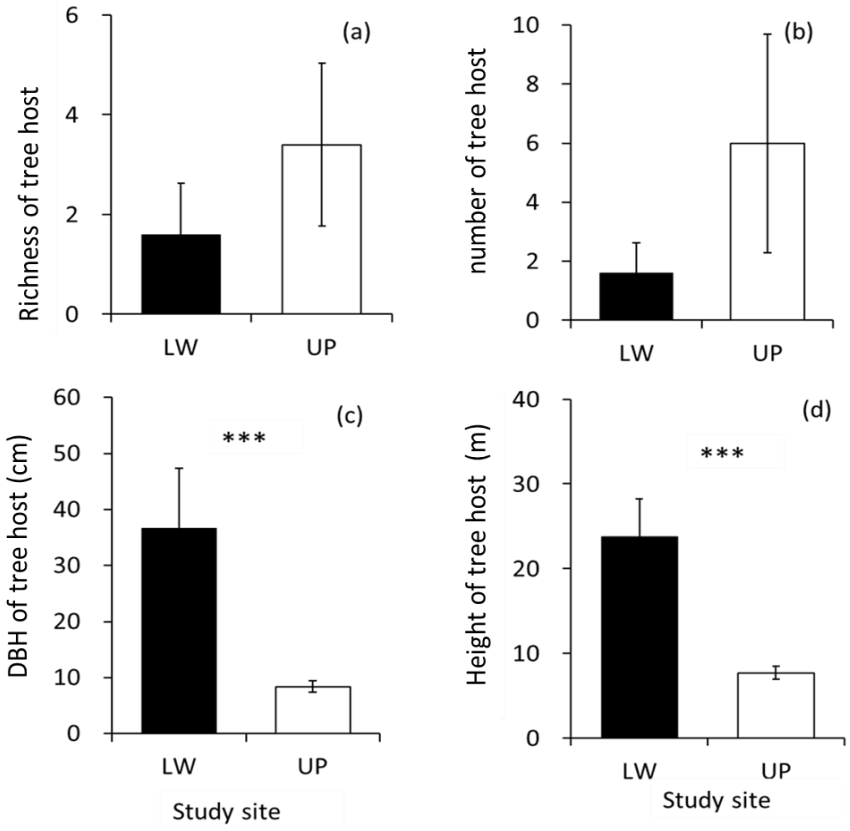

Figure 2.13 Mean (±SE) of (a) species, (b) number of individuals, (c) diameter at breast height (DBH) and (d) high of tree host at lower sites (LW) and upper site (UP)

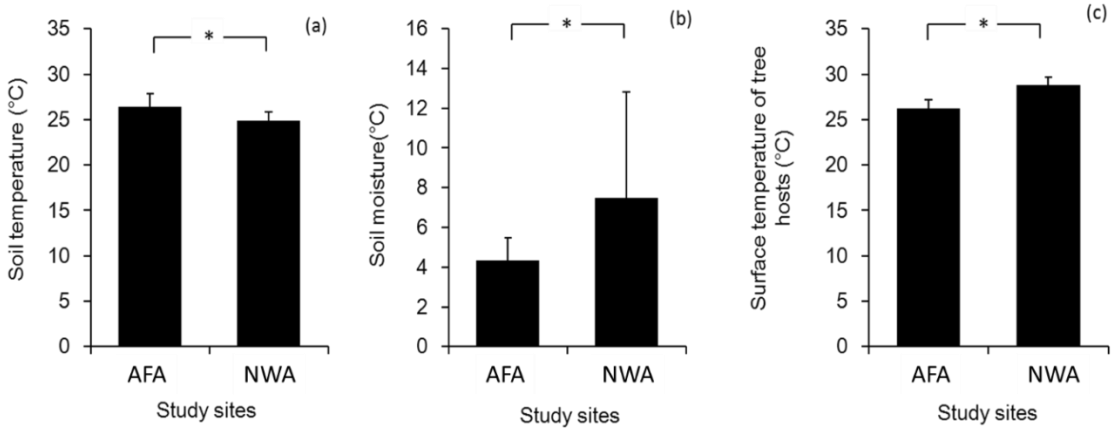

Figure 2.14 Mean (±SE) of (a) soil temperature, (b) soil moisture and (c) surface temperature of host tree at adjacent forest areas (AFA) and near waterfall areas (NWA). Significant values are indicated with an asterisk for * = P<0.001

5. Discussion

The plant communities where *Vanilla siamensis* occurs contain a diverse flora. However, there were no differences in tree and shrub species composition between areas near waterfalls (NWA) and adjacent forest areas (AFA). At the same time, a significant difference in the number of individuals of *V. siamensis* was found.

The results showed that more individuals of *V. siamensis* were found at the upper areas when compared with the lower areas, which was the case for both NWA and AFA. These results suggest that the level of species richness and number of individual Vanilla plants may not explain the distribution of *V. siamensis*. On the other hand, a larger number of *V. siamensis* vine can be found at higher elevations and near waterfalls, especially in association with the host tree *Pterocymbium tinctorium*. From the data collected, these areas have a higher level of soil moisture content compared with adjacent forest areas. These results show that the distribution of *V. siamensis* in the study areas could be related to a higher level of moisture content near waterfalls and also to the position of host trees, as has been reported for other hemi-epiphytes (Patel 1996; Harrison et al. 2003; Bergstrom et al. 2008), and Muñoz et al. (2003) who reported that three epiphytic ferns showed preferences for large-sized trees, whereas frequency of occurrence of three common vines was independent of host tree size.

According to the present study, micro-site factors, in particular species of host tree and their bark characteristics, are among the key variables that relate to the occurrence of *V. siamensis*. A greater incidence of *V. siamensis* was found on 19 woody plant species of the plant families Malvaceae, Annonaceae and Achariaceae. These trees have thick and soft bark on the lower part of tree trunk and smooth bark in the upper part of tree trunk. Bark structure might be a factor influencing the climbing habit of *V. siamensis* as found in other groups of vascular epiphytes (Wyse and Burns 2011; Zhao et al. 2015).

The most common host tree is *Pterocymbium tinctorium* (Malvaceae: Sterculioidae), known in Thailand as Phor E Gae, where it is a significant timber tree growing to about 45 metres tall. The straight, cylindrical bole can be 90 cm in diameter and unbranched for up to 30 metres (Brown 1920). The trees are grown in plantations as well as in the wild for local use as a source of fibre, wood and dyestuff. The fibres obtained from the bark are used to make rope. The bark is used to improve the dying of cotton cloth black, due to the presence of tannins. The white wood is light and very soft, and is used for construction, matches, veneer and pulp (Brown 1920).

All host trees found in this study have a large diameter at breast height (DBH). In the study area, *V. siamensis* can attains lengths of 8 to 25 m, with internodes 5 to 10 cm long. These results suggest that larger DBH and height of host trees are potentially important factors that influence the presence of *V. siamensis* in natural conditions (Köster et al. 2011)

Chapter 3: Floral morphology and Potential Pollinator of *Vanilla siamensis* Rolfe ex Downie (Orchidaceae: Vanilloideae) in Thailand

1. Abtract

The orchid genus *Vanilla* has a pantropical distribution. It includes notable species, particularly *Vanilla planifolia* Jacks. ex Andrews and *V. pompona* Schiede from the tropical Americas, and the first commercial hybrid *V. tahitensis* (Moore 1933). Both species are used in various industries, including food, pharmaceuticals, cosmetics, tobacco, insect repellent and traditional crafts. Less known is *V. siamensis* Rolfe ex Downie from Thailand.

Here we investigate the flower morphology, fruit set and natural pollinator of *V. siamensis*. Flower morphology and pollination experiments indicate that the species is pollinator-limited. Thus undergoing pollination via deception. During observations of visiting insects, eight insect groups were collected (Blattodea, Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera, Mantodea and Orthoptera), representing twenty-eight insect morpho-species. The most frequent visiting group was the Hymenoptera that account for the largest value of frequency of occurrence with 80% of the total visitations. The main visitor, *Thrinchostoma* sp. 2 is most likely the pollinator as observed at Kao Soi Dao in the eastern region of Thailand. This species was observed entering a flower carrying Vanilla-like pollen before exiting with the pollen having been removed.

It is therefore suggested that pollination of *V. siamensis* is by the *Thrinchostoma* bees, under natural conditions, however visitations are infrequent, resulting in pollinator-limitation when compared to hand pollination experiments.

2. Inrtoduction

The family Orchidaceae, possibly the second largest plant group in the world (Bory et al. 2008; Chase et al. 2015), is renowned for its diverse and often remarkable pollination strategies (Micheneau et al. 2009). About 27000 species are known, or roughly one out of every 15 plants currently described (Dressler 1981; Dressler 1993; Tremblay et al. 2005). At the same time, for many orchid species, pollination events have never been recorded. This is especially true for epiphytic species where the lack of access to flowering plants makes observation difficult.

In many tropical areas, species of the pantropical orchid genus *Vanilla* occur as lianas and hemi-epiphytes (Cameron 2011). The genus is best known for the widely cultivated tropical crop plant *V. planifolia*, the main source of natural vanilla flavor. While most of the ca. 120 species of *Vanilla* have relatively large and conspicuous flowers, these are invariably ephemeral, usually lasting a single day, or only a few hours (Cameron 2011). Four species are native to Thailand, *V. aphylla, V. pilifera, V. griffithii*, and *V. siamensis*, not including the widely cultivated *V. planifolia* that is native to Mexico (Díaz-Bautista et al. 2018). They all occur as trailing vines along tree trunks and in some cases on rock. Very little is known about the biology of *V. siamensis* and it was thought, at the beginning of the century, to be an endemic species of Thailand. The first recorded collections of *V. siamensis* (1905, 1907 and 1909) were by the British botanist Dr. Arthur Francis Kerr (1877–1942). He was the first scientist to be sent to, then, Siam, by the British government and catalogued many plants species. All his specimens were then sent to the Herbarium collection at the Royal Botanic Gardens, Kew, UK; although, significant collections are also held in the Herbarium of the University of Aberdeen.

Vanilla siamensis Rolfe ex Downie is a robust species found in Thailand, China (Yunnan) and probably Cambodia (Schuiteman, pers. comm.). Here we describe the results of pollination experiments and observations of this striking orchid species. A few *Vanilla* species have been suggested to be self-pollinated through stigmatic leakage or reduced rostellum (Soto-Arenas and Cameron, 2003; Householder et al. 2010; Soto-Arenas and Dressler 2010; Lubinsky et al. 2006); however, the majority are dependent on pollinators (Peakall 1994; Proctor et al. 1996; Soto-Arenas and Cameron 2003; Petersson 2015). The most common reward for orchid pollinators is nectar (Dressler 1993; Roubik and Ackerman 1987); although, no Vanilla species are known to produce a floral reward (Proctor et al. 1996). Several Vanilla species in the American tropics are pollinated by *Euglossine* bees (Dressler 1981), a tribe that is known to collect fragrance from non-nectar producing orchids (Dodson et al. 1969). So far, there is no direct evidence for this scent collecting behavior in any Vanilla species (Proctor et al. 1996; Gigant et al. 2011). Many orchids have also developed different types of deceptive systems to attract pollinators (van der Pijl and Dodson 1966), a strategy known to be used by several Vanilla species in the American tropics; although, the exact form of the deception remains unclear (Soto-Arenas and Cameron 2003).

Vanilla siamensis is of particular interest as extracts and have been shown to have pharmaceutical properties in the treatment of osteoporosis. It is therefore important to have an understanding of the species pollination biology, not only for the conservation of

the species, but also for the exploitation of the species through breeding in relation to its pharmaceutical properties. This study therefore examines the 1) flower morphology of *V. siamensis*, 2) natural pollination, and 3) pollinator behavior observations of *V. siamensis.*

3. Materials and Methods

The study was conducted at Khao Soi Dao Waterfall, located in Khao Soi Dao Wildlife Sanctuary (KSD-WS), Chanthaburi, Thailand (13.1041889°N, 102.1945329 °E), and at a second site in Chiang Mai Province, Thailand (18.7767366°N, 99.2470935 °E). Both study sites are classified as tropical moist evergreen forest and were chosen due to the accessibility of *V. siamensis* at both sites. The study was undertaken in the flowering season, May 2019 to June 2019.

3.1 Floral morphology of *Vanilla siamensis*

Nine inflorescences were randomly selected at plots 10 m apart for the study. Five flowers were randomly selected on each inflorescence for floral measurements. The floral characters were measured within one hour after collection using a digital caliper to the nearest 0.1 mm. The number of individual flowers were counted for each inflorescence. These data were used to calculate the average number of flowers per inflorescence.

3.2 Natural pollination and pollinator behavior observations

A combination of two techniques was used for collecting the data on diversity of insect pollinators, which were the sweep-net method, and digital video recording (DVR) devices (McCravy 2018). Wherever *V. siamensis* flowers were observed, insects which visited the flowers were caught by sweep-net.

Three Brinno 200TLC digital cameras, binocular, and extra supporting long lens Canon were set up by using tripods. The DVR has the potential to greatly benefit the recording of pollinator diversity, activity, and breeding systems in natural ecosystems. Moreover, the DVR can objectively improve the direct data collection of pollinator diversity and activity over direct human observation. All sample points were labelled when insects visited on inflorescence as recorded by video. They were caged with fine muslin cloth to protect the flower from the other visiting insects. Observations were conducted for 6 weeks during the period 06.45-12.00 h from May 2019 to June 2019

3.3 Identification of visitors

All insect specimens were identified to the species or family level. Identifications were performed using taxonomic keys (Triplehorn and Johnson 2005; Michener 2000). The Thrinchostoma species was identified by Dr. Alain Pauly at the Royal Belgian Institute of Natural Sciences in Brussels and other species were identified by entomologists at the University of Chiang Mai. Some insect species unable to collect because of high location and period time of the day. Insects were classified through the help of digital cameras.

3.4 Data Analysis

A frequency of occurrence (FQ) was used to quantify the probability of finding an insect group in study site. Frequency of occurrence of each insect group was calculated by; FQ (%) = (number of times in which the insect group is found/ total number of sampling times) ×100.

T-test was used to compare the means of frequency of occurrence of insect (classified by morphospecies) visits on *V. siamensis* flowers. Before applying parametric tests, we tested for normality and homogeneity of variances. All data were log10(x+1) transformed to meet conditions of normality, homogeneity of variances, and sphericity. Statistical analyses were performed with SPSS Ver. 20.0.0 for Windows (SPSS Inc., Chicago, IL, USA).

4. Results

4.1 Morphology of *Vanilla siamensis* flower

Inflorescences emerge as a light green protuberance from the leaf axil, measuring 5–8 cm. long, most often as an un-branched (rarely branched) raceme. Anthesis (flower opening) occurs in the early morning. Flowers open acropetalic *i.e.*, from the base of the inflorescence upwards. Each inflorescence has only a single flower open at any one time, with each flower lasting up to three days. Flowers measure 8–9 cm across, are pale green with a cream-colored or yellow and red-pink labellum (Fig. 3.1A-E). Flowers are pedicellate and each flower is subtended by a small pointed bract. The flower is resupinate, with the labellum being lowermost. Interestingly, we found the "white bearded" *V. siamensis,* which has a white labellum, is the most common (98 %; n = 98 vines of vanilla), and the "red bearded" *V. siamensis*, with a red-pink labellum, is extremely rare (2 %; n = 2 vines of vanilla).

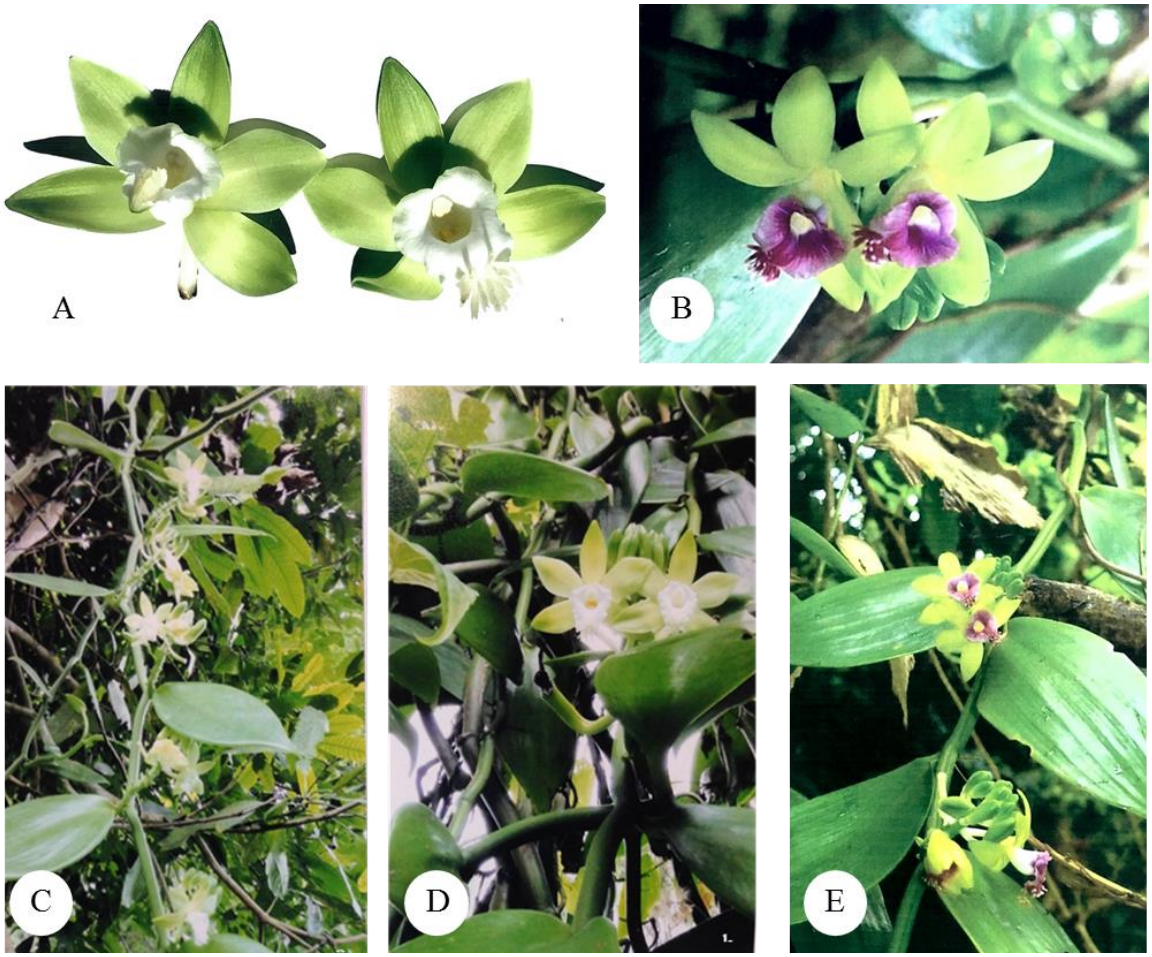

Figure 3.1 (A), (C) and (D) showing the common "white bearded" *Vanilla siamensis*. (B) and (E) showing the much rarer "red bearded" *V. siamensis*.

The corolla consists of two upper petals that resemble the sepals but are slightly smaller, and a trumpet shaped labellum. The apex of the labellum bears a tuft of fat, elongate hairlike projections, producing a beard-like appearance, while about halfway the labellum, underneath the anther, there is a patch of backward-pointing lamellae, producing a hump on the labellum surface. The column, which is adnate to the basal sides of the labellum, has long hairs on its ventral surface (Fig. 3.2). A single anther is attached to the column, containing four pollen masses (resembling pollinia but of less definite shape and of crumbly texture) covered by a cap/hood. The concave stigma is situated below the anther and is separated from it by a thin flap-like membrane (the rostellum), preventing self-pollination. The ovary is inferior and elongate (Fig. 3.3).

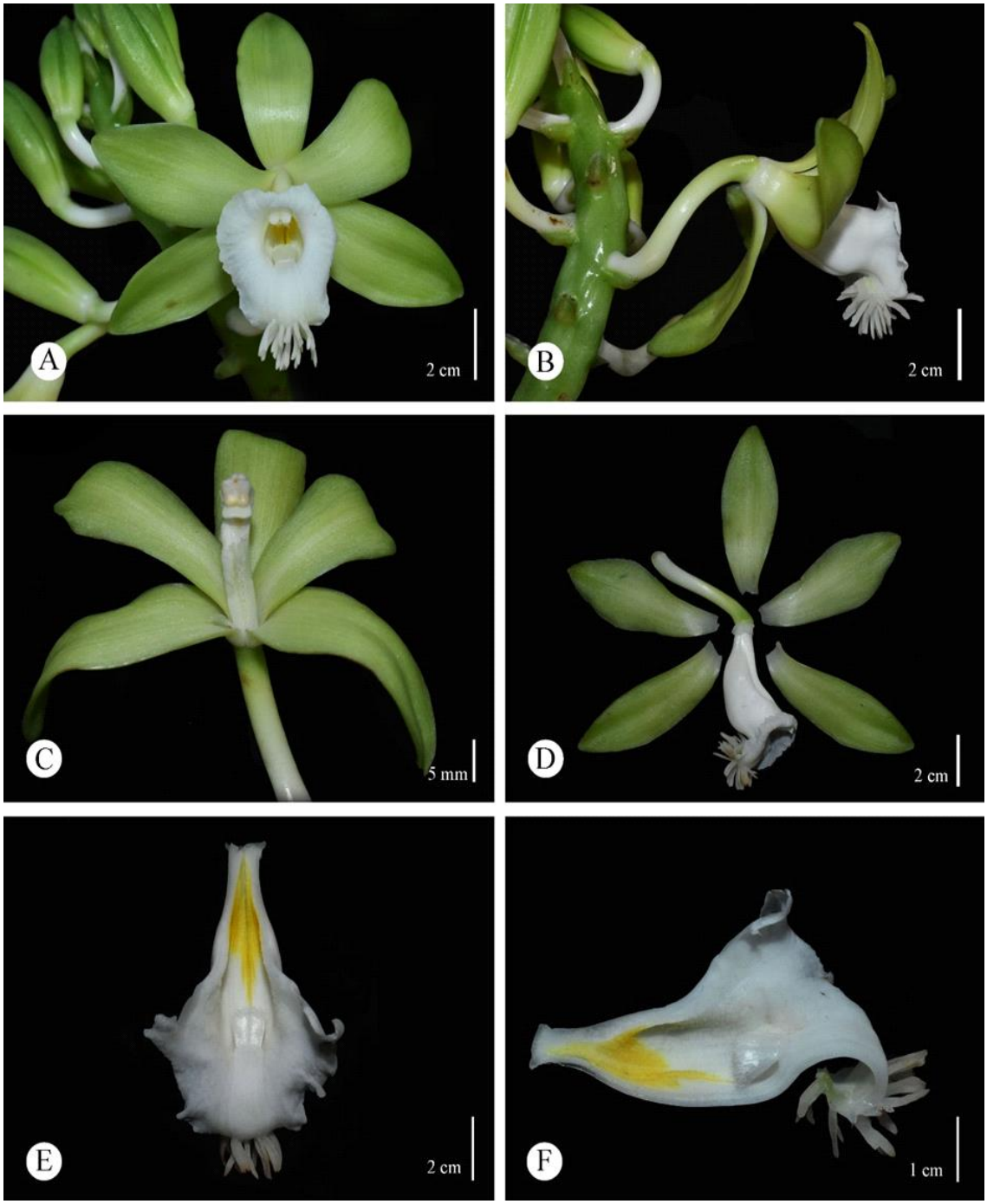

Figure 3.2 Floral morphology of *Vanilla siamensis*. (A) Flower front view; (B) Flower side view; (C) Flower front view and without labellum; (D) The detail of floral parts showing the sepals, petals, labellum and ovary; (E) Labellum dorsal view; (F) Longitudinal section of the labellum (cont.)

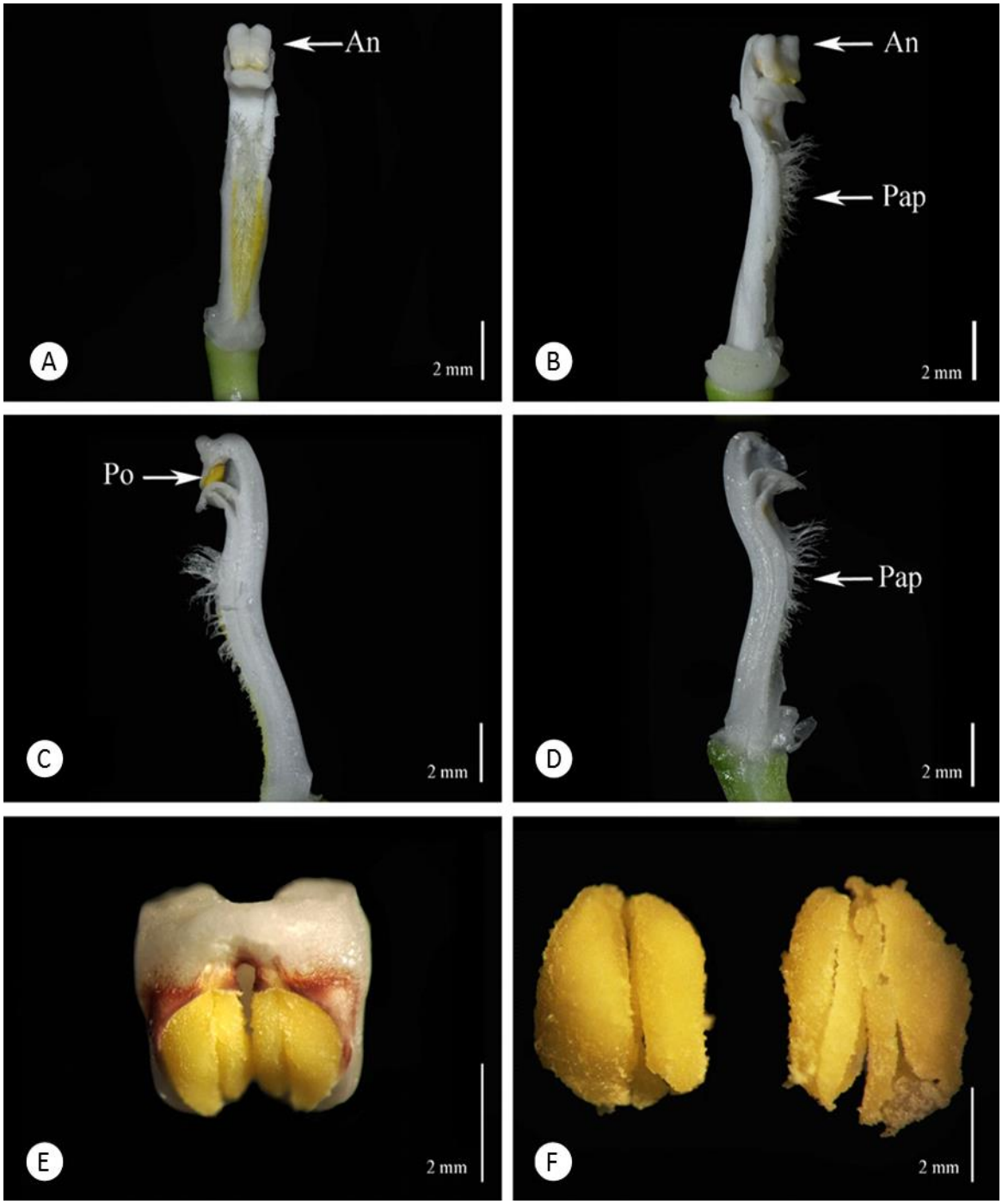

Figure 3.3 Floral morphology of *Vanilla siamensis*. (A) Column front view; (B) Column side view; (C) Longitudinal section of column with pollen-mass; (D) Longitudinal section of column without pollen-mass; (E) Anther cap and pollinia; (F) Pollinia; An = Anther cap; Pap = Papillae hair; Po = Pollen-mass.

4.2 Flower visitor diversity

A total of 8 orders, representing 28 morphospecies, were collected during visitation to flowers of *V. siamensis* (Table 3.1; Fig.3.4). Hymenoptera made up the majority of the species (80 %), followed by Diptera (50%) and Coleoptera (33%).

Observations were made of insects that visited the flowers of *V. siamensis*, including their activity and frequency of occurrence. Bee species visited *V. siamensis* with significantly higher frequency than all other insect groups combined (Table 3.1; T-test; F=4.56, P<0.05).

Table 3.1 Diversity of insects caught during visits to the flowers of *V. siamensis*.

Order (Family; species)	**Mophospecies**	**Occurrence** (%; N=30 days)
1. Blattodea (Blatellidae; *Blatella* sp. 1, *Chrysocoris* sp. 1) – cockroaches	2	10
2. Coleoptera (Chysomellidae; *Chrysomyia* spp.) – beetles	4	33
3. Diptera (*Chryops* spp.) i.e. flies	3	50
4. Hemiptera (Pantamiidae; *Chrysocoris* sp., Callidea sp.) – true bugs	2	6
5. Hymenoptera: (Apidae; *Apis cerana*, *Trigona* sp., *Thrichostoma* sp. 1, *Thrichostoma* sp. 2 Halictudae; *Nomia* sp. 1, *Nomia* sp. 2) – bees; (*Dolichoderus* sp. 1, *Camponotus* sp. 1, *Camponotus* sp. 2, *Camponotus* sp. 3, *Polyrachis* sp. 1, *Crematogaster* sp. 1, *Crematogaster* sp. 2, *Crematogaster* sp. 3) – ants	14	80
6. Lepidoptera (Papilionidae; *Graphium* sp.) – butterflies	1	10
7. Mantodea (Mantidae sp.) – mantis	1	3
8. Orthoptera (Acrididae sp.) – grasshoppers	1	3
Total	28	

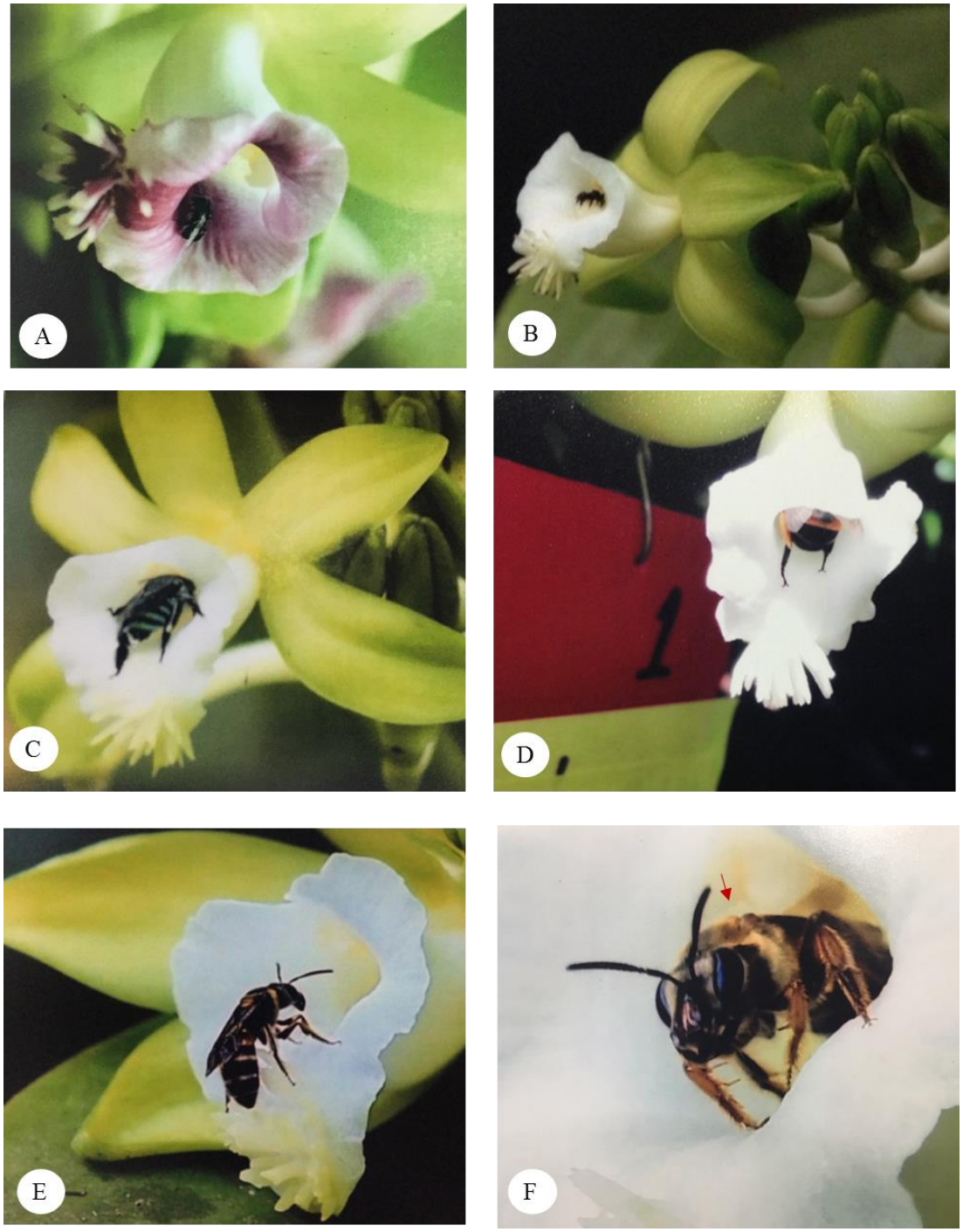

Figure 3.4 Bee species of Hymenoptera visiting the Vanilla flower, captured on digital video recording (DVR) devices; (A-B) the *Trigona* sp. (C) *Nomia* sp. 1, (D) *Nomia* sp. 2 and (E) *Thrinchostoma* sp. 1. (F) Red arrow indicating pollen deposited on the thorax of *Thrinchostoma* sp. 2. All captured pollinator specimens have been lodged at The Royal Botanic Gardens in Chiang Mai, Mae Rim.

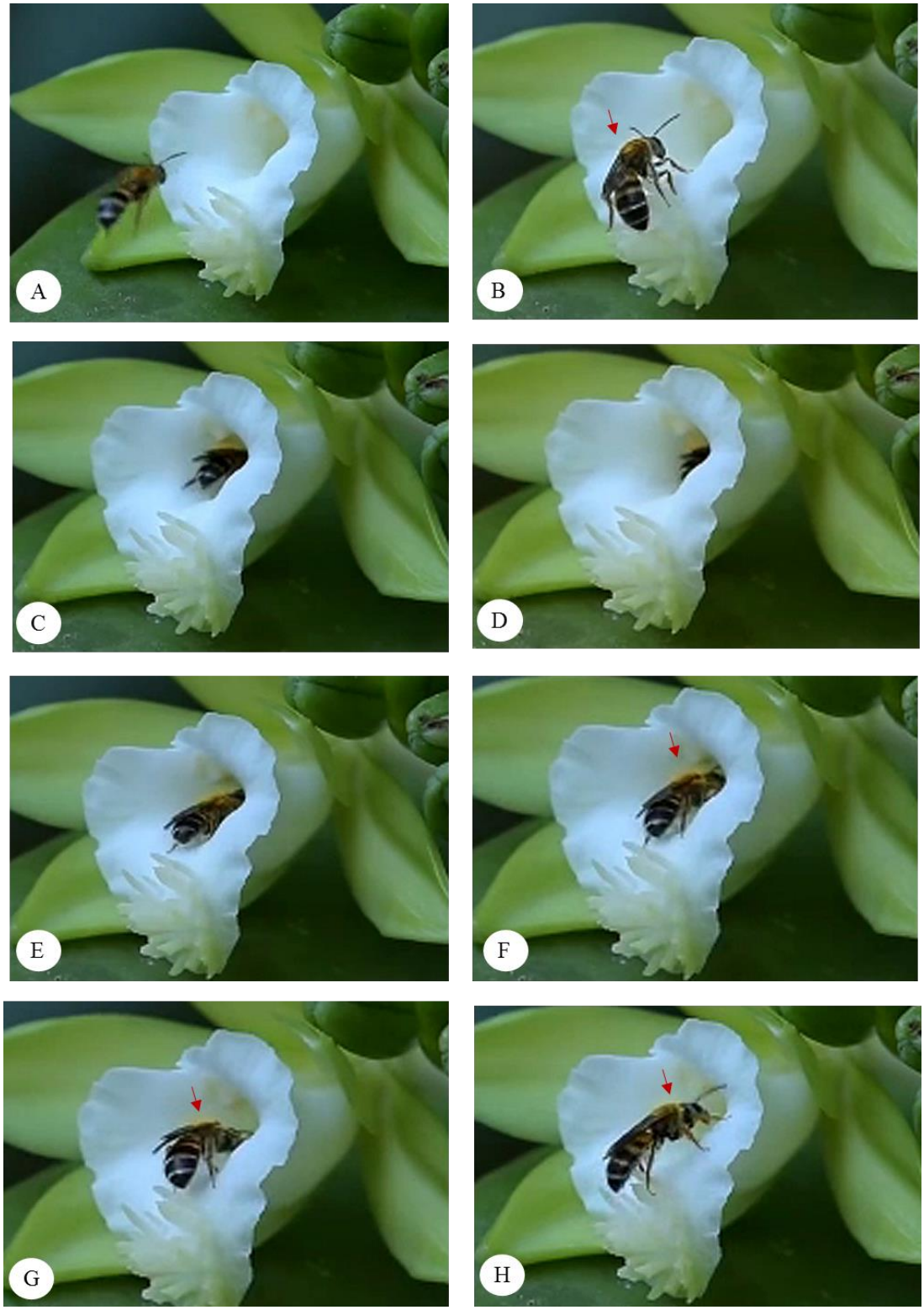

Figure 3.5 A series of video stills (A-H) showing *Thrinchostoma* sp. 1 visiting the flower of *Vanilla siamensis*; (B) Red arrow indicates the absence of the pollen grains on the thorax of *Thrinchostoma* sp. 1, before entering the *Vanilla* flower. (F-H) The red arrows indicate the pollen grains on the thorax of *Thrinchostoma* sp. 1, after leaving the Vanilla flower

Vanilla-like pollen was observed on the thorax of *Thrinchostoma* sp. 1 and *Thrinchostoma* sp. 2 as they visited the flowers of *V. siamensis* (Fig. 3.4E, 3.4F and Fig. 3.5). *Thrinchostoma* bees caught on *V. siamensis* were measured. Morphological measurements of the operative size of *Thrinchostoma* sp. 1 showed these bees to have a functional height of 5 mm. They had a total body length of 13 mm, including a thorax length of 3.9 mm, with a thorax width of 3.2 mm and a thorax height of 2.9 mm (Fig. 3.6). *Thrinchostoma* sp. 2 also had a functional height of 5 mm. They had a total body length of 12 mm, of which the thorax length is 4 mm, with a thorax width of 3 mm and a thorax height of 2 mm (Fig. 3.7).

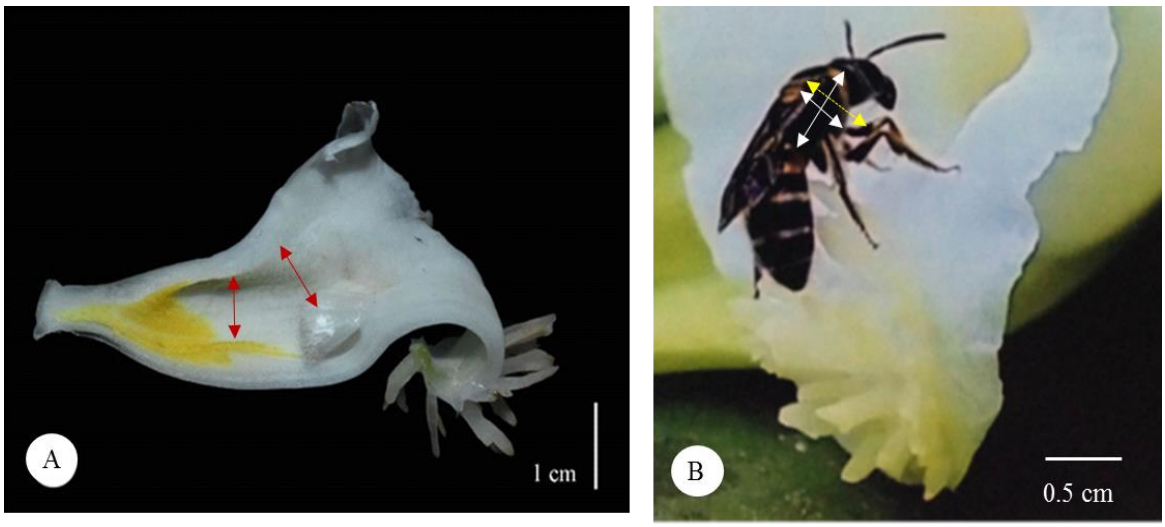

Figure 3.6 Morphological measurements of (A) longitudinal section of labellum, and (B) *Thrinchostoma* sp. 1 with morphological measurements of thorax length (TL) and thorax height (TH) indicated with white arrows, and the functional height approximated by a yellow arrow.

Figure 3.7 *Thrinchostoma* sp. 2 with morphological measurements of thorax length (TL) and thorax height (TH) indicated with arrows, and the functional height approximated by a dotted arrow.

Following visitation of *V. siamensis* flowers by a *Thrinchostoma* sp.1 and *Thrinchostoma* sp.2 that was carrying *Vanilla*-like pollen, the ovary developed rapidly, doubling in length in a few weeks. It was observed that when pollination did not occur, then within one to three days, the flower would detach from the ovary, and later drop off entirely. The sizes of *V. siamensis* fruit by insect visitors were recorded. Fruit development resulted in the elongation of the pedicel, with the pedicel changing color from white to light yellow and eventually to green, showing the presence of photosynthesis (Fig. 3.8). Only one flower out of 28 flowers (3.6%) was pollinated by *Thrinchostoma* sp. 1 and *Thrinchostoma* sp.2 and developed into a fruit.

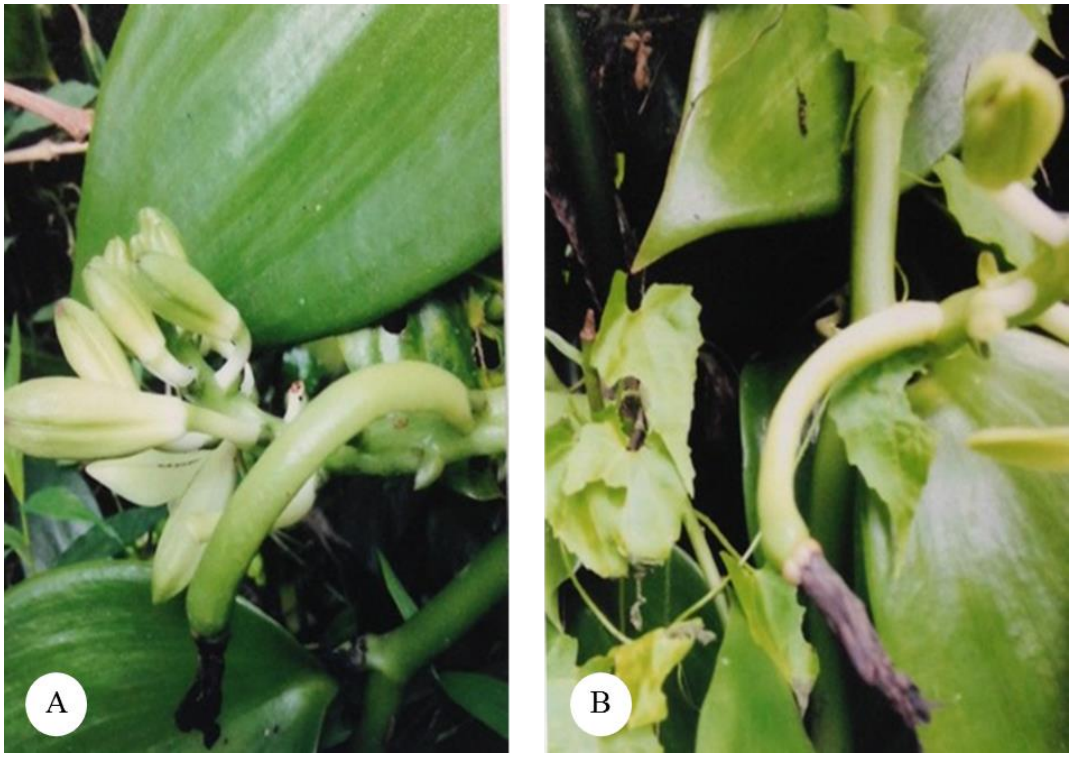

Figure 3.8 (A-B) A fruit of *Vanilla siamensis* after visitation by *Thrinchostoma* sp. 2

4.3 Pollinator behavior observations

The behavior of the *Thrinchostoma* sp. 1 and *Thrinchostoma* sp. 2 was observed in as much detail as possible, including their approach entering and leaving the flower, and the time it spent in the flower (Fig. 3.9). This can be seen from various still picture frames. The observation for pollinators began on 14 May and continued until 5 June 2017. Every day equipment was set up at 06.15 before sunrise and stayed in place until late afternoon. On a few occasions, it was not possible to observe any pollination taking place due to constant rain and morning mist. On the ninth day, perfect conditions appeared. Few visitors approached the flower when it was fully open and did not make any attempt to enter the flower. At 07.45 on 22 May, approximately 3±0.4 (SD; N=30) *Thrinchostoma* bee specimens per day visited the flowers

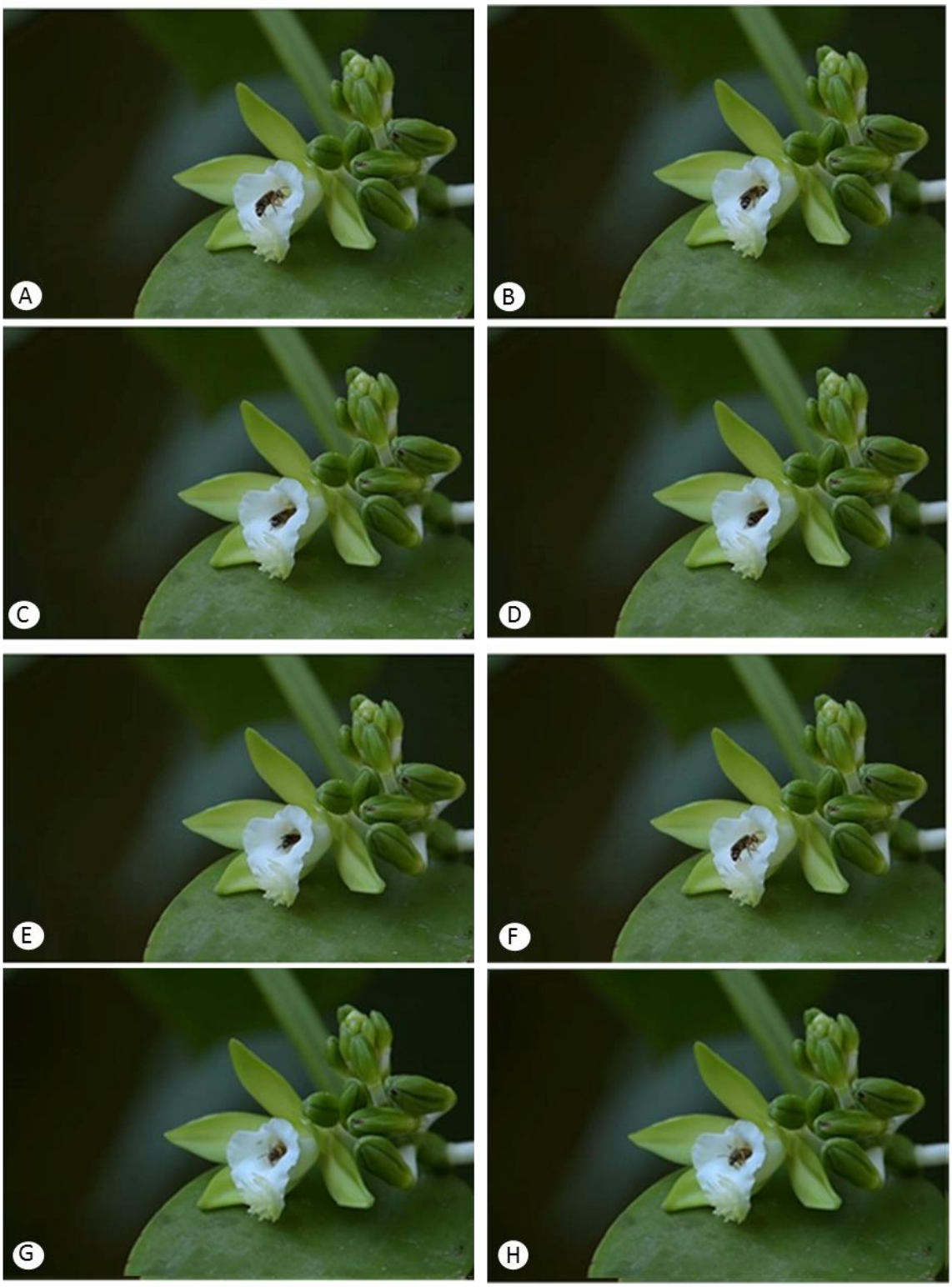

Figure 3.9 Entering Stage. Showing different stages in slow motion in various frames of pollination by *Thrinchostoma* bee. (A, B) Upon landing on the labellum, the bee moves toward the center of the flower, drawn by the fragrance attraction. (C, D) The bee lowers its body, as the inner flower is becoming narrower. (E, F) Moving slowly with yellow-orange lines inside the flower as guideline. (G, H) The bee turns itself around inside the flower. Moving gradually towards the exit and positioning its body and ready to fly away.

As the first *Thrinchostoma* bee approached a white-flowered *V. siamensis*, it spent over two minutes hovering round the open flower, before landing on the tuft of white hairs onto the labellum. The function of the white hairs on the labellum was possibly to act as a supporting pad as well as guiding line the insect into the labellum (Vargas et al. 2019; Johnson et al 1998; Johnson et al. 2003). Within a few seconds, the bee started to move along the labellum and into the flower. On high magnification of the video, pollen masses can be clearly seen attached to its back. The bee stayed for ninety seconds inside before turning its body around. The distance between the anther and the nearest part of the labellum was at least 3.2±0.2 (SD; N=30) mm (Fig. 3.3A and 3.3B), which was related to the functional height of the bee (Fig 3.7).

It proved difficult to observe the behavior once the bee was inside the labellum tube, particularly because the flower was situated more than 2.5 m above ground level, and the observation was made facing a bright sky. The bee crawled into the labellum and spent the majority of the time crawling on and around the central crest. Before leaving, it turned around the labellum with its head lifted. No direct field observation could be made on mechanical interactions between the bee and the anther or stigma. The interpretation by Rasmussen (1985) suggested the act in the genus as follows "*The flower is gullet-shaped and often provided with a tuft of retrorse hairs or crests, forcing visiting insects, when retreating, to raise their body and thereby touch the versatile anther.*" The effective pollinator is expected to have a functional height that is sufficient to be able to activate the pollination mechanism.

5. Discussion

Observation of natural pollination including the pollinator event by a *Thrinchostoma* species bee was captured on DVR. This resulted in the formation of a fruit suggesting the species was potentially pollinated by a *Thrinchostoma* bee species. This was the only case of pollination observed out of 28 flowers, giving a fruit set of 3.6%. This indicates that the species is pollen-limited, possibly due to low pollinator visitation. As a very low fruit set at 3.6% in this study by *Thrinchostoma* sp. 1 and *Thrinchostoma* sp. 2 compared with the non-fruit set for controlled flower pollination by the muslin covered. The low fruit set might be explained by three possible causes for pollen limitation: 1) low pollinator abundance, resulting in some flowers never being visited; 2) low amounts of pollen reaching the stigma, even when the pollinator visits are frequent; 3) low pollen quality, when the weather is too hot or dry, or even in wet conditions, the pollen may lose

its viability (Tremblay et al. 2005). However, this may not always be the case, as on occasion unusually high fruit set can be seen in the wild. Autogamy has been suggested to occur in a few *Vanilla* species, all of which have unusually high natural fruit sets (Householder et al. 2010; Soto- Arenas and Dressler 2000; Lubinsky et al. 2006), but there is no evidence that *V. siamensis* is autogamous; flowers that are shielded from insect visitors do not set seed (pers. obs.).

Results presented here suggest that two species of Hymenoptera, *Thrinchostoma* sp. 1 and *Thrinchostoma* sp. 2 are pollinators of *V. siamensis*. Morphological measurements of the operative size of *Thrinchostoma* sp. 2 filmed on *V. siamensis*, with functional height, which is the approximate height at which the insect passes by the column, were estimated based on the leg length and overall size of the insect. Observations of visitors and the floral morphology of *V. siamensis* indicate that the species is bee pollinated. This is consistent with the present knowledge of pollinators for other *Vanilla* species, all of which are pollinated by different bee species and genera (Ackerman 1983; Ackerman 1986; Johnson and Steiner 1997; Soto-Arenas and Cameron 2003; Soto-Arenas and Dressler 2010). From the observations of the floral morphology, an effective pollination of *V. siamensis* would be a bee that is of a specific size, such as *Thrinchostoma* sp. 2. No other smaller insects were observed as potential pollinators, as they would be too small to activate the pollination mechanism (Roubik and Ackerman 1987). The species produces extra-floral nectaries as an incentive for ants to patrol the flowers as a defense from herbivores. Similar to previous research, the most frequent group of non-pollinating insects found interacting with the buds of *V. siamensis* was ants, which is also true for other orchid species, (Johnson et al. 1998; Johnson and Steiner 2000; Jersáková et al. 2006; Schiestl and Johnson 2013).

False nectar guides such as fine spots and lines can stimulate visiting and food searching reaction by potential pollinators (Nilsson 1980). It is possible that the white and red tufts of hairs on the surface of the labellum contribute to the flower's attraction in a similar manner. However, floral fragrance has been shown to be more important than visual cues for bees when orientating at short distances to adjust their approach and landing (Free 1970). The *Vanilla* genus is known for its fragrant flowers, with no species lacking a fragrance currently known. Fragrance seems to play an important part in attracting pollinators for many *Vanilla* species (Soto-Arenas and Cameron 2003; Lubinsky et al., 2006 Householder et al. 2010; Soto-Arenas and Dressler 2010; Pansarin 2016). For example, the unusual high fruit set produced by *V. chamissonis* Klobschis was attributed to the strong floral fragrance (Gigant et al. 2011). Householder et al. (2010) described how particularly fragrant flowers of *V. pompona grandiflora* attract a greater number of pollinators than

individual flowers with a faint fragrance. To humans, the faint fragrance of *V. siamensis* is only perceptible at close distance. Most likely, the pollinating bees are also attracted to the flowers of *V. siamensis* because of its conspicuous size, color and placement in the canopy (Roubik and Ackerman 1987). The orange-yellow inner surface of the labellum of *V. siamensis* creates a color contrast from the rest of the flower that likely contributes to the close-up orientation of the pollinator.

There was a clear indication of the pollinia attached to its back before the bee took off. As soon as the bee left, the flower was immediately covered with a muslin cloth to prevent other insects going in. After ten days, the presence of an abscission zone between the ovary and the remainder of the flower appeared. It is at this point, that the flower fell away and the ovary started to swell within a few days. However, unsuccessful pollination results in the whole flower falling off much sooner, leaving a scar (Soto-Arenas and Dressler 2010).

6. Conclusions

This study suggests that bees of the genus *Thrinchostoma* are the pollinators of *V. siamensis*. The pollination mechanism is quite straightforward. Upon leaving the flower, the bee passes the central bump on the labellum and is forced to raise its body, activating the pollination process by coming into contact with the rostellum before the anther, and later with the stigma in another flower. However, pollination of *V. siamensis* by the *Thrinchostoma* bee, under natural conditions, appears to be less favorable when compared to artificial pollination techniques such as hand pollination (Johnson 2011)

Chapter 4: In vitro seed germination and plantlet regeneration of *Vanilla siamensis*: An endemic species in Thailand

1. Abstract

This study reports the *in vitro* germination of self-pollinated pod of *Vanilla siamensis*, native to Thailand. The vanilla seeds were asymbiotically germinated on difference culture media under aseptic conditions. The results showed that, New Dogashima medium (NDM) supplemented with 2% sucrose, 15% coconut water (CW), and 0.7% agar gave the highest levels of seed germination at 10.1%. The fastest and highest percentage seed germination was achieved using NDM supplemented with 2 mg/L gibberellic acid (GA_3). Seeds on this culture medium germinated within 7–8 weeks in comparison with 10–11 weeks on culture medium without plant growth regulators. Protocorms were transferred to different culture media and various concentrations of 6-benzyladenine (BA) to assess the protocorm development.

The results revealed that ½ MS medium gave the higher result in survival rate of protocorm than NDM. Culture media with BA showed the survival rate higher than that without BA. BA at 2 mg/L gave the highest shoot formation at 100% significantly difference from culture medium without BA. Plantlet development was completed after subculturing on ½MS medium supplemented with 0.5 mg/L α-naphthaleneacetic acid (NAA) for 8 weeks and successfully acclimatized at 91.7%. We conclude that MS medium, at half strength of nutrients, supplemented initially with 2 mg/L BA, was ideal for protocorm development, and later with NAA (0.5 mg/L), supported plantlet development. The protocol outline here and potentially variations on it can be used for mass propagation of this endangered species and used as guideline for improvement of *V. siamensis* and conservation *in vitro*.

2. Introduction

The genus Vanilla is a cosmopolitan genus of orchid found throughout tropical regions. Although the genus is comprised of 110 species, it is *V. planifolia* originally from Mexico that makes up the vast majority of commercial vanilla production. Vanilla is commercially cultivated for its pods, from which the world's most popular flavoring substance, vanillin, is extracted[1]. While *V. planifolia* originated in Mexico, it is now widely cultivated in many countries including Madagascar, Mexico, Indonesia, the Philippines,

Papua New Guinea, Fiji, Jamaica, Costa Rica and Peninsular India; Indonesia and Madagascar, however, make up 90% of the world's production (Sujatha and Bhat, 2010). The product of Vanilla, vanillin, is widely used in a variety of industries including food, pharmaceuticals, and cosmetics (Renuga and Kumar, 2014). It is the second most expensive spice in the world after saffron. In addition, vanilla is unique within the Orchidaceae as it is the member of the family to produce edible fruits.

Within South East Asia, *V. siamensis* (Rolfe ex Dawnie) is one of the many thick-leafed species of vanilla, and is one of five species found in Thailand (others being *V. albida*, *V. aphylla*, *V. griffithii* and *V. pilifera*). Flowers of *V. siamensis* can be differentiated from other species by the presence of finger-like hairs at the apex of the labellum. Within Thailand, *V. siamensis* is locally abundant in evergreen, dry evergreen and tropical forests, at 500 to 1200 masl *V. siamensis*. Like other species of vanilla, it produces a climbing vine that will climbs up the trunk into the canopy before cascade downward. However, anthropogenic pressures have resulted in the rapid decline in the natural habitat and as a consequent a reduction in the number of *V. siamensis* within its habitat. While the decline of the species is of conservation, the lost of the species could lead to the lost of important biomedical products. Recently it has been shown that extracts from the fruits of *V. siamensis* exhibit characteristic effects of a natural bone promoting compounds such as phytoestrogen (Renuga and Kumar, 2014).

Generally, vanilla is propagated by stem cutting of mature vine. However, the propagation rate by this method is slow, labour intensive and time consuming. At present, the application of biotechnological tools to questions around vanilla has focused mainly the development of *in vitro* multiplication methods as alternative propagation and germplasm storage *in vitro*. *In vitro* seed germination is necessary due to low seed production, hard seed coat and low germination under natural conditions.

Standardization of tissue culture technique can, therefore, be used to help solve problems of propagation (Chugh et al. 2009; Gonzalez et al. 2009; Gu at al. 1987; Knudson 1950; Morwal et al. 2015). At present, the *in vitro* germination and plantlet regeneration of *V. siamensis* are still limited, however an understanding of technique can be used for the improvement and *in vitro* conservation of vanilla. Further, successful *in vitro* germination of hybrids (*V. siamensis* x *V. planifolia*) is still in its early germinating stages of developement. Here we studied the effect of culture media and plant growth regulators (PGRs) on suitable germination of vanilla seeds and development of protocorms.

3. Material and Methods

3.1 Effect of culture media on seed germination

To determine the influence of culture media on seed germination subsequent to protocorm development, vanilla pod (bean) was cleaned by washing with running tap water for a few minutes, then cut open vertically and the seeds removed on to filter paper. The seeds were surface sterilized by dipping in 15% (v/v) sodium hypochlorite (NaOCl) solution containing 0.5% (v/v) Tween 20 for 10 minutes, after which the seeds were then rinsed three times with sterile distilled water. Sterilized seeds were placed onto New Dogashima medium (NDM) or ½ Murashige and Skoog (½ MS) or Vacin and Went (VW) medium supplemented with 2% sucrose, 15% coconut water (CW) and 0.7% agar, with or without 0.2% (w/v) activated charcoal (AC).

All cultures were maintained at 25±1°C under a 16 h photoperiod with light supplied by cool-white fluorescent lamps at an intensity of 10 µmol $m^{-2}s^{-1}$ photosynthetic photon flux density (PPFD). After culture for 10 weeks percentage of seed germination was calculated. All experiments consisted of three independent replicates with 10 culture bottles per replicate.

3.2 Effect of plant growth regulators (PGRs) on seed germination

Based on the results of the previous experiments, the most suitable medium was selected and used for further investigation. To determine the influence of PGRs on enhancement of seed germination frequency, vanilla pod was clean by the same processes or protocols as mentioned in the previous experiment.

The sterilized seeds were then placed onto NDM supplemented with 2% sucrose, 15% CW, 0.7% agar and various concentrations of gibberellic acid (GA_3) or 6-benzyladenine (BA). All cultures were maintained under the same conditions as mentioned in the previous experiment. After culture, percentage of seed germination was recorded every week. All experiments consisted of three independent replicates with 10 culture bottles per replicate.

3.3 Effect of culture media on protocorm development

To determine the influence of culture media on protocorm development, the protocorms were transferred to NDM or ½ MS medium supplemented with 2 mg/L BA, 2% sucrose, 15% CW and 0.7% agar. All cultures were maintained under the same conditions as mentioned in the previous experiment. After culture, percentage of survival

rate of protocorms was recorded every week. All experiments consisted of three independent replicates with 8 culture bottles per replicate.

3.4 Effect of concentrations of BA on shoot induction

To determine the influence of concentration of BA on shoot induction, the protocorms were transferred to ½MS medium supplemented with various concentrations of BA (0, 0.125, 0.25, 0.5, 1.0, 2.0 mg/L), 2% sucrose, 15% CW and 0.7% agar. All cultures were maintained under the same conditions as mentioned in the previous experiment. After 8 weeks of culture percentage of protocorm forming shoots was calculated. All experiments consisted of three independent replicates with 8 culture bottles per replicate.

3.5 Effect of concentrations of naphthaleneacetic acid (NAA) on root induction

To determine the influence of concentrations of NAA on root formation, the shoots of 1.5-3 cm in height were transferred to ½MS medium supplemented with various concentrations of NAA (0, 0.25, 0.5, 1.0, 2.0 mg/L), 2% sucrose, 15% CW and 0.7% agar. All cultures were maintained under the same conditions as mentioned in the previous experiment. Percentage of shoot with roots and number of roots per shoot were recorded after 8 weeks of culture. All experiments consisted of three independent replicates with 8 culture bottles per replicate.

3.6 Acclimatization of plantlets

The in vitro plantlets with well-developed roots were removed from culture vessel, washed thoroughly in running tap water to remove residual agar medium before transfer to sand: compost mixture: coconut husk (1:1:2) in 4-inch plastic pots. The plants were grown in a shaded house under 80% shading. Percentage of survival plantlets was recorded after 8 weeks of transplanting.

3.7 Statistical analysis

The experiments were set up in completely randomized design (CRD) The test of significant differences among treatments were determined using Duncan’s multiple range test (DMRT)

4. Results

4.1 Effect of culture media on seed germination

After 12 weeks of culture, seed germinated from all tested culture media was observed, but the germination percentage significantly differed. The seeds cultured on NDM supplemented with 2% sucrose, 15% CW, and 0.7% agar gave the highest level of seed germination (10.1%). In the same culture medium, seed germination rate in the medium without AC was higher compared to medium containing 0.2% AC (Fig. 4.1). The result indicates that, the seeds cultured on this medium could imbibe water the best leading to soften of seed coat. In addition, oxygen could enter the seed together with water caused the seed germinate as early as possible. The germination process started approximately about 9 weeks after culturing. After this period the embryo continued growing larger, seed coat bursted, embryo emerged from the seed coat and rhizoids appeared (arrow) (Fig. 4.2a). Twelve weeks after culturing, protocorm with shoot apex and leaf primordial continued to grow (Fig. 4.2c). When the protocorm reached about 2 mm in length, the protocorm turned into green color (Fig. 4.2c-d).

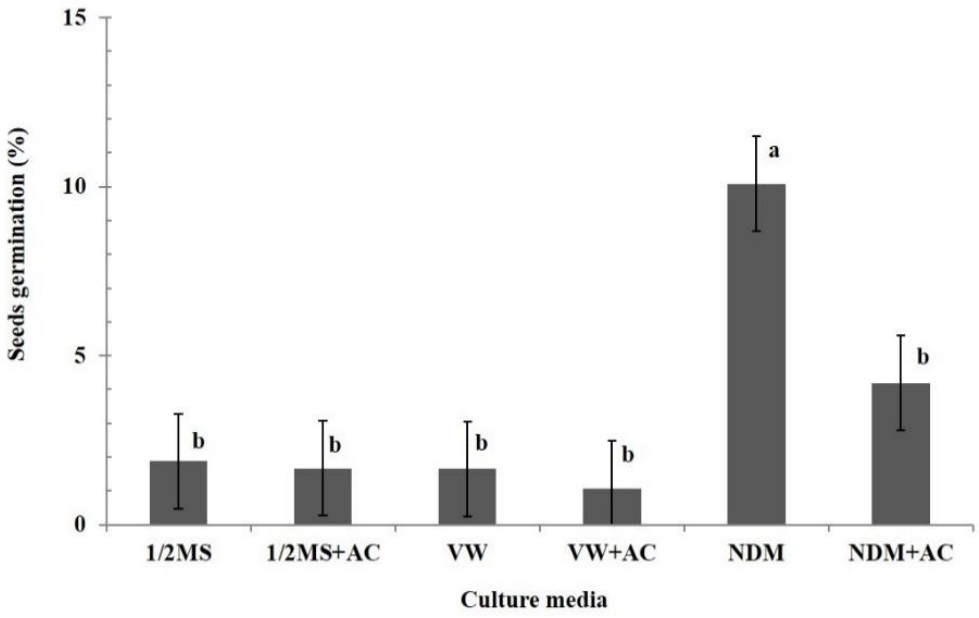

Figure 4.1 Percentage seed germination after culturing on different culture media with and without activated charcoal (AC) for 12 weeks. Bars correspond to SE of means. Values with different letters are significantly different ($P \leq 0.01$)

Figure 4.2 Germination of vanilla seeds on NDM showing the enlargement of embryo andrhizoids (arrows) formed on the surface of the protocorm (a), 10 weeks of culture (b) 11 weeks of culture (bar = 2 mm) (c-d) development of protocorm with shoot apex (arrow) after 12 weeks of culture (bar = 2 mm)

4.2 Effect of plant growth regulators (PGRs) on seed germination

The effect of PGRs on seed germination after culturing on NDM supplemented with 2% sucrose, 15% CW, 0.7% agar were shown in Figure 4.3. The fastest and highest levels of seed germination were obtained on this medium supplemented with 2 mg/L GA_3. Seeds on GA_3 containing medium germinated within 7–8 weeks in comparison with 10–11 weeks on the same medium without plant growth regulators. After 10 weeks of culture, the highest percentage of seed germination was observed (18.2%). Both concentrations of GA_3 gave nearly the same effective on seed germination but far different to that obtained from BA and PGR-free containing medium. The seed germination rate in the medium without PGRs was the lowest (5.6%) (Fig. 4.3). Although the highest germination level was observed on GA_3 containing medium, however protocorm remained yellow with some turned into brown color and died after germination for 4 weeks (Fig. 4.4).

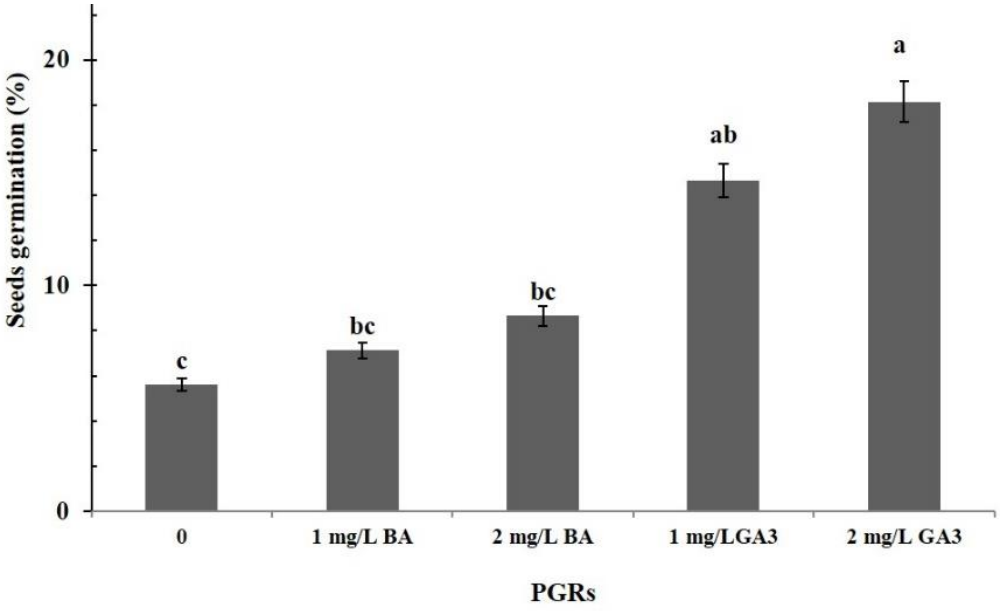

Figure 4.3 Percentage of seed germination after culturing on NDM supplemented with various PGRs at different concentrations for 10 weeks. Bars correspond to SE of means. Values with different letters are significantly different ($P \leq 0.01$)

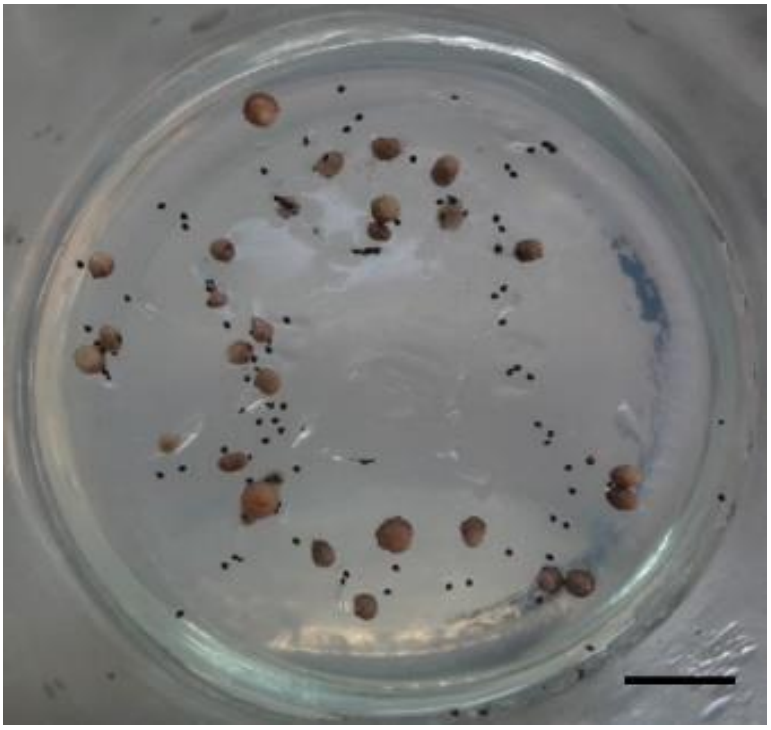

Figure 4.4 Seed germination on NDM with 2 mg/L GA_3 after 10 weeks of culture with some turning brown and dying after culture for further 4 weeks

4.3 Effect of culture media on protocorm development

The role of culture media on protocorm development was presented in Figure 4.5 The component of nutrient in culture media showed a significant effect on the survival rate and growth of protocorm. At 6 weeks after culturing, protocorms cultured on ½MS medium had a survival rate of 79%, higher than those on NDM (21%) (Fig4.5). In ½MS medium, protocorms showed the development of the scale-like leaf primordial and developed into shoot, whereas protocorms on NDM had not change. The sizes of protocorms still the same as initial culture (Fig. 4.6). Thus, ½MS medium was found to be the best choice for promoting the development of protocorm into shoot formation.

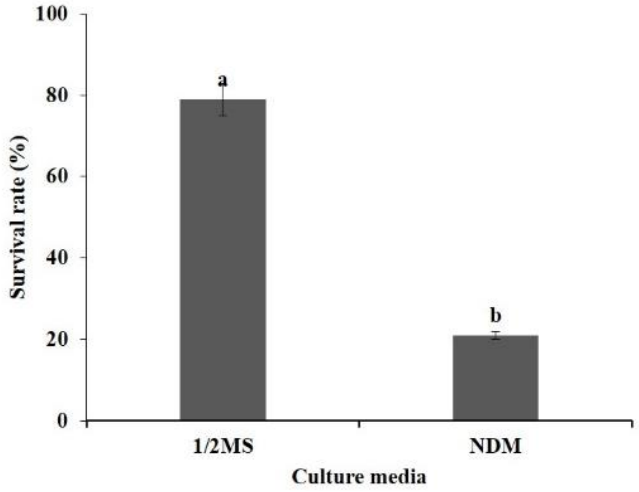

Figure 4.5 Survival rate of protocorms after culturing on ½ MS and NDM for 6 weeks**.** Bars correspond to SE of means. Values with different letters are significantly different ($P \leq 0.01$)

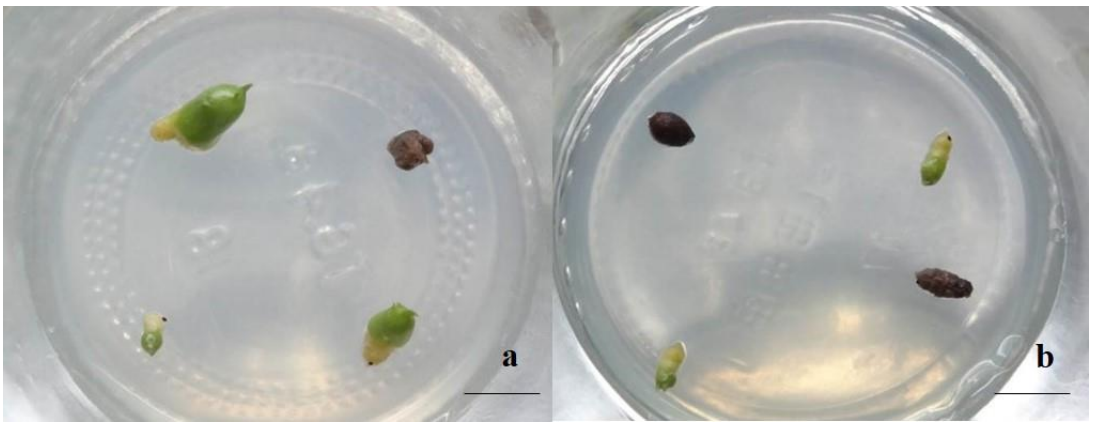

Figure 4.6 Comparison of protocorm development after transferring onto ½ MS (a) and NDM (b) for 6 weeks (bar = 0.5 cm)

4.4 Effect of concentrations of BA on shoot formation

After 8 weeks of culture, protocorm on a culture medium with BA showed a higher survival rate than culture medium without BA. Significant differences were observed among concentrations of BA for survival rate, shoot formation and number of shoots (Table 4.1). The highest survival rate (96.7%) was observed when protocorms were cultured on ½ MS medium supplemented with 0.5-1 mg/L BA. BA at 2 mg/L exhibited the highest multiplication of shoot at 100%, followed by 0.5 and 1.0 mg/L BA (93.3 and 96.7%, respectively). The number of shoots per explant was not significantly different. The culture medium with 1.0 mg/L BA was suitable for shoot formation (Table 4.1).

Table 4.1 Effect of concentrations of BA on survival rate and shoot formation of *V. siamesis* after culturing on ½ MS for 8 weeks

BA (mg/L)	Survival rate (%)	Shoot formation (%)	Number of shoots/explant (shoots)
0	73.3c	0d	1.33
0.125	76.7bc	23.3c	1.67
0.25	83.3abc	73.3b	2.00
0.5	96.7a	93.3a	2.00
1.0	96.7a	96.7a	2.67
2.0	93.3ab	100.00a	2.33
F-test	**	**	ns
C.V. (%)	11.5	11.6	37.3

**=significantly different at $P \leq 0.01$, ns = not significantly different
Mean values followed by the same letters within a column are not significantly different ($P \leq 0.01$).

4.5 Effect of concentrations of NAA on root induction

After 8 weeks of culture, root induction on culture medium with NAA showed better results than culture medium without NAA. Significant differences were observed among concentrations of NAA for root formation. The highest root formation (100%) was observed when shoots were cultured on ½MS medium supplemented with 0.5 mg/L NAA. There was not significantly different for number of roots among concentrations of NAA (Table 4 . 2). Complete plantlets were obtained after culturing on ½MS medium supplemented with 0.5 mg/L NAA for 8 weeks and successfully acclimatized at 91.7% (Fig. 4.7).

Table 4.2 Effect of concentrations of NAA containing ½ MS on root formation and number of roots of *V. siamensis* after culturing for 8 weeks

NAA (mg/L)	Root formation (%)	Number of roots/shoot (roots)
0	58.3b	1.33
0.25	83.3a	1.45
0.5	100a	1.55
1.0	100a	1.67
2.0	100a	1.67
F-test	**	ns
C.V. (%)	10.3	42.0

**=significantly different at $P \leq 0.01$, ns = not significantly different

Mean values followed by the same letters within a column are not significantly different ($P \leq 0.01$).

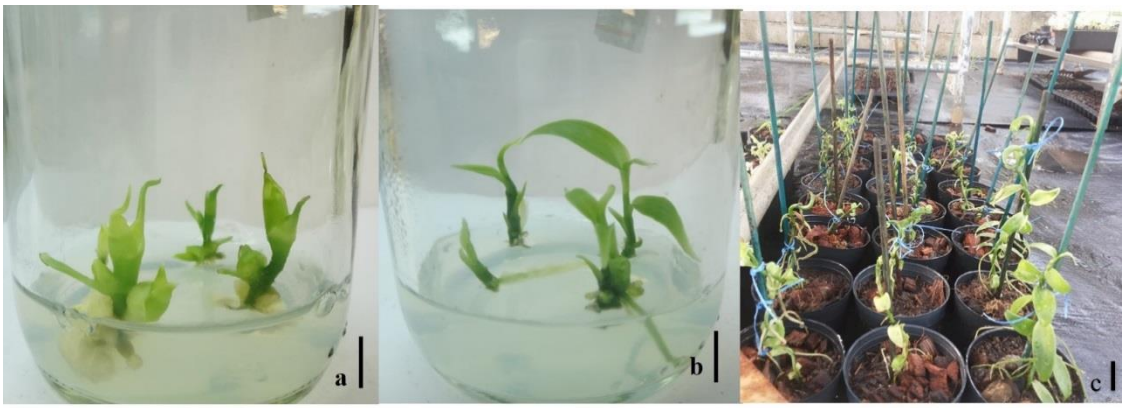

Figure 4.7 Shoot formation on ½ MS medium containing 2 mg/L BA (a), complete plantlets afterculturing on ½ MS with 0.5 mg/L NAA for 8 weeks (b) and survival plantlets after 4 months of acclimatization (c) (bar = 1 cm)

5. Discussion

There are many different culture media used for asymbiotic germination of orchid, including MS, VW, Kundson (KC), and NDM. Since orchid seeds, including vanilla, contain very little or no food storage tissues, and as such there is a nutrient requirement for initiation of germination. The results from this study showed that NDM medium provided a good germination rate of *V. siamensis* seed while prolonging survival rate of the protocorm probably due to the high contents of mineral in medium. MS medium with a reduction of constituents to half its original strength (½MS) has been reported to give a good response in protocorm development (Zuraida et al. 2013). Zuraida et al. (2013) studied the effect of growth on shoot formation, the result showed that ½MS medium gave a better response on shoot formation than full strength MS medium. However, Minoo (2002) reported that full strength MS medium gave better results than KC medium, for in vitro culture of vanilla. The minimum germination (26%) was observed in ½MS and maximum (85%) was recorded in full strength MS medium supplemented with 2 g/L tryptone.

The requirement of PGRs for germination is considered to be related to the utilization of lipids that constitute the primary storage material in most orchid seeds. It has been observed that unless storage lipids are utilized, germination does not continue (Pauw et al. 1995; Paul et al. 2012). The phytohormones, BA and GA_3 are important in regulating numerous plant growth and developmental processes. Adding GA_3 in culture medium increased the percentage of germination. Seeds on this medium germinated within 7–8 weeks compared to 10–11 weeks on medium without plant growth regulators. GA_3 plays a significant role in flower development, fruit development, shoot elongation and is known to stimulate seed germination in a wide range of plant species (Thomas et al. 2005). The result is similar to the findings of Mello (2009) who reported that GA_3 increases the rate of seed germination. This might be due to effectiveness of GA_3, which at higher concentrations overcome dormancy, causing rapid germination of seed. Higher concentration of GA_3 proved to be more effective from their respective than lower concentration. However, the present study indicated that percentage of germination was low, which might be cause by the hard seed coat of vanilla seeds. To active a high percentage of seed germination in the further, research should focus on pre-treatment of seed as a pre-culture method.

For shoot induction, the culture medium with BA was significantly difference from culture medium without BA. BA is a cytokinin hormone, which affects cell division and

shoots multiplication. Moreover, this study also showed the same corresponding result as of (Zuraida 2013). They reported that BA play role in shoot formation of *V. planifolia*. After 45 days of culture, 1 mg/L BA showed the highest shoot multiplication at 6.06 shoots/explant and shoot length at 4.5 cm Similar to Tan et al. (2011), a mean number of 4.2 shoots per callus was produced on medium containing 1.0 mg/L BA and 0.5 mg/L NAA with a mean shoot length at 3.8 cm after 8 weeks of culture. Janarthanam and Seshadri[21] reported that shoots were only formed from the callus of *V. planifolia* when the medium was supplemented with BA in addition to NAA, and no shoots were induced when BA or NAA were applied alone. BA at 1 mg/L combined with 1.5 mg/L kinetin (KN) gave the best result in shoot induction of *V. planifolia* (Abebe 2009).

Chapter 5: Development of cryopreservation protocol for *vanilla siamensis*: an endangered orchid species in Thailand.

1. Abtract

Vanilla siamensis is listed in Appendix-II of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) as endangered species in Thailand. To develop optimum cryopreservation protocol for *V. siamensis*. Protocorms were precultured on solid ½ MS medium with 0.5 M sucrose for 0-7 days. For encapsulation-dehydration, encapsulated protocorms (beads) were dehydrated for 0-6 hours. In case of encapsulation-vitrification, the beads were loaded with a plant vitrification solution 2 (PVS2) at 0 ˚C for 0-90 min. Protocorms precultured for 3 days gave the highest post-cryopreservation survival of 17%. Dehydration of the encapsulated protocorm beads for 4 hours gave the highest survival of 33% and a regrowth of 25%. Protocorms from encapsulation-vitrification method did not survive at all. Protocorms precultured with 0.5 M sucrose for 3 days, encapsulated with 3% sodium alginate and dehydrated to moisture content c. 14% before plunging into LN was suitable method for cryopreservation of *V. siamensis* protocorms.

2. Introduction

Vanilla siamensis (Rolfe ex Dawnie), is one of the five species of vanilla found in Thailand (others being *V. graffithii, V. pilifera, V. aphylla* and *V. albida*). *V. siamensis* is locally abundant in the hill of evergreen forests, in the dry evergreen forest and tropical forest where they are situated at high elevation from 500 -1200 masl. The mature *V. siamensis* vine will climb as high as possible and will cascade downward from the canopy, which will eventually stimulate the stems to flower. Recent studies on the extract from the fruits exhibited the characteristic effects of a natural bone promoting compound such as phytoestrogen (Wanachantararak et al. 2012). *V. siamensis* is listed in Appendix-II of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) as endangered species in Thailand.

Vanilla germplasm is conserved ex situ as whole plants in the field at both various institutes and private collections in Central America, the Caribbean, Asia, the Pacific Islands and Oceania, and Madagascar (Poobathy et al. 2013). In vitro slow growth storage of vanilla plantlets is also employed in several institutes. For example, the Indian Institute of Spices Research maintains 100 vanilla accessions and several self progenies of *V.*

tahitensis under in vitro storage conditions (Divakaran et al. 2006). The slow growth procedure developed for *V. planifolia* has been effectively applied to four other endangered *Vanilla* species, allowing their in vitro storage for more than 7 years with yearly subcultures (Divakaran et al. 2006). Cryopreservation is the most efficient and cost-effective strategy for long-term storage of genetic resources of vegetatively propagated plants such as vanilla and is also becoming an increasingly important tool for ex situ conservation of germplasm of endangered plant species (Keller et al. 2008; Touchell and Walters 2000).

In vitro cryopreservation is currently widely experimented on members of the Orchidaceae. Many different cryopreservation methods have been proposed for successful conservation of Thai orchids, including vitrification (Hu et al. 2013; Mohanty et al. 2013a; Mohanty et al. 2013b; Thammasiri 2000; Thammasiri and Soamkul 2007) encapsulation-dehydration (Hongthongkham & Bunnag 2014; Maneerattanarungroj et al. 2007; Surenciski et al. 2013; Worrachottiyanon and Bunnag 2018) and encapsulation-vitrification (Gogoi et al 2012; Thammasiri 2008). The successful methodologies using different orchid explant, including seed, pollen, protocorms and meristematic tissues have been reported. Immature seed and mature seed are the preferred material for conservation due to required small storage space. However, vanilla seeds are covered by a hard and dark coat that makes it difficult to determine the embryo viability. Thus, the protocorm was selected as plant material. Unfortunately, there have been no reports about cryopreservation of *V. siamensis*. Thus, the objective of this study was to develop optimum cryopreservation protocol for cryopreserve of *V. siamensis.*

3 Material and Methods

3.1 Plant Materials

Seeds were cultured on ½MS medium supplemented with 3% sucrose and adjusted pH to 5.7 with 0.1 N HCl before adding agar and autoclaving at 1.05 kg/cm^2, 121ºC for 15 min. The cultures were placed at 26±2ºC under 14 h photoperiod at density of 40 μmol^{-2}s^{-1} photosynthetic photon flux density (PPFD). After germination, protocorms were used for cryopreservation. (Fig. 5.1)

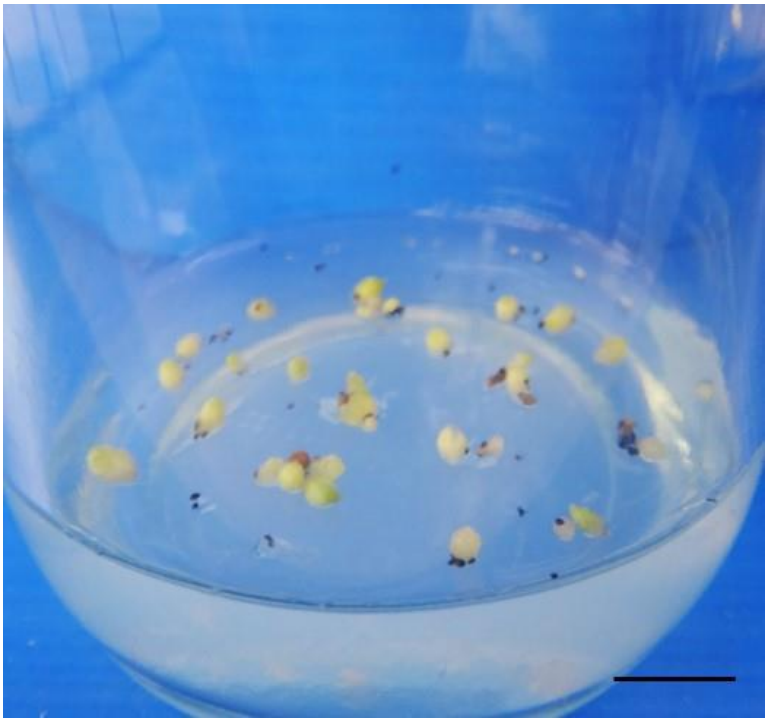

Figure 5.1 Germinated protocorms after culturing on ½ MS medium supplemented with 3% sucrose for 8 weeks (Bar = 1 cm)

3.2 Effect of pre-conditioning periods on survival rate of protocorms

Protocorms were precultured on solid ½MS medium supplemented with 0.5 M sucrose in proliferation medium (MS supplemented with 1.0 mg/l BA) for 0, 1, 3, 5 and 7 days. After the pre-conditioning protocorms were directly plunged into liquid nitrogen (LN) for 24 hours. After rapid warming in a water bath at 40˚C for 3 min, protocorms were transferred to solid ½MS medium supplemented with 1.0 mg/l BA, 3% (w/v) sucrose and 0.75% (w/v) agar. The survival rate was determined by counting the number of protocorms that remained green after 8 weeks of culture. Regrowth was evaluated by counting the number of protocorms that developed new shoots after 16 months of culture.

3.3 Encapsulation-dehydration

The precultured protocorms were encapsulated in sodium alginate beads by suspending them in 3% sodium alginate solution followed by 0.1 M $CaCl_2.2H_2O$ solution for 30 minutes to allow polymerization of the beads with approximately 4 mm in diameter. The beads were put on the sterilized Petri-dishes and dehydrated in laminar air flow for different durations ranging from 0, 2, 4 and 6 hours. For the determination of beads moisture content, fresh weight of beads was measured after each dehydration period; then beads were dried at 70 ºC in an hot air Oven for 12 hours and then reweighed. Moisture

Content (MC) was calculated using the following formula:

$$MC\ \% = [(\text{Beads fresh weight} - \text{Beads dry weight}) / \text{Beads fresh weight}] \times 100$$

After the dehydration the beads were directly plunged into LN for 24 hours. After rapid warming in a water bath at 40˚C for 3 min, protocorms were transferred to solid ½MS medium supplemented with 1.0 mg/l BA, 3% (w/v) sucrose and 0.75% (w/v) agar. The survival rate was determined by counting the number of protocorms that remained green after 8 weeks of culture. Regrowth was evaluated by counting the number of protocorms that developed new shoots after 16 months of culture.

3.4 Encapsulation-vitrification

Protocorms were encapsulated in 3% sodium alginate. Protocorms that were dispersed in 0.1M $CaCl_2.2H_2O$ solution and allowed to harden for 30 minutes to form beads approximately of 4 mm in diameter. The beads were sufficiently dehydrated by modified plant vitrification solution 2 (PVS2) at 0˚C for various durations (30-90 min). The solution consisted of 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) dimethyl sulfoxide without sucrose in liquid MS medium. The beads were transferred into cryo-tube containing fresh PVS2 solution and plunged into LN for 24 hours. About 10 dehydrated beads were suspended in 0.7 ml PVS2 solution in a 2.0 ml cryotube which is plunged in LN. For rapid warming, cryotubes were immersed in a water bath at 40°C for 3 min. The beads were unloaded with basal culture medium supplemented with 1.2M sucrose for 20 min, and then transferred to solidified ½MS medium. The survival rate was determined by counting the number of protocorms that remained green after 8 weeks of culture. Regrowth was evaluated by counting the number of protocorms that developed new shoots after 16 months of culture.

3.5 Statistical analysis

The experiments were set up in completely randomized design (CRD) with 8 replications. Significance of difference in mean values among treatments was assessed by analysis of variance (ANOVA) with Duncan's multiple range test ($P < 0.05$).

4. Results

4.1 Effect of pre-condition of on survival rate of protocorms

Protocorms were precultured on ½MS medium supplemented with 0.5M sucrose for various times and freezing. After 8 weeks of culture, the result indicated that survival rate of protocorms without plunging into LN (-LN) was gradually decreased by increasing duration of preconditioning. After plunging protocorms into LN (+LN), the result revealed that protocorms precultured with 0.5M sucrose for 3 days gave the highest survival rate at 16.67% (Fig. 5.2).

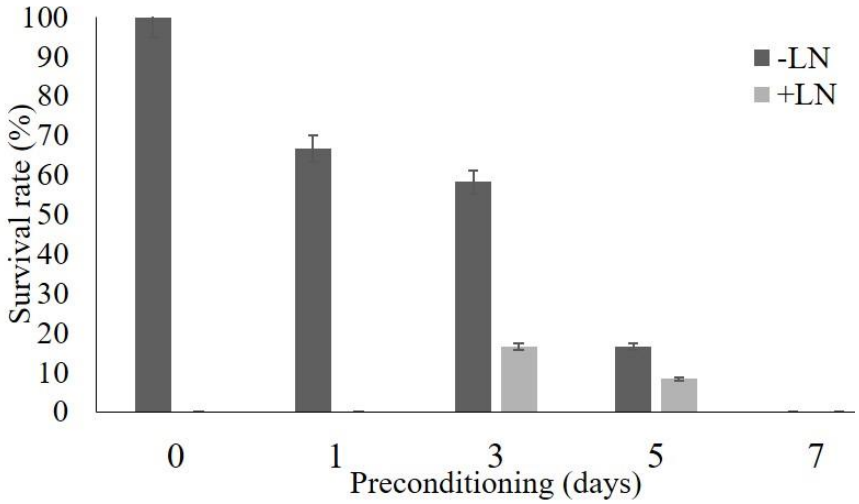

Figure 5.2 Effect pre-conditioning period on survival rate of protocorms without plunging into LN (-LN) and plunging into LN (+LN) for 24 hours after culturing on regrowth medium for 8 weeks.

4.2 Encapsulation-dehydration

Encapsulated protocorms were dehydrated for 0 to 6 hours in laminar air flow before immersion into LN. Cryopreserved protocorms showed significant difference among the dehydration periods. The moisture content of encapsulated-protocorms decreased with dehydration periods. The beads dehydrated for 4 hours and plunged into LN showed the best survival rate of protocorms at 33.33%. When protocorms were dehydrated for more than 4 hours, there was a decrease in viability. (Table 5.1). After culturing for 16 weeks, the highest percentage of protocorms regrowth obtained was around 25% when encapsulated protocorms were dehydrated for 4 hours. Protocorms showed initial growth by development of shoot and root (Fig. 5.3). Protocorms with non-dehydration (MC=40.55%) and long dehydration periods with moisture content less than 7% did not survive. range test (DMRT) at 5% confidence level.

Table 5.1 Effect of dehydration periods on survival rate and regrowth of PLBs without plunging into LN (-LN) and plunging into LN (+LN) for 24 hours after culturing on regrowth medium for 8 and 16 weeks

Dehydration (h)	MC (%)	**Survival rate (%)[1]		**Regrowth (%)[2]	
		-LN	+LN	-LN	+LN
0	40.55	100.00a	8.33cd	83.33a	0.00c
2	18.12	83.33ab	16.67cd	41.67b	8.33bc
4	14.10	66.67b	33.33c	33.33bc	25.00bc
6	7.03	0.00d	0.00d	0.00c	0.00c

[1, 2] The data was recorded after 8 and 16 weeks of culturing respectively.
**=significantly different at $P \leq 0.01$, MC = Moisture content
Mean values followed by the same letters within a column are not significantly different ($P \leq 0.01$).

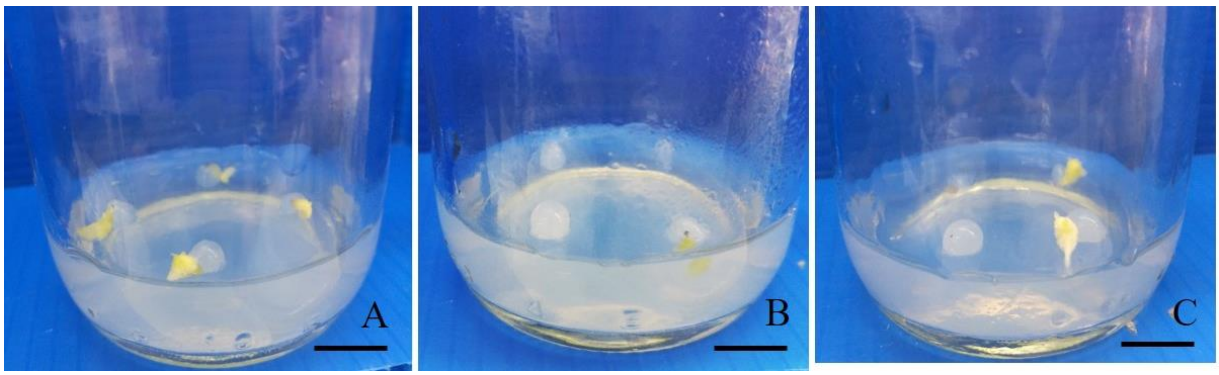

Figure 5.3 Protocorms development after cryopreservation by encapsulation-dehydration method (A) non-cryopreserved protocorms; (B) cryopreserved protocorms from dehydration for 2 hours; (C) cryopreserved protocorms from dehydration for 4 hours. (Bar = 1 cm)

4.3 Effect of exposure time to plant vitrification solution

Effect of PVS2 on survival of encapsulation, cryopreserved protocorms was presented in Table 5.2. Encapsulated protocorms were dehydrated with various time of PVS2 before plunging into LN. Results obtained from encapsulation-vitrification experiments indicated that the immersion in PVS2 is damaging to the protocorms. Survival rate was gradually decreased by increasing duration of immersion in PVS2. No growth was observed for cryopreserved protocorms that were subjected to the encapsulation-vitrification treatment, even after 16 weeks of recovery. Only non-cryopreserved protocorms showed initial growth by development of shoot and root (Fig.5.4). This indicated that the

encapsulation-vitrification method may need to be further optimised for successful cryopreservation of this plant.

Table 5.2 Effect of exposure time to plant vitrification solution 2 (PVS2) at 0 ºC on survival rate and regrowth of protocorms without plunging into LN (-LN) and plunging into LN (+LN) for 24 hours after culturing on regrowth medium for 8 and 16 weeks

PVS2 (min)	**Survival rate (%)[1]		**Regrowth (%)[2]	
	-LN	+LN	-LN	+LN
0	100.00a	0.00c	100.00a	0.00b
30	16.67b	0.00c	8.33b	0.00b
60	8.33bc	0.00c	0.00b	0.00b
90	0.00c	0.00c	0.00b	0.00b

[1, 2] The data was recorded after 8 and 16 weeks of culturing respectively.
**=significantly different at $P \leq 0.01$
Mean values followed by the same letters within a column are not significantly different ($P \leq 0.01$).

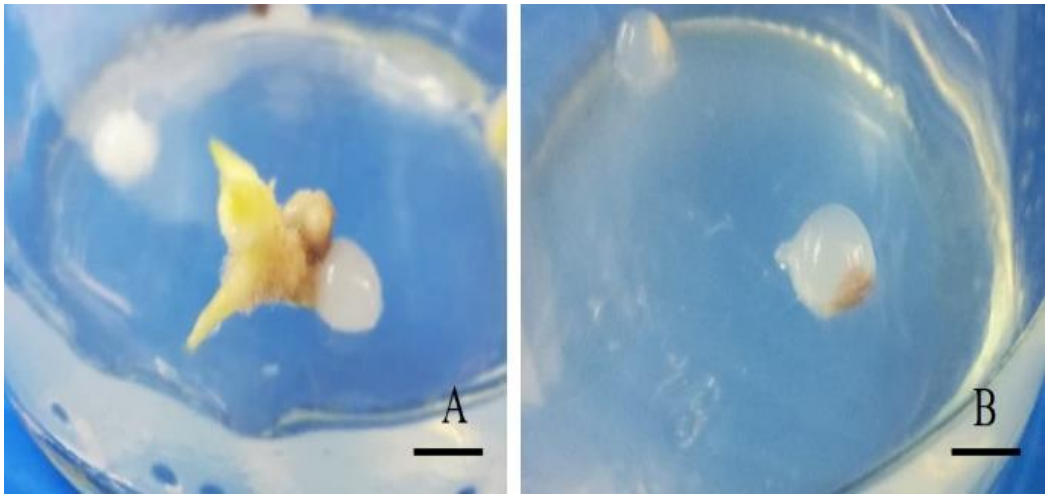

Figure 5.4 Encapsulated protocorms after dehydration with plant vitrification solution 2 (PVS2) at 0 ºC without plunging into LN (A) and after plunging into LN (B) after culturing on regrowth medium for 16 weeks (bar = 5 mm)

5. Discussion

5. 1 Effect of pre-condition of on survival rate of protocorms

In vitro cryopreservation is currently widely experimented on members of the Orchidaceae (Bukhov et al. 2006; Gogoi et al 2012; Hu et al. 2013; Popov et al. 2004; Popova et al. 2016; Pritchard and Seaton,1993; Yin et al. 2011; Zainuddin et al. 2011). Preconditioning of samples by culturing on sucrose-enriched medium was shown to be very important to produce high level of recovery growth after cryopreservation. The results from this study showed that protocorms precultured with 0.5M sucrose for 3 days significantly improved the recovery of protocorms which gave the highest survival rate at 16.67%. Preculture of encapsulated beads with medium containing 0.5M sucrose in the dark for one day and dehydrated for 8 hours before plunging into LN was successfully cryopreserved of protocorms of Grammatophyllum specinocum BL. (Wongrasee and Bunnag 2018). The role of sucrose in the present study could be interpreted as both nutritional and osmotic regulatory functions of this carbohydrate. A sugar treatment of plants with sucrose was shown to be very important in improving the survival of cryopreserved PLBs from several orchids such as Cymbidium finlaysonianum (Worrachottiyanon and Bunnag 2018), Bletilla formosana (Hayata) Schltr (Hu et al. 2013) and Dendrobium Bobby Messina (Zainuddin et al. 2011). The accumulation of sucrose inside tissues helps maintain cell viability during dehydration and cryopreservation by stabilization of membranes (Gonzalez-Arnao et al. 2009). In addition, the absorbed sugar stabilizes the membranes by replacing water and forming hydrogen bonds with the phospholipids (Turner 2001). The balance between the exit of intracellular water, ice formation and cell solute concentration is important for successful cryopreservation (Benson 2008). In agreement with above, in the present study none precultured protocorms did not survive at all. The reduction in survival and regrowth may be due to the osmotic shock at higher sucrose concentrations.

5. 2 Effect of dehydration periods on the survival rate of encapsulated protocorms

Encapsulation-dehydration is widely practiced on plants, because it is easily handled and maintains high rates of recovery after cryopreservation, this is due to the beads surrounding the explant which reduces the shock of exposure to LN (Shibli 2006). The encapsulation step protects the explant while allowing exposure to extreme conditions that are usually deleterious to unencapsulated explants, for instance pre-treatment on high sucrose concentrations and dehydration to low moisture contents. The dehydration process removes most or all freezable water from the encapsulated explants, allowing vitrification

of the intercellular solutes as an explant is exposed to LN and avoiding lethal intracellular ice crystallization (Engelmann 1997; Engelmann et al. 2008; Poobathy et al. 2013; Worrachottiyanon and Bunnag 2018). The present study showed the significant difference among the dehydration periods. Survival and regrowth of the non-cryopreserved encapsulated protocorms was decreased with increasing the dehydration period. The beads dehydrated for 4 hours and plunged into LN showed the best survival rate of protocorms. And the result demonstrated that no survival rate was achieved after dehydration for 6 hours. However, dehydration for 12 hours yielded the highest viability and regrowth of cryopreserved *Cymbidium finlaysonianum* (Worrachottiyanon and Bunnag 2018). The optimum MC of dehydrated beads for cryopreservation varied between 14.10-18.12% was achieved in this study.

5.3 Effect of exposure time to plant vitrification solution

In case of encapsulation-vitrification, vitrification refers to the physical process by which a highly concentrated aqueous solution solidifies into a glassy solid at sufficiently low temperatures without crystallization (Hong et al. 2009). The keys of success with cryopreservation by vitrification are to carefully control the dehydration procedures and to prevent injury by chemical toxicity or excessive osmotic stress during treatment with the PVS2 solution (Lambardi et al. 2008; Tsukazaki et al. 2000). The most common approach involves the immersion time of samples with PVS2. Several reports have shown that dehydrated at 0˚C can reduce the toxicity of vitrification solutions, usually yielding higher survival (Wang et al. 2002; Yin and Hong 2009). This was evident in the case of non-cryopreserved explants. High recovery rates were also obtained in many orchids (Gogoi et al 2012; Hu et al. 2013; Thammasiri 2008; Watanawikkit et al. 2012) However, in this study PLBs did not survive after plunging into LN by encapsulation-vitrification procedure at all. Because, the toxicity of vitrification solution could occur to the samples due to extended incubations in the cryoprotectant. The result of encapsulation-vitrification from this study was related with Gonzalez-Arnao (Hongthongkham and Bunnag 2014) reported that vanilla tissues proved to be very sensitive to PVS2 and were either killed (apices derived from clusters of in vitro plantlets obtained from microcuttings) or damaged before cooling (apices derived from clusters of in vitro plantlets obtained from IFEs), vanilla apices could not be cryopreserved using the vitrification method.

Among available cryopreservation techniques, encapsulation-dehydration has received much attention in recent years because of its simple, easy-to-handle and cost-effective procedures which allow for high genetic stability of cryopreserved explants.

Moreover, this technique causes no toxicity and is even applicable to dehydration-sensitive explants (Popova et al. 2016; Zainuddin et al. 2011). In conclusion, the results obtained from both the control encapsulation-dehydration and encapsulation-vitrification experiments indicated that the encapsulation-dehydration treatment is less damaging to the non-cryopreserved protocorms when compared to the encapsulation-vitrification treatment. Thus, protocorms precultured with 0.5M sucrose for 3 days, encapsulated with 3% sodium alginate and dehydrated by laminar air flow for 4 hours before plunging into LN was suitable method for cryopreservation of protocorms of *V .siamensis*.

Chapter 6: General Discussion

1. Distribution of *Vanilla siamensis*

In the observation of various Vanilla vines in the wild, as well as in cultivation, there seem to be no host-specific trees, but host preference trees. However, at Kao Soi Dow the Vanilla vine will adapt and chose their host preference tree in their natural sursoundings. The *V. siamensis* in the wild is predominantly located near the edge of a waterfall areas, within a clear forest. Chosen for its high content in organic matter as well as excessive water areas-moisture and high humidity. To the edge of the waterfall areas, within a clear forest, the branches of the *Pterocybium tentorium*, 45 metre trees are high to maintain constant shade, neither too intense, nor too weak in order to preserve the development and blooms of *V. siamensis*.

In some way *V. siamensis* do not have to compete with the *P. tentorium* for water areas and its nutritive requirements, but obtain these from the bark of *P. tentorium*, where it is always moist. This encourages *V. siamensis* further development. The gradual habitation is sloped below the wooden trees to benefit the humus generated by the trees at the top. At the Kao Soi Dow, the ground is flat, moving away from the nearest edge of the water areas fall where the soil is well drained so as to clear up the excessive water areas. The preference choice of the tree is important as it must protect the Vanilla vines from the heat of the sun or from the wind, and so as not to compete for water areas supply and compost.

Where as, in comparison to Tahiti, the traditional way of cultivation has been used for several generations where the Vanilla vine is planted at the base of a tree. The tree provides support and shader for the climbing orchid. The two favourite host trees for *Vanilla tahitiensis* are the “piti” (*Gliricidea maculata*) and the “pignon d’ inde” (*Jatropha curcas*). Gliricidea is a leguminous plant which does not compete with the Vanilla for nitrogen supply. It grows quickly and requires frequent pruning where (Jatropha) requires less pruning to limit its growth, as leaves fall naturally during the dry season. It is during this time when flower induction occurs in *V. tahitiensis*.

The *Pterocybium tentorium* tree distribution is found predominantly from 10 m x 50 m grid techniques. The trees thrive better on the edges of the waterfall areas. The bark of *P. tentorium* is soft and can retain its moist surface which *V. siamensis* prefer in the wild. Few *P. tentorium* trees have been observed to have fallen down from the excess weight of the *V. siamensis* vines, tightly packed over the years. Once the *V. siamensis* vine reaches the

top of the tree canopy, the vines cascade down and develop new flower buds. Eventually, all flower buds are ready for pollination. At Kao Soi Dow waterfall areas, on the upper level, two to three metres of aerial roots hang down from the nodes, and also present are the flat roots with fringed edges used to cling on to the *P. tentorium* bark, offering extreme support.

This study can be used for integrated strategy for the conservation and sustainable use of wild species of *V. siamensis* in Thailand. In part of In situ conservation of *V. siamensis,* there were clearly presented that (1) environmental factors are important for established their population and disperse, and (2) wild honey bee species play a role as natural pollinator of *V. siamensis* under natural condition. Finally, from the result of aerial photograph, it is clearly presented that *V. siamensis* vine distribution can be observed using drone in particularly along the water route. This study can be shown that using drone for inventory and conservation applications, including preparing real-time mapping of local land cover, monitoring and inventory on distribution of *V. siamensis* in natural ecosystems, can be applied by Conservationist and Ecologist. The ability of the drone to take off autonomously with a light touch by the operator. Landing of the drone in a constrained space does require some manual control to avoid trees and other obstacles, as the drone circles down to the ground. Over many missions flown during the in situ conservation study the drone was found to be one hundred percent reliable in terms of flying its mission and returning to its launch pad. However, on one occasion only two months into buying the first drone which I operated crashed into the forest canopy and was unable to retrieve it. It was left above 45 metres above ground as a reminder of my work there. The second drone operated by a drone expert, was also lost in the second year. This was due to sudden, strong storm which carried the drone with it and disappeared into the tree canopy as well. The third hexagonal drone was hired and operated by the owner. The results were amazing, so full of details and supported my data research. Many stages of the video frame were printed and presented in my thesis.

2. Pollinators of *Vanilla siamensis*

This study suggests thatbees of the genus *Thrinchostoma* bees are the pollinators of *V. siamensis*. The pollination mechanism is quite straightforward. Upon leaving the flower, the bee passes the central crest on the labellum and is forced to raise its body, activating the pollination process by coming into contact with the rostellum before the anther and later with the stigma in another flower. However, pollination of *V. siamensis* by *Thrinchostoma*

bee under natural condition is less favourable when compared to hand pollinated. The conclusion for pollination success in a deceptive *Vanilla* flower with similarityin flower, colour, shape and fragance may through natural selection increase the probability that a pollinator may temporarily shift from a non- rewarding plant to a nectar- producingplant (Johnston et al. 2003).

3. In vitro seed germination and plantlet regeneration of *Vanilla siamensis*

In conclusion, ½MS is suggested for seed culture. BA containing medium was suitable for shoot formation and ½MS supplemented with 0.5 mg/L NAA gave the best result in root induction. This is the first report of the successful in vitro germination of *Vanilla siamensis*. This protocol ensures the high survival rate and vigorous growth of in vitro plantlets when transferred to greenhouse and can be used for mass propagation of the endangered species and used as guideline for improvement of *V. siamensis* and conservation in vitro.

In the case of root induction, culture medium with NAA showed the higher results than culture medium without NAA. This present study was in concurrence with Tan et al.(2008) who reported that, the highest rooting response (88.3%) of *V. planifolia* was achieved on the medium supplemented with 1.0 mg/L NAA alone with a mean number of 3.3 roots per shoot and a mean length of 4.4 cm Moreover, this finding was similar with the study carried out by Janarthanam and Seshadri (2009), where the rooting response in *V. planifolia* was suppressed with an increase in concentrations of NAA. Complete plantlets were successfully acclimatized at rate of 91.7%.

4. In Situ and Ex Situ Conservation of *Vanilla siamensis* in Thailand

This study can be used for integrated strategy for the conservation and sustainable use of wild species of *V. siamensis* in Thailand (Fig. 6.1). In part of In situ conservation of *V. siamensis,* there were clearly presented that (1) environmental factors are important for established their population and disperse, and (2) wild honey bee species play a role as natural pollinator of *V. siamensis* under natural condition. Finally, according to the result of aerial photographs, which it clearly presented that *V. siamensis* vine can be took photo by using drone in particularly along waterfall route. While, there are not clearly see *V. siamensis* vine from high position by aerial photographs. This study can be shown that using drone for inventory and conservation applications, including preparing real-time

mapping of local land cover, monitoring and inventory on distribution of *V. siamensis* in natural ecosystems.

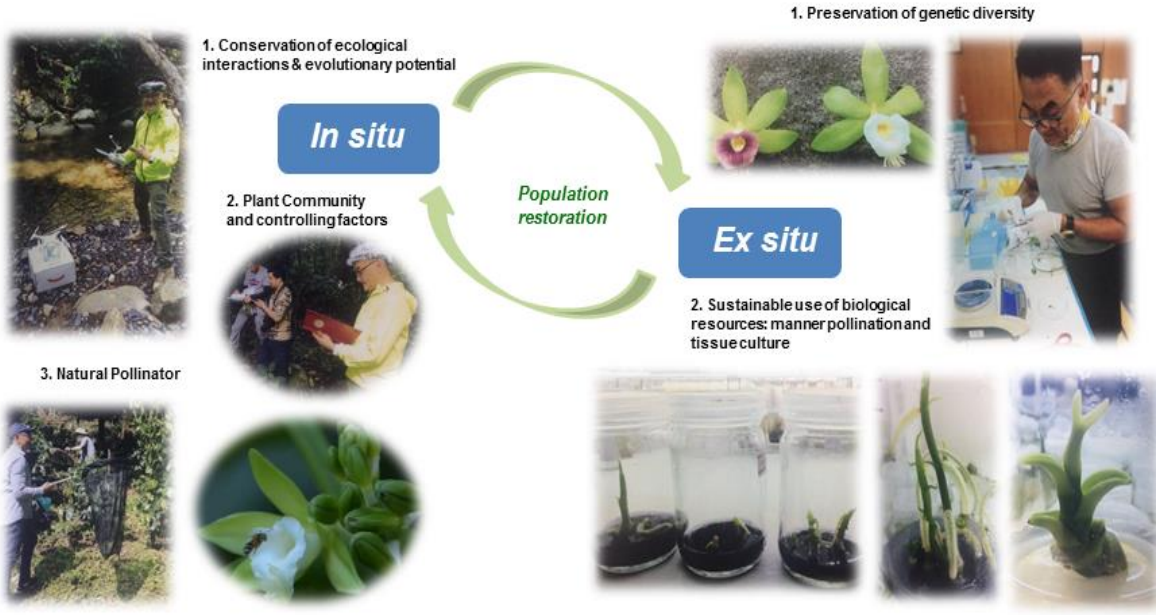

Figure 6.1 Integrated strategy for the conservation and sustainable use of wild species of *Vanilla siamensis*

5. Future research of *Vanilla siamensis*

Many plant species are threatened with extinction or genetic erosion and this is probably true for *Vanilla siamensis*, which occurs in declining numbers. Initiatives for in-situ conservation of this charismatic species must therefore be encouraged, while ex situ conservation is also to be recommended to safeguard genetic diversity. In vitro and cryopreservation techniques need to be tailored towards this species. In-situ conservation is, moreover, vital to enable further research into the ecology of *V. siamensis*, which is still imperfectly understood, for example with respect to seed dispersal and recruitment.

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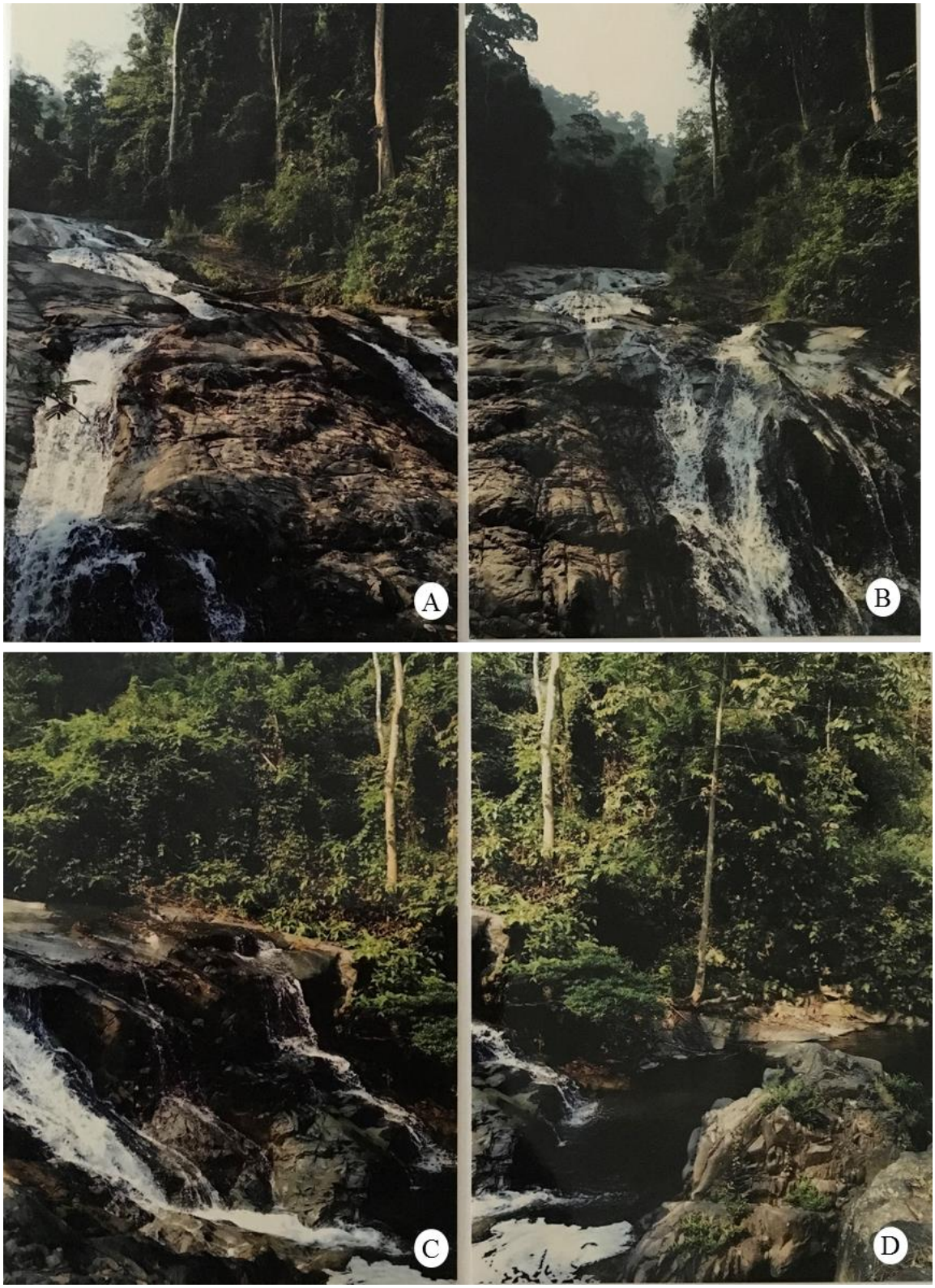
Appendices I Recher location

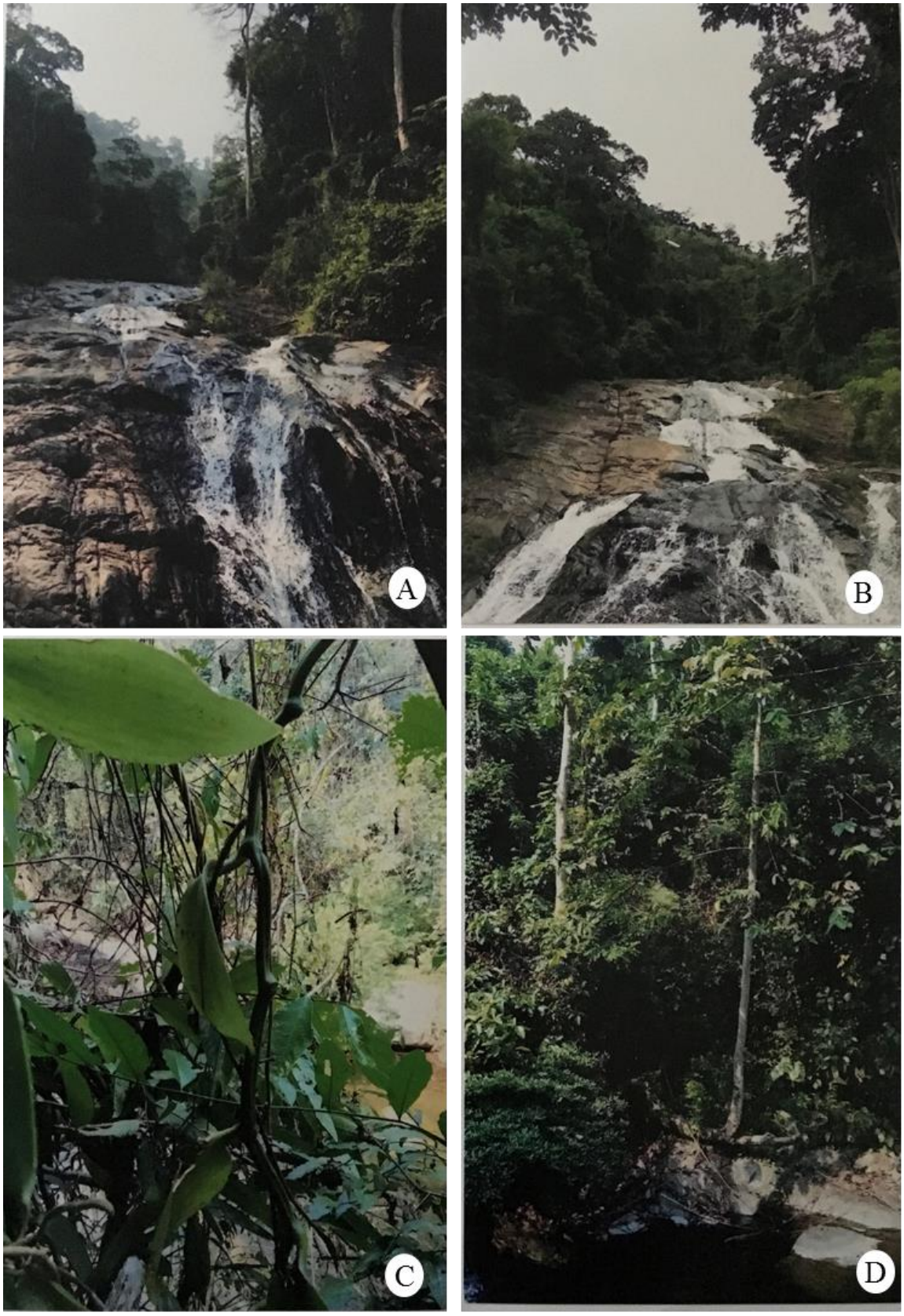
Appendices Figures 1 (A – D) This is the famous waterfalls at Kao Soi Dow where *Vanilla siamensis* can be found growing. The dominant host support trees are the *Pterocymbium* trees, a soft bark trees which they prefer to grow on the edge of the waterfalls for water. Research location: Kao Soi Dow.



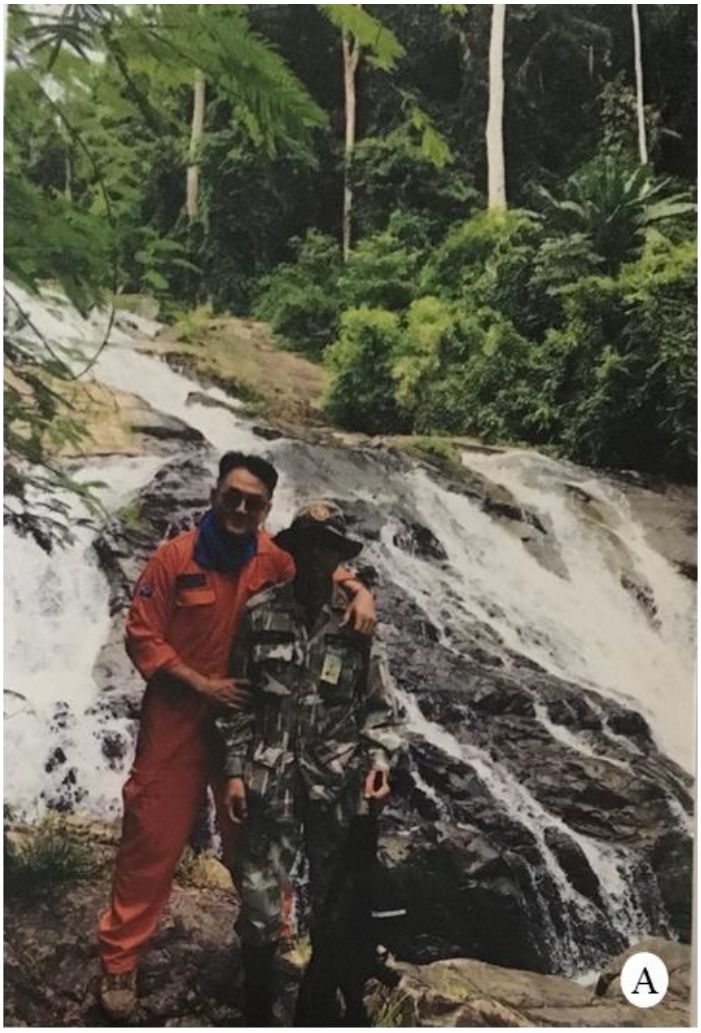
Appendices Figures 2 (A-D) The drought year of 2016-2017. The level and flow of water at the waterfalls was very low then normal. These pictures were taken in 2016 to 2017 when severe drought, forest fire, short and warm winter, destroyed much of the *Vanilla siamensis.*

Appendices Figures 3 (A-B) The same occurrence in depletion as the waterfalls moved downwards to 300 metre above sea level.

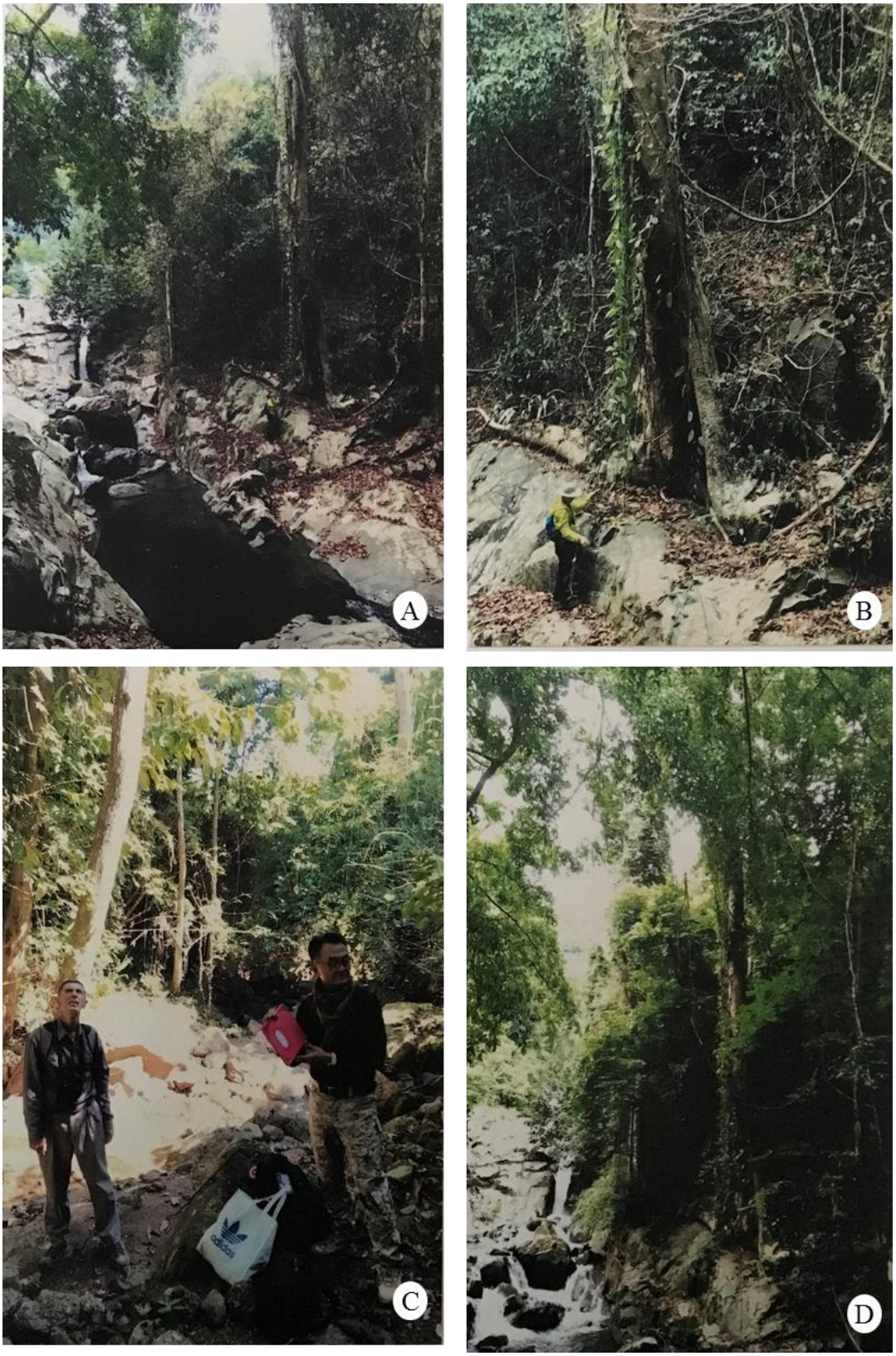
Appendices Figures 4 (A-B) Very dried parched land in 2016-2017.



Appendices Figures 5 (A-D) Extreme low flow of water from the waterfalls due to extreme drought of 2016-2017. Showing the dried and shrivelled roots of *Vanilla siamensis* clinging onto the host tree. The leaves became pale green in colour and drupped to forty five degree downwards. This is to reduce the transpiration rate.

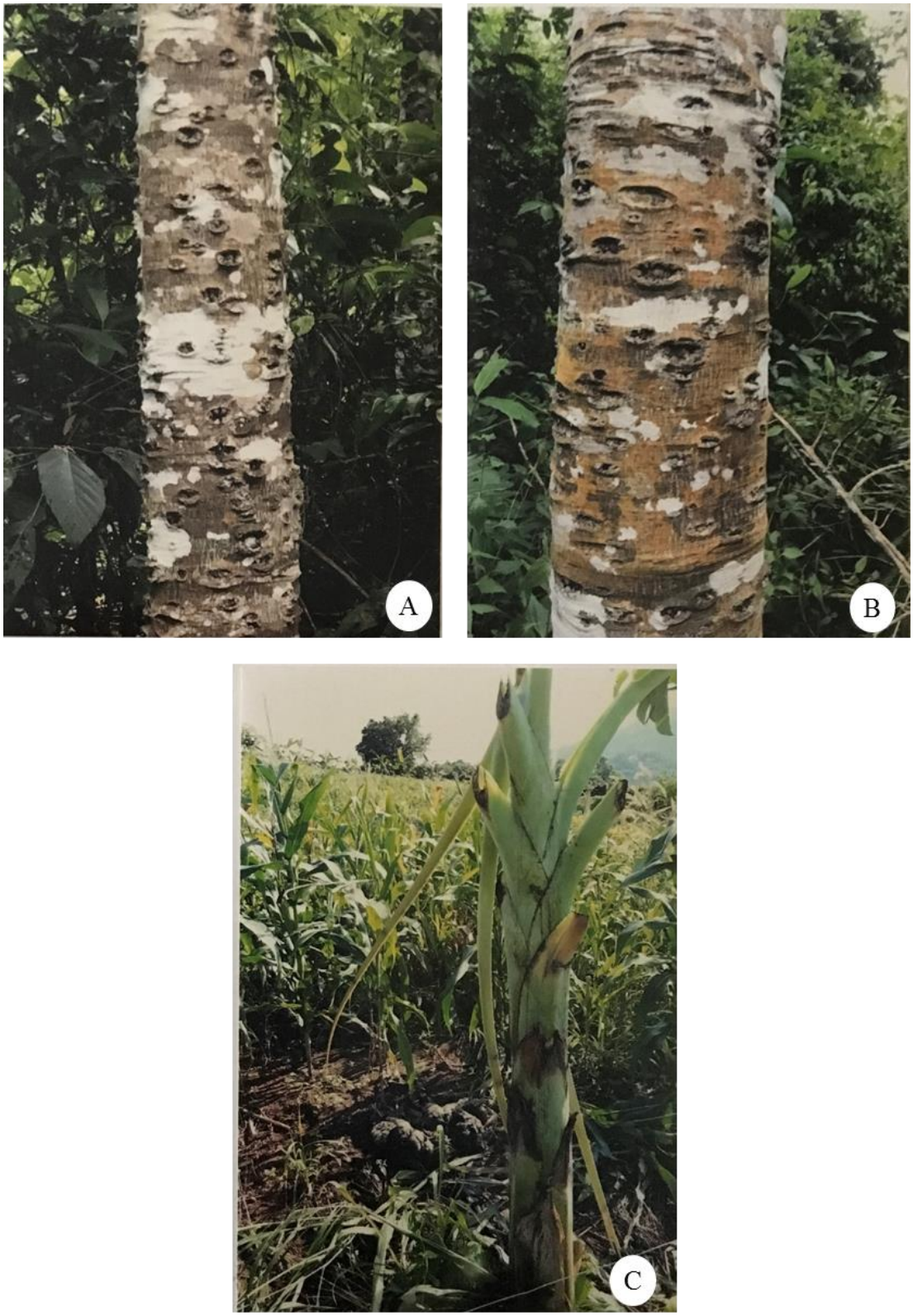


Appendices Figures 6 (A-B) The forest ranger at Kao Soi Dow on patrolling to warn people about the wild elephants movement.



Appendices Figures 7 (A-D) Many trees died resulting from the severe drought towards the lower region of the waterfalls. As the trees and bushes died and eventually dried up, this lead to them being easily prone to fire starter. Many floura and fauna were destroyed as well as wild animals.

Appendices Figures 8 (A-B) Showing sign of dying *Pterocymbium* spp. Two weeks later, the forest fire destroyed many of the trees with what remaining of the Vanilla vines attached to them.



Appendices Figures 9 (A-C) The tree barks of *Pterocymbium* or "Por E Gay" showing a remarkable characteristic of "Pineapple eye" on the outer bark at KSD. They are commonly found growing on the edges of the waterfalls up to 1000 metre above sea level as shown from the aerial drone .Destruction of the banana plantation and the elephant" s dropping are enough proof.

Appendices Figures 10 (A-C) Preparing to set up the Hobo Data logger onto the tree trunk in order to measure the moisture, relative humidity, and temperature for fourteen days.

Appendices Figures 11 (A-C) In 2016-2017, showing severe draught and gradual dying of many trees.

Appendices Figures 12 (A-C) Further down, at the elevation of 300 metres the flow of the waterfall path.Very slow, little remaining water and showing the decolouration of the forest canopies. Extreme low water flow level further down at 200 metres above sea level.

Appendices Figures 13 (A-C) This was one of my first year research regular visits .The waterfalls showed a mighty water flow downward .

Appendices Figures 14 (A-D) The remaining scenes of destruction on banana plantation left by the wild elephants are a common sighting near to the waterfalls.

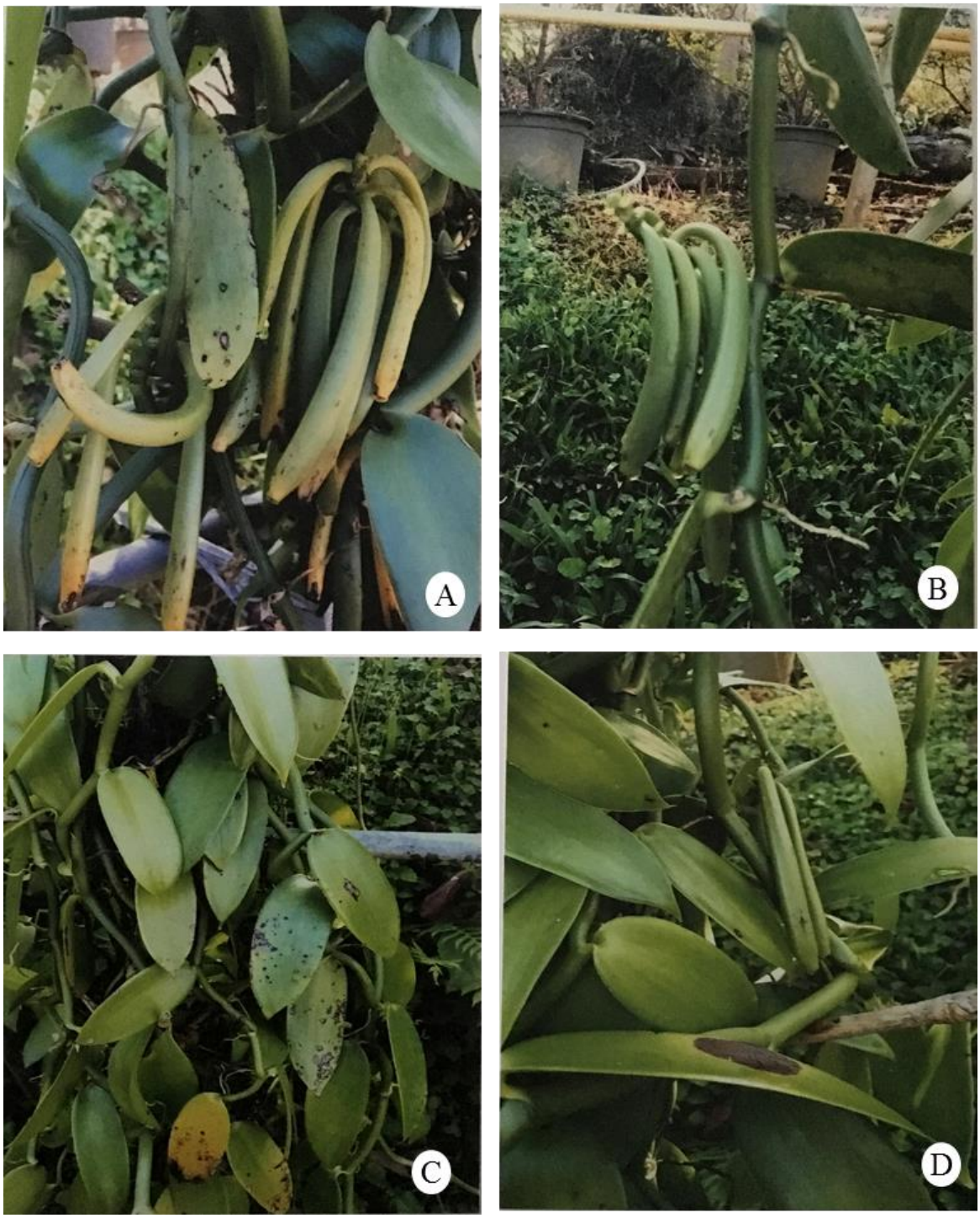
Appendices Figures 15 (A-D) This is the actual proof of the wild elephants coming down to feast on anything that is edible. An unexpected visitor in front of my house and orchard. Water pipes for irrigation were broken and the elephants have left their foot prints every where.

Appendices Figures 16 (A-C) These recently pictures were taken in June of 2018 at the Kao Soi Dow mountain range. In the evening around 6 to 8 pm, around five to ten wild elephants come down to feast on their favourite meal. After that they will make their way back to the Waterfall area. Last year, many local casualties and few death happened in this area due to the startled elephants . Sometimes, some of the farmers use fire work and shot gun to scare them. Now nearly all houses and orchards are fenced with single soft electric wire. Still this does not prevent them from going into the orchard with so many different fruits and plenty of them everywhere to their watchful temptation. Now these intelligent elephants have come up with a new short cut access version to the orchards and that is by pulling up a tree trunk and place them onto the single wire. There is nothing the farmers can do about the problem, as these wild elephants are protected and have been here well before human habitation arrived. This area at one time have been full of wild animals like deer, gibbons, snakes, elephants, small cats as well as wild fauna and flora.

Appendices II Morphology of *Vanilla siamensis*

Appendices Figures 17 (A - D) the morphology of Vanilla siamensis in bloom, Showing flowering and two pollinated pods.
Research location: Chiangmai.
Date: June-July 2018

Appendices Figures 18 (A - D) Showing the gradual disappearing of the wild *Vanilla siamensis* in this private coffee plantation. This is the only two trees left standing with host trees supporting several trailing vines.
Research location: Chiangmai



Appendices Figures 19 (A – D) Showing the healthy in situ environment with lots of mulsh.
Research location: Kao Soi Dow.

Appendices Figures 20 (A – D) Showing the healthy in situ environment with lots of mulsh. Research location: Kao Soi Dow.

Appendices Figures 21 (A – D) *Vanilla siamensis* growing on rock as support. Research location: Kao Soi Dow.

Appendices Figures 22 (A - C) The flowers are in full bloom starting from lower raceme. The fruits are larger and straighter than the upper raceme.
Research location: Chiangmai.

Appendices Figures 23 (A - D) These hand pollinated *Vanilla siamensis* beans are coming to a maturing stage after eighteen months from the point of pollination, fertilization and maturation.
Research location: Chiangmai.

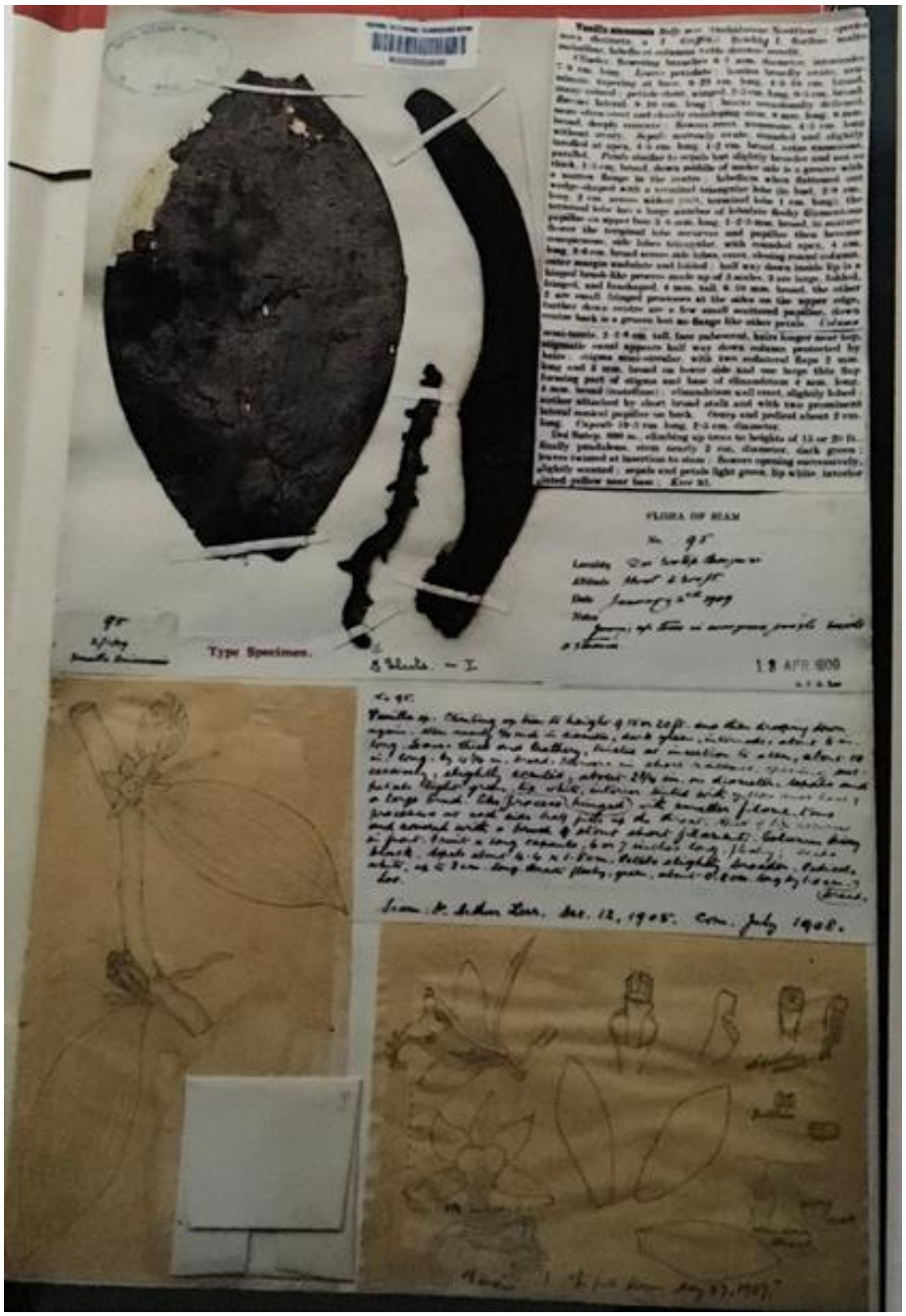
Appendices Figures 24 (A – C) The pods are getting to the ripening stage, by the darkening at the tips of the pods and gradual moving upwards. The longest pod ever measured was 24 cm long and 5.5 cm in width. (D) Pollens are held together as pollinium .Data on the early growth are measured every fifteen days.

Appendices Figures 25 (A - B) Differences in Vanilla leaves are reflected in their size, shape, colour and texture. This is an oval in outline, a shade of dark emerald green, thick, leathery and extreme waxy. The thick succulent leaves are filled with a sticky, liquid mucilage of calcium oxalate and calcium carbonate.
Research location: Chiangmai.

Appendices Figures 26 (A - D) The harvest of 2015-2017. Study of phytoestrogen in the Vanilla fruits with Dr. Phenphichar Wanachatararak, Faculty of Dentistry, Chiangmai University for helping me. The sign was put up which read “Please do not pick these pods as they are under research”
Research location: Chiangmai.

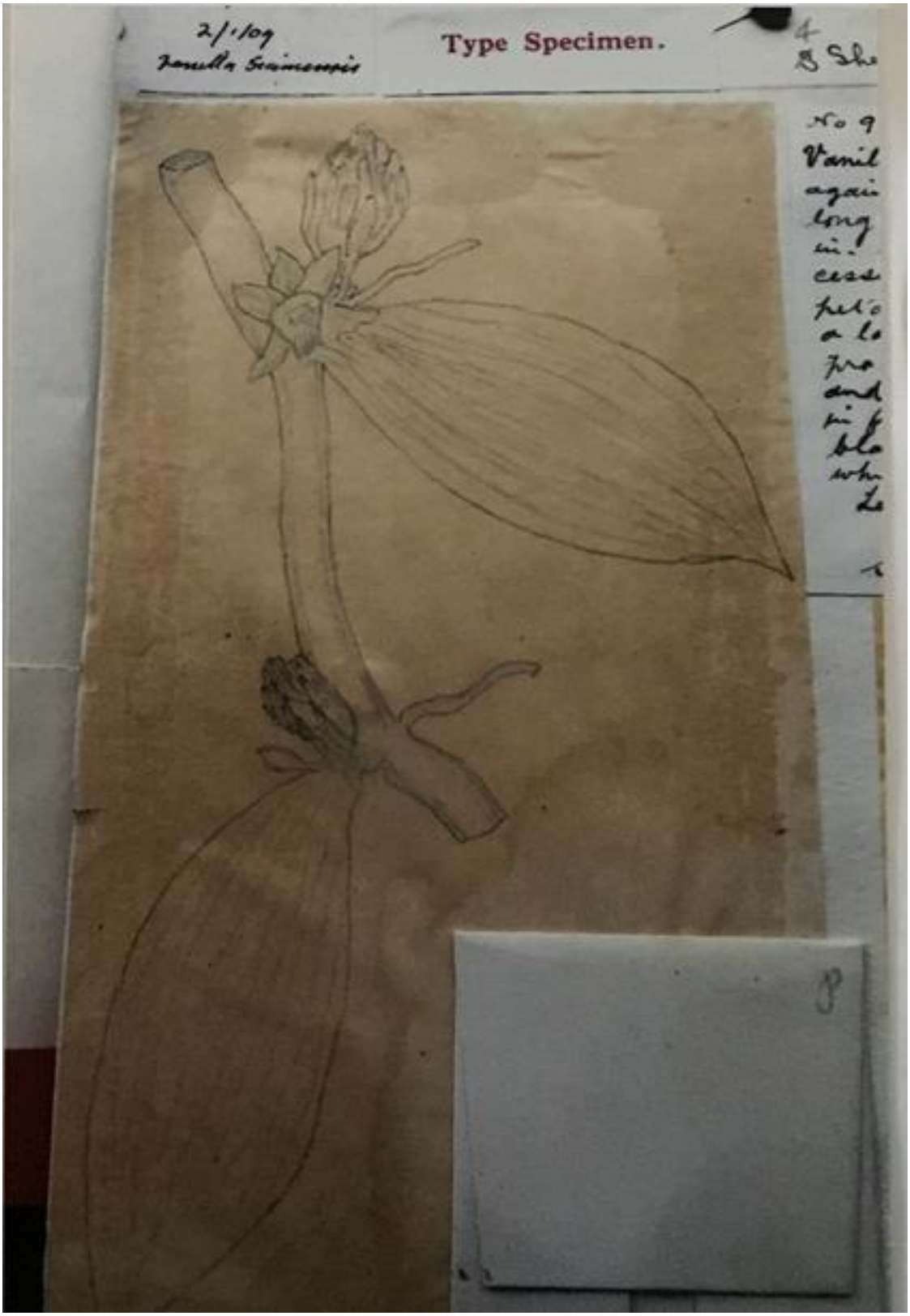
Appendices III Herbarium

The first recorded Vanilla siamensis specimen was discovered and collected in 1905, 1907 and 1909 by the scientist Dr. Arthur Francis George Kerr (1877 to 1942) and are being kept at the Royal Botanic Garden, Kew. He was a pioneerinr botanist of the twentieth century who collected extensively in, then, Siam now the modern Thailand. He was the first scienctist to be sent to Siam by the British government and cataloged many plants and were then sent to the Herbarium Collection at Kew. Photographs taken and are presented as part of my thesis from the Herarium Collection at the Royal Botanic Gardens, Kew with permission. (Appendics Figures 27, 28, 29,30,31,32)

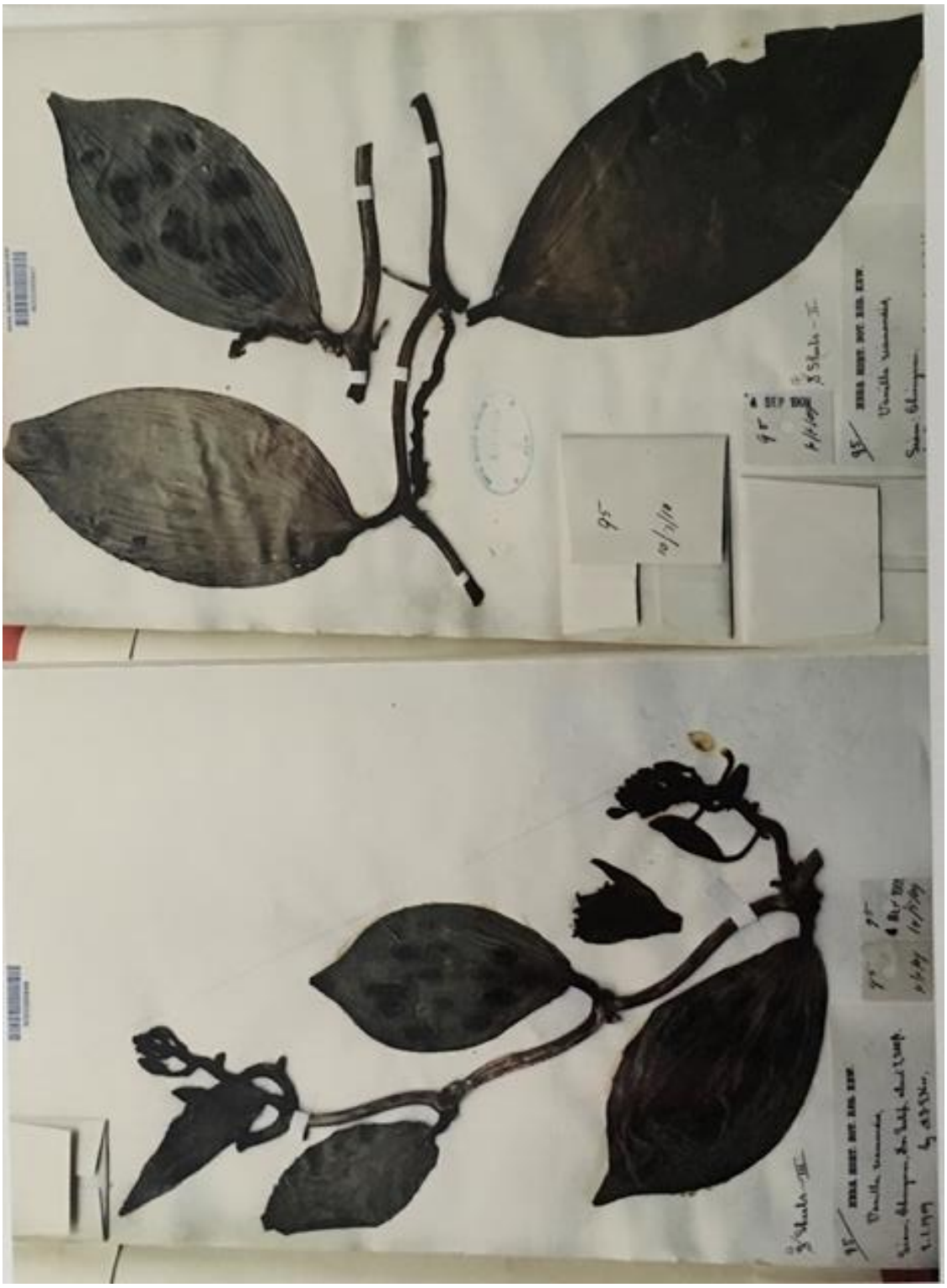


Appendices Figures 27 These pictures are taken from the Herbarium collection at the Royal Botanic Gardens, Kew in London.

Appendices Figures 28 These pictures are taken from the Herbarium collection at the Royal Botanic Gardens, Kew in London.

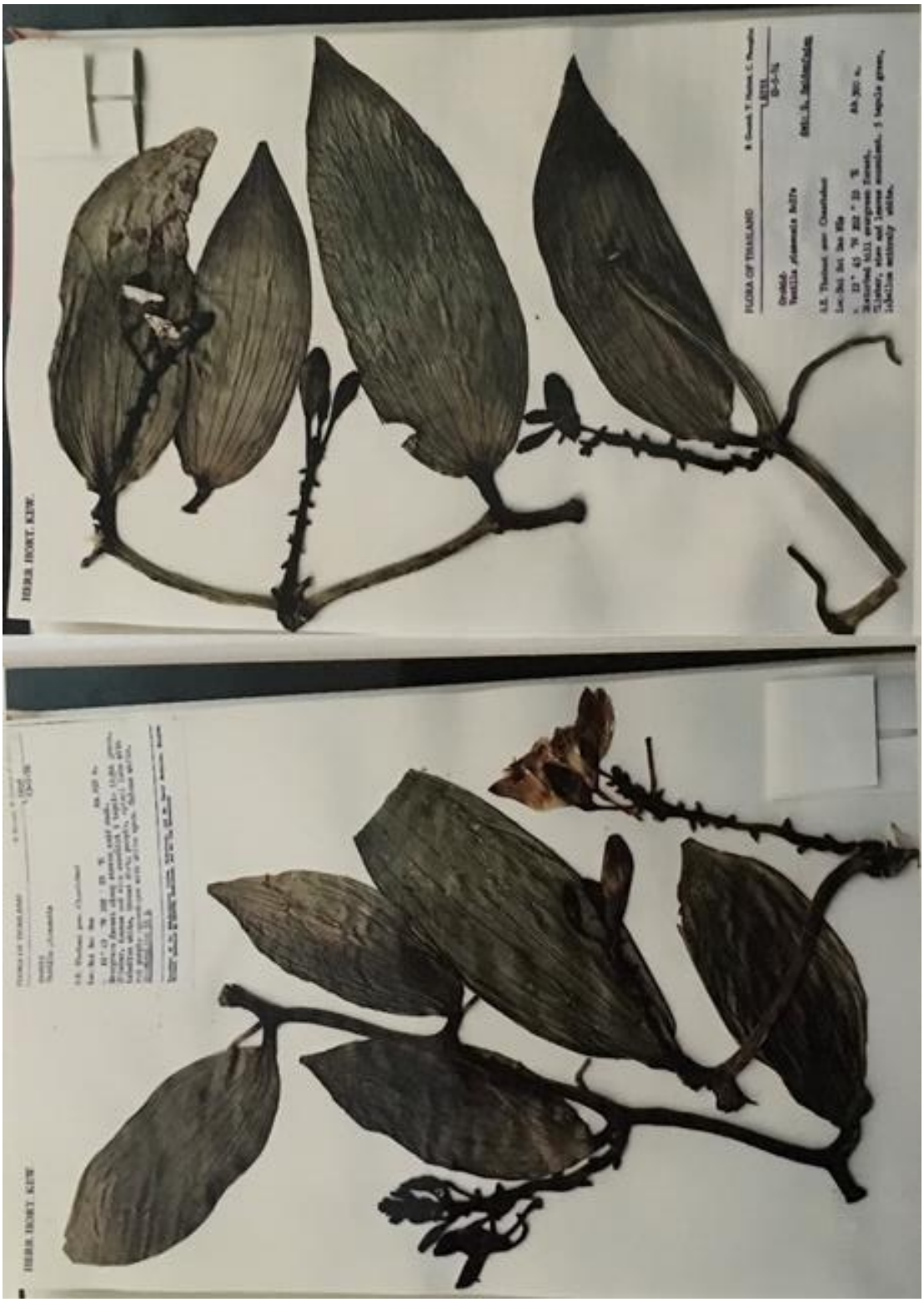


Appendices Figures 29 These pictures are taken from the Herbarium collection at the Royal Botanic Gardens, Kew in London.



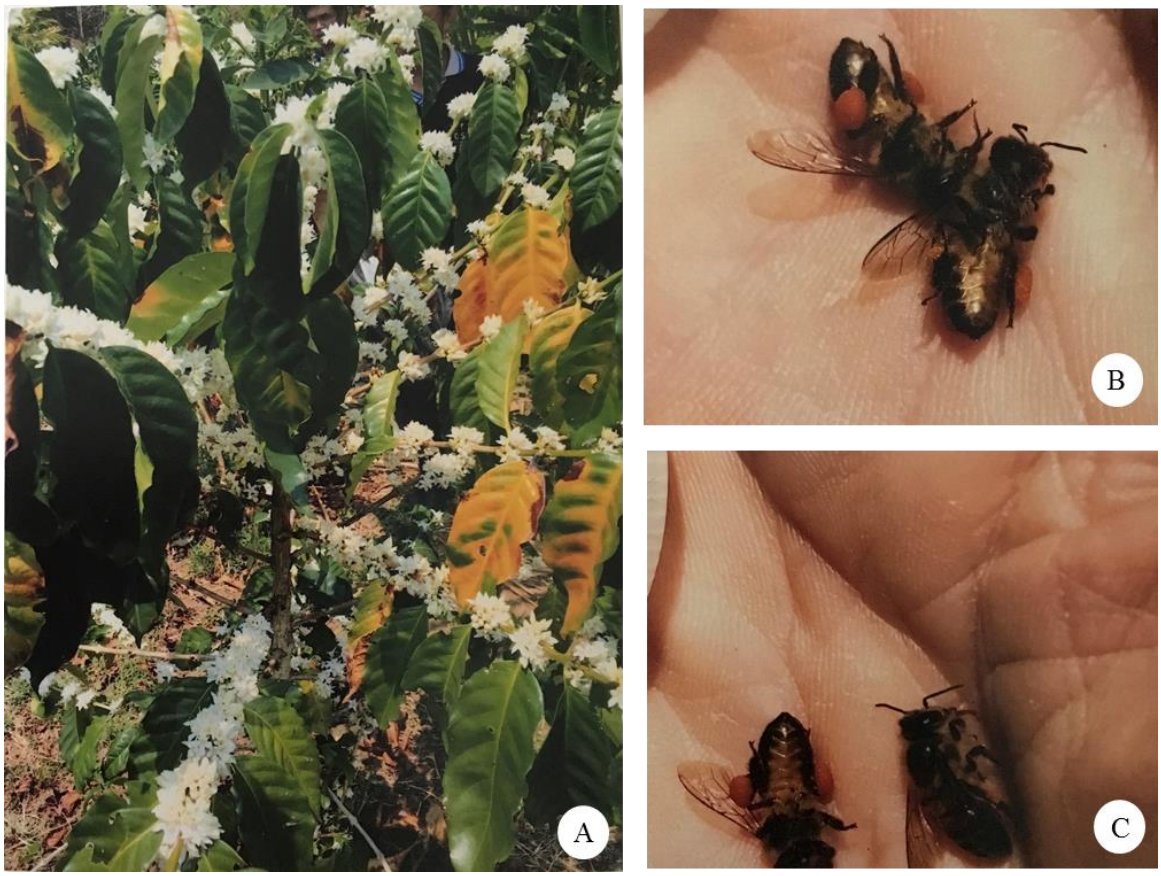
Appendices Figures 30 Dr.Arthur Francis Kerr who collected the first *Vanilla siamensis* specimen in then Siam in 1905.

Appendices Figures 31 Dr.Arthur Francis Kerr who collected the first *Vanilla siamensis* specimen in then Siam in 1905.



Appendices Figures 32 Dr.Arthur Francis Kerr who collected the first *Vanilla siamensis* specimen in then Siam in 1905.

Appendices IV Pollination procedure

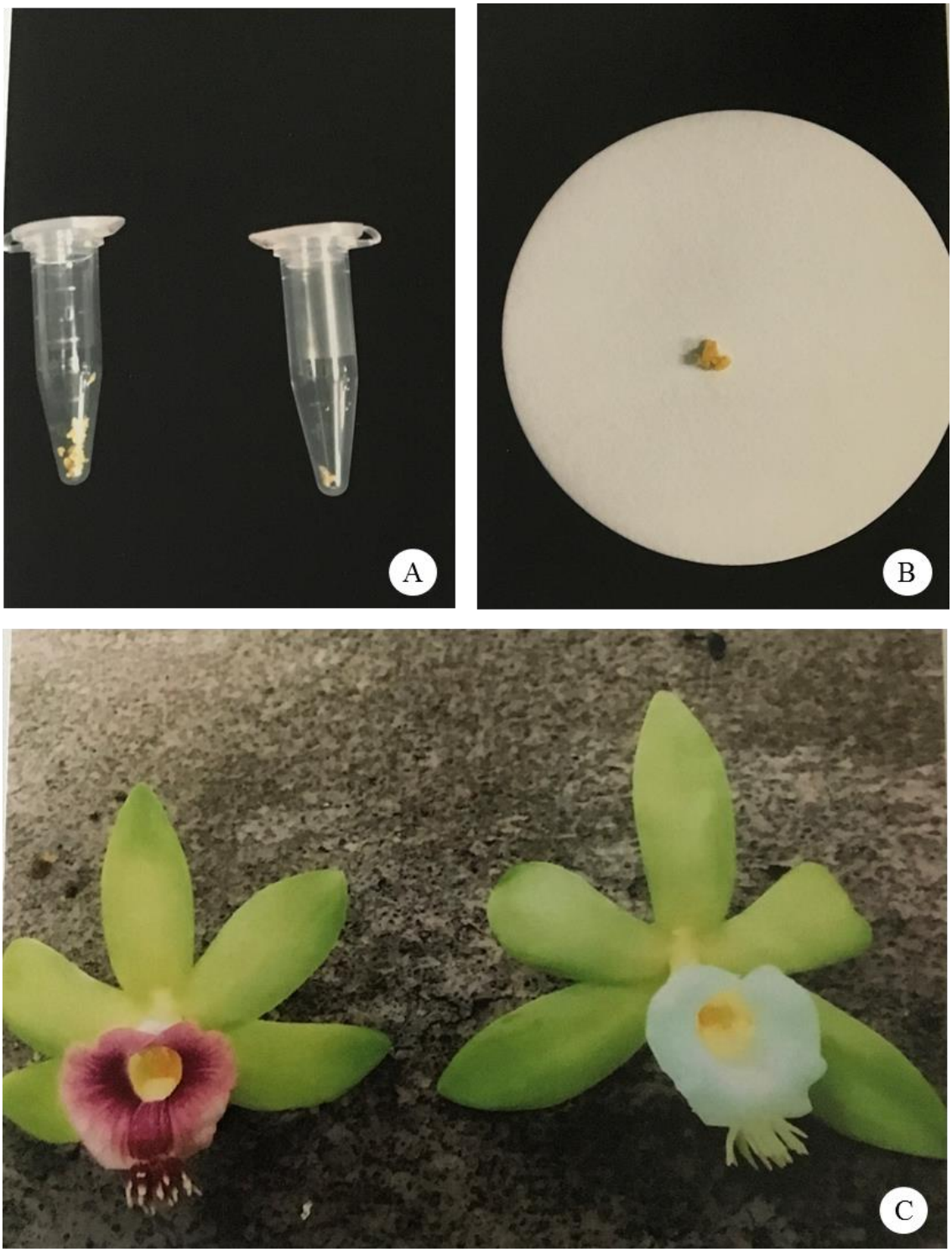


Appendices Figures 33 (A) The coffee plants are all in full flowerng stage and giving off beautiful aromatic fragrance to attract the pollinator bees. (B - C) These are the pollinator bees for coffee plants.
Research location: Chiangmai.

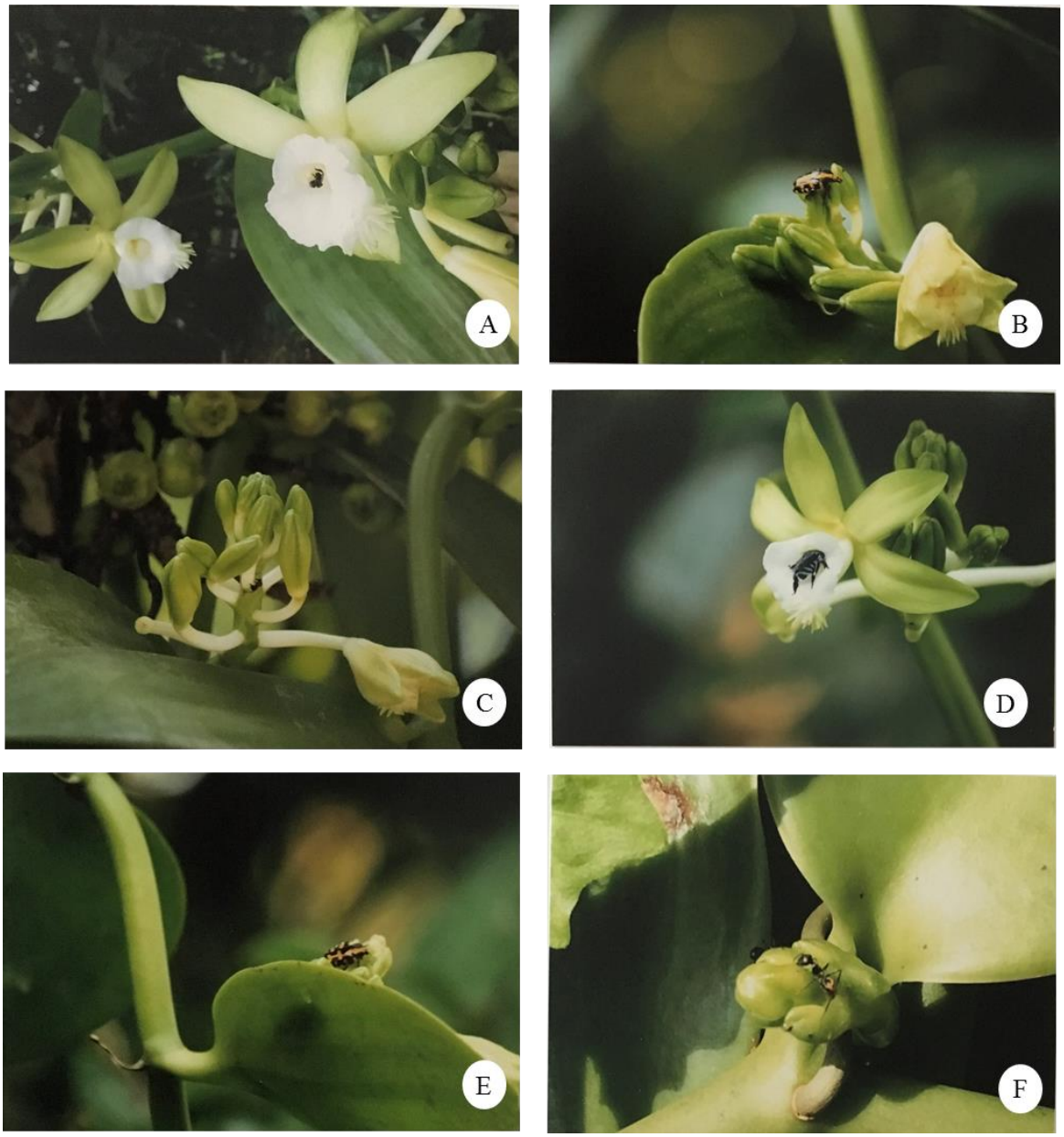
Appendices Figures 34 (A – C) Unlike most orchids, following pollination of the flowers and fertilization of the ovules, the sepals and petals of the *Vanilla siamensis* flower whither and eventually fall away from the ovary as it develops into a seed bearing fruit. Research location: Chiangmai.

Appendices Figures 35 (A – C) This is the only ever successful recorded insect pollination process obtained. The sign says “please do not touch and remove”. Research location: Kao Soi Dow.

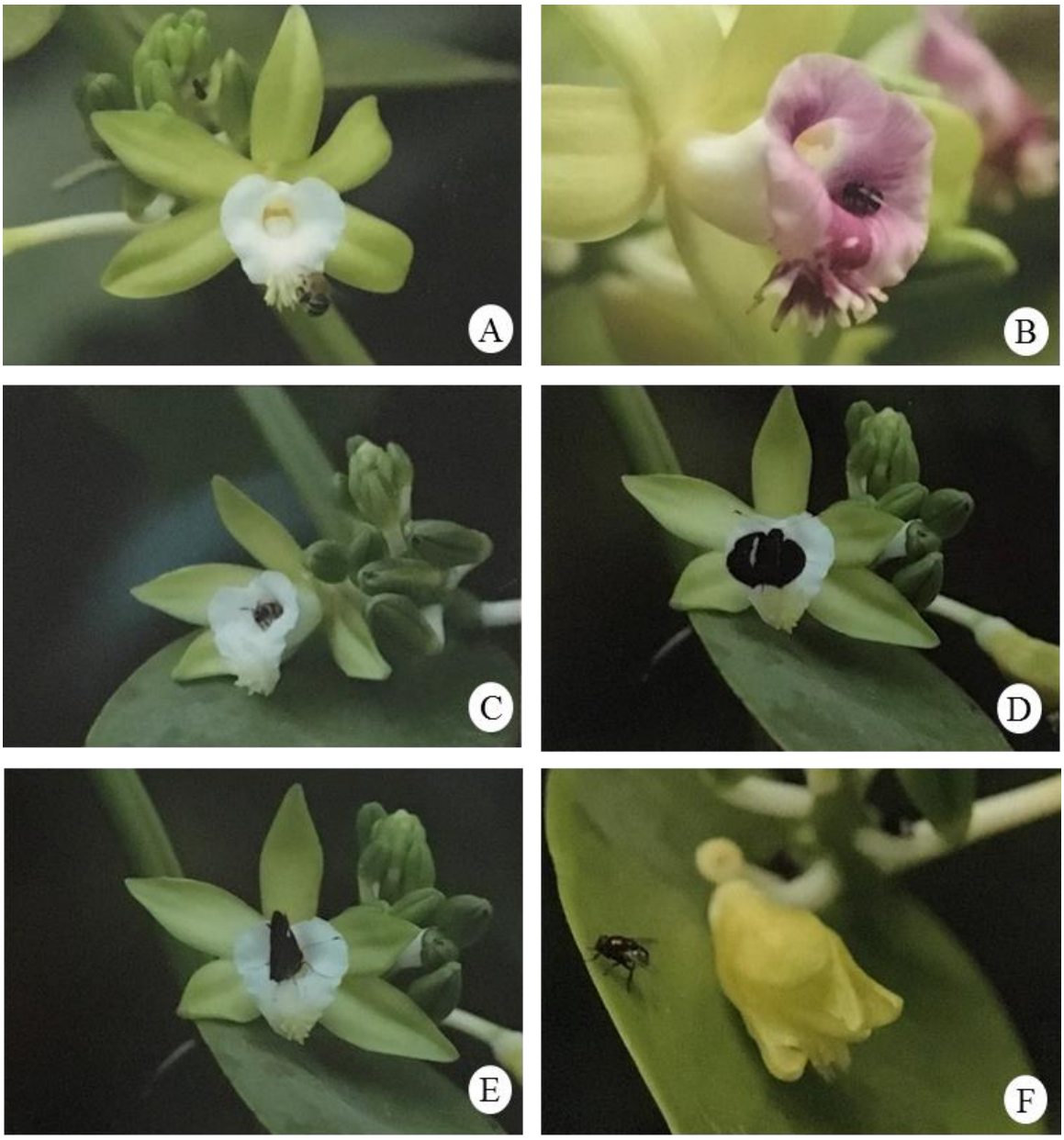
Appendices Figures 36 (A – C) This is the first ever interspecific hybridisation between *Vanilla planifolia* (parental parent) and *Vanilla siamensis* (maternal parent) accomplished. It was carried out in Chiangmai and took nine months to develop. A strong indication that the pods have taken on the *Vanilla planifoia* trait. The pods are much smaller and took less time to mature comparing to eighteen months for *Vanilla siamensis*. Data of growth and width were taken every fifteen days.
Research location: Chiangmai.



Appendices Figures 37 (A - B) Pollens were collected from the *Vanilla siamensis* and kept in the plastic vile to use for next year pollination on another vanilla species. (C) Two different colors of *Vanilla siamensis* found namely white and red.
Research location: Chiangmai.

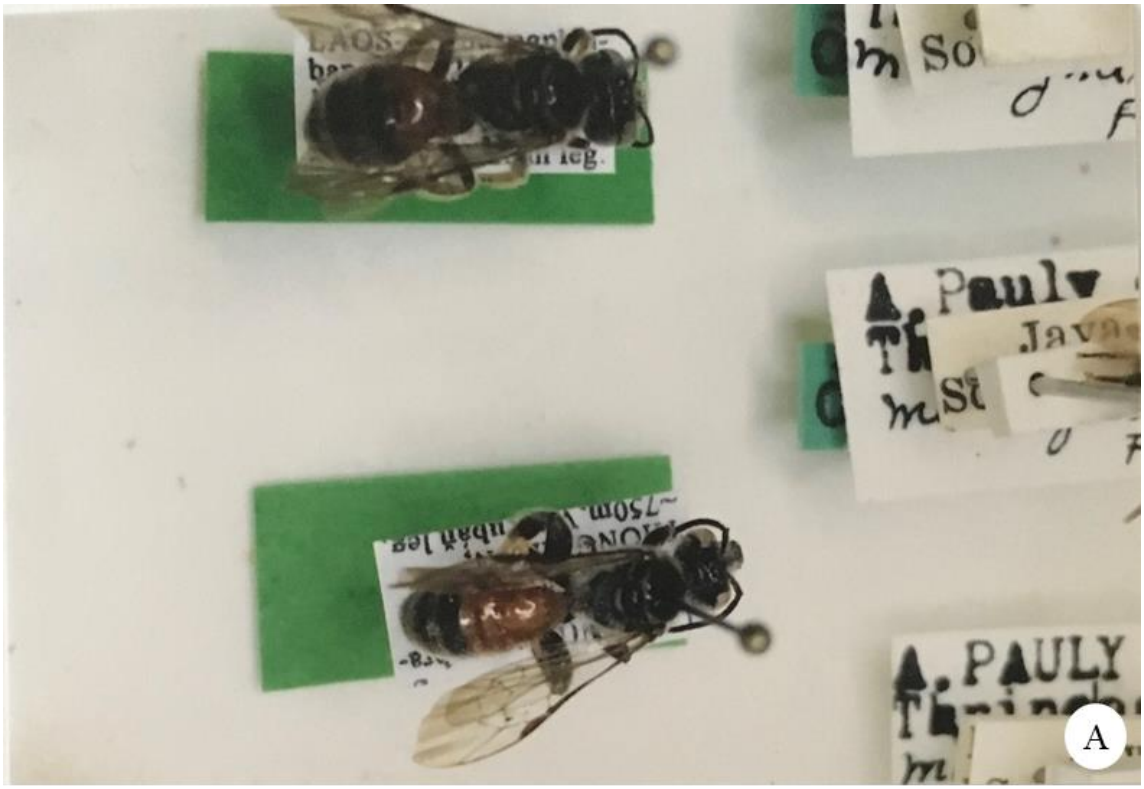


Appendices Figures 38 (A – F) Visitation of other non pollinator insects.



Appendices Figures 39 (A – F) Collection of various non pollinator insects taken from three Brino TLS200 (time lapsed camera) during the flowering season at Kao Soi Dow.

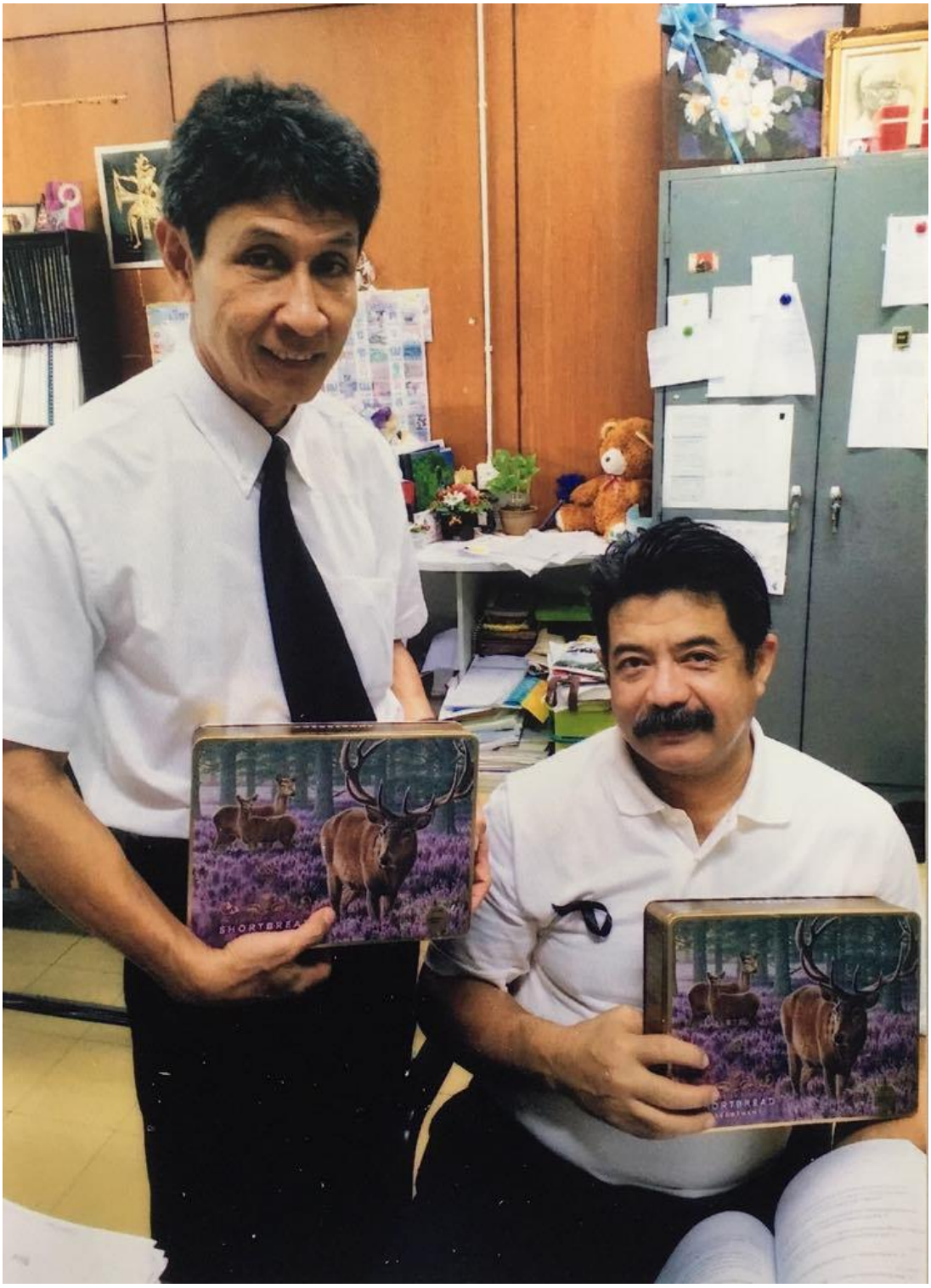
Appendices Figures 40 (A - B) Waiting patiently for the pollinator to arrive early in the morning, which it did eventually captured in the four minutes video at Kao So Dow, Date: 2016



Appendices Figures 41 (A - B) A high magnification picture of this identified pollinator bee as Thinchostoma bee, of little known Asian species, but commonly found in Madagascar.
Research location: Kao Soi Dow.

Appendices V Personal gratitude acknowlengment

Appendices Figures 42 (A - B) Two pollinator experts namely Dr. Hans Banziger at the Entemology Department in Chiangmai University. Also Dr.Alain Pauly at the Royal National History Museum in Brussels. A thank you dinner was set in Brussels.



Appendices Figures 43 Professor Sompong Techato and Dr. Ayut Nissapa University of Songkhra, Deparment of Agricultural Development.

Appendices Figures 44 Associat Professor Michel Grisoni at CIRAD, Reunion Islands. Unite Mixte de Recherche. Expert on pathogents and diseases of Vanilla.

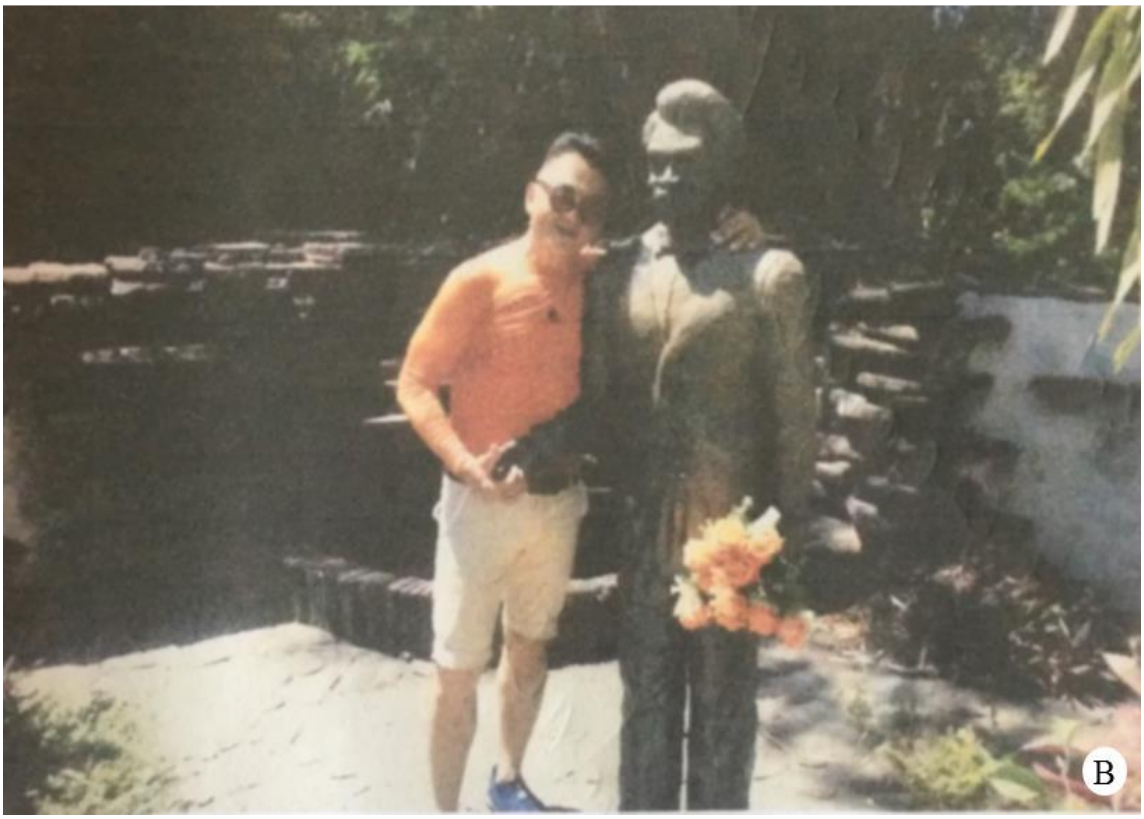
Appendices Figures 45 Dr.Sureerat Yenchom.Micropropogation and Cryopreservation, University of Songkhla, Department of Plant science.

Appendices Figures 46 Assiciate Professor Nicola Flanagan at World Orchid Canference Guayaquil, Equador 2017. University of Pantificia Universidad Javeriana Cali, Colombia.

Appendices Figures 47 Professor Kennth M. Cameran, world famous Vanlla specialist Department of Botany, University of Wiseanser in Equador.

Appendices Figures 48 Conference held recently in Bangkok, Thailand.

Appendices Figures 49 Assoiate Professor Kanchit Thammasiri at University of Mahidol, Deparment of Plant Science, Bangkok, Thailand.



Appendices Figures 50 (A-B) quiet moment of thoughts and contemplation to Edmond Albius, a 12 years old black slave who was the first person to practice hand pollination of the Vanilla planifolia on the island of Reunion.

Appendices Table 1 Plant species list.

	Botanical Name	Species code	Family_Name	Habit
1	*Hydnocarpus ilicifolia* King	*Hydnocarpus ilicifolia*	Achariaceae	ST
2	*Mangifera cochinchinensis* Engl.	*Mangifera cochinchinensis*	Anacardiaceae	T
3	*Alphonsea boniana* Finet & Gagnep.	*Alphonsea boniana*	Annonaceae	T
4	*Miliusa mollis* Pierre var. *mollis*	*Miliusa mollis*	Annonaceae	ST
5	*Orophea polycarpa* A. DC.	*Orophea polycarpa*	Annonaceae	S/ST
6	*Uvaria wrayi* (King)	*Uvaria wrayi*	Annonaceae	C
7	*Willughbeia edulis* Roxb.	*Willughbeia edulis*	Apocynaceae	C
8	*Wrightia coccinea* (Roxb.) Sims	*Wrightia coccinea*	Apocynaceae	T
9	*Markhamia stipulata* (Wall.)	*Markhamia stipulata* var. *pierrei*	Bignoniaceae	T
10	*Mayodendron igneum* (Kurz) Kurz	*Mayodendron igneum*	Bignoniaceae	T
11	*Capparis* sp.	*Capparis* sp.	Capparaceae	C
12	*Gonocaryum lobbianum* (Miers) Kurz	*Gonocaryum lobbianum*	Cardiopteridaceae	ST
13	*Glyptopetalum sclerocarpum* M. A. Lawson	*Glyptopetalum sclerocarpum*	Celastraceae	S/ST
14	*Garcinia vilersiana* Pierre	*Garcinia vilersiana*	Clusiaceae	ST
15	*Combretum album* Pers.	*Combretum album*	Combretaceae	C
16	*Terminalia nigrovenulosa* Pierre	*Terminalia nigrovenulosa*	Combretaceae	T
17	*Neuropeltis racemosa* Wall.	*Neuropeltis racemosa*	Convolvulaceae	C
18	*Dipterocarpus alatus* Roxb. ex G. Don	*Dipterocarpus alatus*	Dipterocarpaceae	T

Appendices Table 1 Continued.

	Botanical Name	Species code	Family_Name	Habit
19	*Dipterocarpus turbinatus* C. F. Gaertn.	*Dipterocarpus turbinatus*	Dipterocarpaceae	T
20	*Shorea guiso* (Blanco) Blume	*Shorea guiso*	Dipterocarpaceae	T
21	*Diospyros defectrix H. R. Fletcher*	*Diospyros defectrix*	Ebenaceae	T
22	*Diospyros transitoria Bakh.*	*Diospyros transitoria*	Ebenaceae	T
23	*Diospyros variegata* Kurz	*Diospyros variegata*	Ebenaceae	T
24	*Alchornea rugosa* (Lour.) Müll. Arg.	*Alchornea rugosa*	Euphorbiaceae	S/ST
25	*Cleidion javanicum* Blume	*Cleidion javanicum*	Euphorbiaceae	S/T
26	*Croton persimilis* Müll. Arg.	*Croton persimilis*	Euphorbiaceae	S/ST
27	*Falconeria insignis* Royle	*Falconeria insignis*	Euphorbiaceae	T
28	*Macaranga indica* Wight	*Macaranga indica*	Euphorbiaceae	ST/T
29	*Mallotus peltatus* (Geisel.) Müll. Arg.	*Mallotus peltatus*	Euphorbiaceae	S/ST
30	*Dalbergia oliveri* Gamble ex Prain	*Dalbergia oliveri*	Fabaceae	T
31	*Dendrolobium lanceolatum* (Dunn) Schindl.	*Dendrolobium lanceolatum*	Fabaceae	S
32	*Erythrina subumbrans* (Hassk.) Merr.	*Erythrina subumbrans*	Fabaceae	T
33	*Millettia leucantha* Kurz var. leucantha	*Millettia leucantha*	Fabaceae	T
34	*Saraca declinata* (Jack) Miq.	*Saraca declinata*	Fabaceae	ST
35	*Cryptocarya albiramea* Kosterm.	*Cryptocarya albiramea*	Lauraceae	T
36	*Strychnos minor Dennst.*	*Strychnos minor*	Loganiaceae	C

Appendices Table 1 Continued.

	Botanical Name	Species code	Family_Name	Habit
37	*Microcos tomentosa Sm.*	*Microcos tomentosa*	Malvaceae	T
38	*Pterocymbium tinctorium (Blanco) Merr.*	*Pterocymbium tinctorium*	Malvaceae	T
39	*Pterygota alata (Roxb.) R. Br.*	*Pterygota alata*	Malvaceae	T
40	*Scaphium affine (Mast.) Pierre*	*Scaphium affine*	Malvaceae	T
41	*Sterculia hypochra* Pierre	Sterculia hypochra	Malvaceae	T
42	*Memecylon caeruleum* Jack var. *caeruleum*	*Memecylon caeruleum*	Melastomataceae	S
43	*Memecylon intermedium* Blume	*Memecylon intermedium*	Melastomataceae	T
44	*Aglaia edulis* (Roxb.) Wall.	*Aglaia edulis*	Meliaceae	T
45	*Aglaia spectabilis* (Miq.) S. S. Jain & Bennet	*Aglaia spectabilis*	Meliaceae	T
46	*Dysoxylum cyrtobotryum* Miq.	*Dysoxylum cyrtobotryum*	Meliaceae	T
47	*Walsura pinnata* Hassk.	*Walsura pinnata*	Meliaceae	T
48	*Artocarpus chama* Buch.-Ham.	*Artocarpus chama*	Moraceae	T
49	*Ficus heterostyla* Merr.	*Ficus heterostyla*	Moraceae	S/T
50	*Knema latericia* Elmer	*Knema latericia* subsp. *Ridleyi*	Myristicaceae	S/T
51	*Syzygium siamense* (Craib) Chantar. & J. Parn.	*Syzygium siamense*	Myrtaceae	T
52	*Syzygium syzygioides* (Miq.) Merr. & L. M. Perry	*Syzygium syzygioides*	Myrtaceae	T
53	*Strombosia ceylanica* Gardner	*Strombosia ceylanica*	Olacaceae	T
54	*Galearia maingayi Hook. f.*	*Galearia maingayi*	Pandaceae	T

Appendices Table 1 Continued.

	Botanical Name	Species code	Family_Name	Habit
55	*Bischofia javanica Blume*	*Bischofia javanica*	Phyllanthaceae	T
56	*Carallia brachiata (Lour.) Merr.*	*Carallia brachiata*	Rhizophoraceae	T
57	*Canthium coffeoides Pierre ex Pit.*	*Canthium coffeoides*	Rubiaceae	S/ST
58	*Hymenodictyon orixense (Roxb.) Mabb.*	*Hymenodictyon orixense*	Rubiaceae	T
59	*Atalantia monophylla (L.) DC.*	*Atalantia monophylla*	Rutaceae	ST
60	*Clausena harmandiana (Pierre) Pierre ex Guillaumin*	*Clausena harmandiana*	Rutaceae	S
61	*Murraya paniculata* (L.) Jack	*Murraya paniculata*	Rutaceae	S/ST
62	*Casearia calva* Craib	*Casearia calva*	Salicaceae	T
63	*Xerospermum noronhianum* (Blume) Blume	*Xerospermum noronhianum*	Sapindaceae	T
64	*Picrasma javanica* Blume	*Picrasma javanica*	Simaroubaceae	T
65	*Tetrameles nudiflora* R. Br.	*Tetrameles nudiflora*	Tetramelaceae	T
66	*Tetrastigma leucostaphylum* (Dennst.) Alston	*Tetrastigma leucostaphylum*	Vitaceae	C

Appendices Table 2 Importance Value (IV) of plant species at upper site

no.	Botanical names	no.tree (tree)	no. plot (plot)	sum Ba (m^2)	density (tree/Rai)	density (tree/ha)	frequency (%)	Dominance (m^2/ha)	RD (%)	RF (%)	RBa (%)	IV
1	*Hydnocarpus ilicifolia*	32	8	0.7490	51.20	320.00	80.00	7.49	20.92	10.81	30.34	62.07
2	*Pterocymbium tinctorium*	19	4	0.2358	30.40	190.00	40.00	2.36	12.42	5.41	9.55	27.38
3	*Dalbergia oliveri*	1	1	0.5771	1.60	10.00	10.00	5.77	0.65	1.35	23.38	25.38
4	*Diospyros variegata*	12	4	0.2311	19.20	120.00	40.00	2.31	7.84	5.41	9.36	22.61
5	*Murraya paniculata*	14	6	0.0901	22.40	140.00	60.00	0.90	9.15	8.11	3.65	20.91
6	*Wrightia coccinea*	11	6	0.0619	17.60	110.00	60.00	0.62	7.19	8.11	2.51	17.81
7	*Orophea polycarpa*	16	4	0.0402	25.60	160.00	40.00	0.40	10.46	5.41	1.63	17.49
8	*Clausena harmandiana*	7	5	0.0213	11.20	70.00	50.00	0.21	4.58	6.76	0.86	12.19
9	*Terminalia nigrovenulosa*	4	3	0.0941	6.40	40.00	30.00	0.94	2.61	4.05	3.81	10.48
10	*Miliusa mollis*	4	4	0.0085	6.40	40.00	40.00	0.08	2.61	5.41	0.34	8.36
11	*Diospyros transitoria*	2	2	0.0867	3.20	20.00	20.00	0.87	1.31	2.70	3.51	7.52
12	*Markhamia stipulata var. pierrei*	3	2	0.0415	4.80	30.00	20.00	0.41	1.96	2.70	1.68	6.34
13	*Memecylon caeruleum*	3	2	0.0281	4.80	30.00	20.00	0.28	1.96	2.70	1.14	5.80
14	*Millettia leucantha*	3	2	0.0190	4.80	30.00	20.00	0.19	1.96	2.70	0.77	5.43
15	*Tetrastigma leucostaphylum*	2	2	0.0213	3.20	20.00	20.00	0.21	1.31	2.70	0.86	4.87

Appendices Table 2 Continued.

no.	Botanical names	no.tree (tree)	no. plot (plot)	sum Ba (m^2)	density (tree/Rai)	density (tree/ha)	frequency (%)	Dominance (m^2/ha)	RD (%)	RF (%)	RBa (%)	IV
16	*Atalantia monophylla*	2	2	0.0210	3.20	20.00	20.00	0.21	1.31	2.70	0.85	4.86
17	*Diospyros defectrix*	2	2	0.0132	3.20	20.00	20.00	0.13	1.31	2.70	0.53	4.54
18	*Memecylon intermedium*	2	2	0.0091	3.20	20.00	20.00	0.09	1.31	2.70	0.37	4.38
19	*Sterculia hypochra*	1	1	0.0387	1.60	10.00	10.00	0.39	0.65	1.35	1.57	3.57
20	*Mayodendron igneum*	1	1	0.0252	1.60	10.00	10.00	0.25	0.65	1.35	1.02	3.02
21	*Hymenodictyon orixense*	2	1	0.0062	3.20	20.00	10.00	0.06	1.31	1.35	0.25	2.91
22	*Falconeria insignis*	1	1	0.0161	1.60	10.00	10.00	0.16	0.65	1.35	0.65	2.66
23	*Erythrina subumbrans*	1	1	0.0075	1.60	10.00	10.00	0.08	0.65	1.35	0.31	2.31
24	*Microcos tomentosa*	1	1	0.0074	1.60	10.00	10.00	0.07	0.65	1.35	0.30	2.30
25	*Canthium coffeoides*	1	1	0.0030	1.60	10.00	10.00	0.03	0.65	1.35	0.12	2.13
26	*Glyptopetalum sclerocarpum*	1	1	0.0047	1.60	10.00	10.00	0.05	0.65	1.35	0.19	2.19
27	*Alphonsea boniana*	1	1	0.0028	1.60	10.00	10.00	0.03	0.65	1.35	0.11	2.12
28	*Strychnos minor*	1	1	0.0024	1.60	10.00	10.00	0.02	0.65	1.35	0.10	2.10
29	*Saraca declinata*	1	1	0.0021	1.60	10.00	10.00	0.02	0.65	1.35	0.09	2.09
30	*Willughbeia edulis*	1	1	0.0019	1.60	10.00	10.00	0.02	0.65	1.35	0.08	2.08
31	*Capparis sp.*	1	1	0.0017	1.60	10.00	10.00	0.02	0.65	1.35	0.07	2.07
	Total	153	74	2.47	244.80	1530.00	740.00	24.69	100.00	100.00	100.00	300.00

Appendices Table 3 Importance Value (IV) of plant species at lower site.

no.	Botanical names	no.tree (tree)	no. plot (plot)	sum Ba (m^2)	density (tree/Rai)	density (tree/ha)	frequency (%)	Dominance (m^2/ha)	RD (%)	RF (%)	RBa (%)	IV
1	*Pterygota alata*	6	5	0.9833	9.60	60.00	50.00	9.83	4.48	5.95	20.60	31.03
2	*Diospyros defectrix*	13	9	0.1627	20.80	130.00	90.00	1.63	9.70	10.71	3.41	23.83
3	*Strombosia ceylanica*	15	5	0.2525	24.00	150.00	50.00	2.52	11.19	5.95	5.29	22.44
4	*Alchornea rugosa*	18	5	0.0928	28.80	180.00	50.00	0.93	13.43	5.95	1.94	21.33
5	*Diospyros transitoria*	12	6	0.2171	19.20	120.00	60.00	2.17	8.96	7.14	4.55	20.65
6	*Dipterocarpus alatus*	1	1	0.7241	1.60	10.00	10.00	7.24	0.75	1.19	15.17	17.11
7	*Atalanti+M:Ra monophylla*	3	2	0.4589	4.80	30.00	20.00	4.59	2.24	2.38	9.62	14.24
8	*Millettia leucantha*	4	3	0.1846	6.40	40.00	30.00	1.85	2.99	3.57	3.87	10.42
9	*Gonocaryum lobbianum*	6	3	0.1099	9.60	60.00	30.00	1.10	4.48	3.57	2.30	10.35
10	*Aglaia edulis*	5	3	0.0798	8.00	50.00	30.00	0.80	3.73	3.57	1.67	8.98
11	*Bischofia javanica*	2	2	0.2118	3.20	20.00	20.00	2.12	1.49	2.38	4.44	8.31
12	*Markhamia stipulata*	3	1	0.1684	4.80	30.00	10.00	1.68	2.24	1.19	3.53	6.96
13	*Saraca declinata*	4	3	0.0124	6.40	40.00	30.00	0.12	2.99	3.57	0.26	6.82
14	*Pterocymbium tinctorium*	1	1	0.2100	1.60	10.00	10.00	2.10	0.75	1.19	4.40	6.34
15	*Galearia maingayi*	2	1	0.1535	3.20	20.00	10.00	1.53	1.49	1.19	3.22	5.90

Appendices Table 3 Continued.

no.	Botanical names	no.tree (tree)	no. plot (plot)	sum Ba (m^2)	density (tree/Rai)	density (tree/ha)	frequency (%)	Dominance (m^2/ha)	RD (%)	RF (%)	RBa (%)	IV
16	*Cleidion javanicum*	3	2	0.0587	4.80	30.00	20.00	0.59	2.24	2.38	1.23	5.85
17	*Dysoxylum cyrtobotryum*	2	2	0.0796	3.20	20.00	20.00	0.80	1.49	2.38	1.67	5.54
18	*Macaranga indica*	1	1	0.1699	1.60	10.00	10.00	1.70	0.75	1.19	3.56	5.50
19	*Xerospermum noronhianum*	2	2	0.0608	3.20	20.00	20.00	0.61	1.49	2.38	1.27	5.15
20	*Artocarpus chama*	3	2	0.0130	4.80	30.00	20.00	0.13	2.24	2.38	0.27	4.89
21	*Knema latericia subsp. Ridleyi*	2	2	0.0423	3.20	20.00	20.00	0.42	1.49	2.38	0.89	4.76
22	*Walsura pinnata*	2	2	0.0123	3.20	20.00	20.00	0.12	1.49	2.38	0.26	4.13
23	*Scaphium affine*	3	1	0.0205	4.80	30.00	10.00	0.20	2.24	1.19	0.43	3.86
24	*Aglaia spectabilis*	1	1	0.0543	1.60	10.00	10.00	0.54	0.75	1.19	1.14	3.08
25	*Croton persimilis*	1	1	0.0479	1.60	10.00	10.00	0.48	0.75	1.19	1.00	2.94
26	*Casearia calva*	1	1	0.0408	1.60	10.00	10.00	0.41	0.75	1.19	0.86	2.79
27	*Mallotus peltatus*	2	1	0.0047	3.20	20.00	10.00	0.05	1.49	1.19	0.10	2.78
28	*Garcinia vilersiana*	1	1	0.0367	1.60	10.00	10.00	0.37	0.75	1.19	0.77	2.70
29	*Tetrameles nudiflora*	1	1	0.0347	1.60	10.00	10.00	0.35	0.75	1.19	0.73	2.66

Appendices Table 3 Continued.

no.	Botanical names	no.tree (tree)	no. plot (plot)	sum Ba (m^2)	density (tree/Rai)	density (tree/ha)	frequency (%)	Dominance (m^2/ha)	RD (%)	RF (%)	RBa (%)	IV
30	*Atalantia monophylla*	1	1	0.0172	1.60	10.00	10.00	0.17	0.75	1.19	0.36	2.30
31	*Dendrolobium lanceolatum*	1	1	0.0123	1.60	10.00	10.00	0.12	0.75	1.19	0.26	2.19
32	*Combretum album*	1	1	0.0064	1.60	10.00	10.00	0.06	0.75	1.19	0.13	2.07
33	*Cryptocarya albiramea*	1	1	0.0061	1.60	10.00	10.00	0.06	0.75	1.19	0.13	2.06
34	*Picrasma javanica*	1	1	0.0057	1.60	10.00	10.00	0.06	0.75	1.19	0.12	2.06
35	*Syzygium siamense*	1	1	0.0041	1.60	10.00	10.00	0.04	0.75	1.19	0.09	2.02
36	*Shorea guiso*	1	1	0.0035	1.60	10.00	10.00	0.04	0.75	1.19	0.07	2.01
37	*Mangifera cochinchinensis*	1	1	0.0034	1.60	10.00	10.00	0.03	0.75	1.19	0.07	2.01
38	*Ficus heterostyla*	1	1	0.0031	1.60	10.00	10.00	0.03	0.75	1.19	0.07	2.00
39	*Willughbeia edulis*	1	1	0.0031	1.60	10.00	10.00	0.03	0.75	1.19	0.07	2.00
40	*Uvaria wrayi*	1	1	0.0030	1.60	10.00	10.00	0.03	0.75	1.19	0.06	2.00
41	*Dipterocarpus turbinatus*	1	1	0.0024	1.60	10.00	10.00	0.02	0.75	1.19	0.05	1.99
42	*Syzygium syzygioides*	1	1	0.0022	1.60	10.00	10.00	0.02	0.75	1.19	0.05	1.98
43	*Neuropeltis racemosa*	1	1	0.0017	1.60	10.00	10.00	0.02	0.75	1.19	0.04	1.97
	Total	134	84	4.77	214.40	1340.00	840.00	47.72	100.00	100.00	100.00	300.00