Shaik Mahammad Khasim
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Orchid Biology: Recent Trends & Challenges

Springer

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Foreword

The family Orchidaceae is the largest family of flowering plants next only to Asteraceae. A great majority of orchids are epiphytic herbs. Although orchids are familiar to the general public due to their ornamental value, they also represent one of the most interesting groups of plants in a number of areas – ecology, morphology, physiology and embryology. Flowers of no other groups of plants show such diversity as orchids; apart from their variations in size, shape and colour, many of them show amazing resemblance to various animals including humans. Also, the flowers of orchids remain fresh for several weeks both on the plants and as cut inflorescences. As it is comparatively easy to raise interspecific and even intergeneric hybrids in orchids, a large number of hybrids, showing unparalleled diversity of flowers, are available in the market. Because of these desirable ornamental features, there is a great demand for orchid plants as well as cut flowers. Unfortunately, though India has great diversity of orchids, our floriculture industry of orchids has remained poor when compared to many Southeast Asian countries such as Singapore, Philippines and Thailand. As orchids are generally slow-growing, their multiplication through conventional means is very time-consuming. Orchids were the first to be commercially propagated using the technique of micropropagation. Now, most of the standard nurseries dealing with orchids use this technology routinely.

Pollination biology of orchids is fascinating and highly variable. Extensive studies have been carried out on pollination of orchids since the time of Darwin. Some species exhibit typical entomophily and a good number of them have also evolved autogamous self-pollination. However, a large number of species exhibit highly specialized pollination system – each orchid species is pollinated by just one specific pollinator. Many of them have evolved deceptive pollination syndrome; they attract animal pollinators by falsely exhibiting the presence of rewards, but do not provide any rewards. Sexual deception is the extreme form of pollination deception evolved to attract species-specific male pollinators. In all the species of *Ophrys*, for example, the flower not only resembles the female of the pollinating insect but also secretes species-specific pheromone to attract male pollinator. The male insect attracted by these features lands on the flower and tries to mate; this is termed as pseudo-copulation, during which it brings about pollination. Seeds of orchids are very small, almost microscopic, and are produced in large numbers. In some species the number of seeds per fruit is reported to be over a million!

In recent decades, overexploitation, human-induced environmental changes, biological invasions and climate change are creating havoc to the sustainability of our biodiversity. These changes have initiated the 'sixth mass species extinction crisis' in which a large proportion of species would become extinct in a geologically short time. As orchids require special habitat, they are highly vulnerable to these changes and require urgent conservation efforts using all available means. Apart from their horticultural importance, in recent years bioactive compounds from plant sources including orchids are gaining importance throughout the world. This again leads to overexploitation of wild species. Many of the overexploited orchid species have already been included in Appendices I and II of CITES. One of the basic requirements for effective conservation is the availability of data on the biology of the species. There is an urgent need to generate such data on most of the wild species of orchids.

Because of their ornamental and other biological importance, orchids have been favorite materials for research for centuries. Extensive studies are being carried out on both fundamental and applied areas of orchids. However, most of the recent literature is scattered in a large number of research papers and reviews. Although there are several books on horticultural aspects of orchids, there are no recent books bringing together the exciting details on other areas of orchid biology in recent years. The present book on *The Orchid Biology: Recent Trends and Challenges* edited by Professor S. M. Khasim and his coeditors from India, Mexico and Thailand is most welcome. This book is the outcome of the proceedings of an international symposium on 'Biodiversity of Medicinal Plants and Orchids: Emerging Trends and Challenges' held during 9–11 February 2018 at Acharya Nagarjuna University, Guntur, India, and also some invited chapters by experts. All the chapters have been grouped under five relevant sections: Cryopreservation and Biotechnology, Orchid Biodiversity and Conservation, Anatomy and Physiology, Pollination Biology and Orchid Chemicals and Bioactive Compounds. I understand that all the chapters have been peer-reviewed before sending to the press. I congratulate the editors for undertaking this compilation. I am confident that various chapters would provide a critical account of the past, present and future trends in diverse areas of orchid biology. Studies on the biology of orchids of India are very limited in spite of the vast diversity it has. Apart from providing consolidated data on various areas of orchid biology, I hope the book would encourage young researchers to take up studies on orchids, particularly of Indian biodiversity hotspots such as North-East Himalaya and the Western Ghats.

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Preface

The Orchidaceae constitutes one of the largest families of flowering plants comprising about 28,484 species. It contributes about 40 percent of monocotyledons. In India, it represents the second largest flowering plant family with 1,141 species in 166 genera and contributes about 10% Indian flora. Orchids comprise a unique group of plants, and their flowers are the most enchanting and exquisite creation of nature. Phylogenetically and taxonomically, the Orchidaceae has been considered as a highly evolved family amongst angiosperms. Orchids show the incredible range of diversity in shape, size and colour of flowers. Orchids with the most attractive and bewitchingly beautiful flowers have commercial importance in floriculture market around the globe. Millions of cut flowers of *Cymbidium*, *Dendrobium*, *Cattleya*, *Paphiopedilum*, *Phalaenopsis*, *Vanda*, etc. besides pot plants of orchids are sold in Western countries, and thus, orchid cut flower industry has now become a multimillion-dollar business in Europe, USA and Southeast Asia.

Besides ornamental value, orchids have got immense pharmaceutical potential. Root tubers of *Habenaria edgeworthii* form an important composition of 'Astavarga' group of drugs in Ayurvedic medicines. It is an established fact that tubers of some terrestrial orchids have been used for treatment of diarrhoea, dysentery, intestinal disorders, cough, cold and tuberculosis. Some orchids, particularly belonging to the genera such as *Aerides*, *Arachnis*, *Cattleya*, *Cymbidium*, *Dendrobium*, *Epidendrum*, *Oncidium*, *Paphiopedilum*, *Phalaenopsis*, *Renanthera*, *Vanda*, etc., have been extensively used to produce the internationally acclaimed hybrids. The Indian orchids are paradoxically victims of their own beauty and popularity. As a result, their natural populations have been declining rapidly because of unbridled commercial exploitation in India and abroad as well. Further this situation has led the orchids to the verge of extinction, e.g. *Renanthera imschootiana*, *Diplomeris hirsuta*, *Paphiopedilum fairrieanum* (already extinct), *Cypripedium elegans*, *Taeniophyllum andamanicum*, etc.

An edited book titled *The Orchid Biology: Recent Trends and Challenges* is the outcome of the proceedings of an international symposium on 'Biodiversity of Medicinal Plants and Orchids: Emerging Trends and Challenges' held during 9–11 February 2018 at Acharya Nagarjuna University, Guntur, India. Besides that, we also invited eminent orchid experts across the globe to contribute to this book, so as to enable us to report on state of the art of scientific investigations that have been going on for the last several decades on orchid biology. All papers contained in this

book were peer-reviewed by international experts. Further, the manuscripts were reviewed by editors and those papers that were judged as suitable for publication following the authors' considerations of reviewer suggestions appeared in this edited book.

In view of the importance of orchids globally for their large-scale production and exploitation for the human health and wealth, we felt that the comprehensive compilation by international experts is the need of the hour.

The present book contains five sections: (I) Cryopreservation and Biotechnology, (II) Orchid Biodiversity and Conservation, (III) Anatomy and Physiology, (IV) Pollination Biology and (V) Orchid Chemicals and Bioactive Compounds. All five sections contain 28 papers authored by eminent orchid experts/professors across the globe. This book serves as a reference book for researchers, teachers, orchid enthusiasts, orchid growers and students of biotechnology, botany, pharmaceutical sciences and ethnomedicine. It would be of equal interest to horticultural industry especially orchid industry, agricultural scientists and policy makers.

We would like to express our sense of gratitude to all contributors from India and abroad for accepting our invitation to contribute chapters and for not only sharing their knowledge but also for admirably integrating expertise in composing the chapters of the various aspects of orchid biology. We greatly acknowledge Dr. So-Young Park (Chungbuk, Republic of Korea), Dr. Apiradee (Bangkok, Thailand), Prof. M. M. Hossain and Prof. M. K. Huda (Chittagong, Bangladesh), Prof. P. Kaushik (Haridwar, India), Prof. S. N. Sinha (Kalyani, India), Dr. A. N. Rao (ORDC, Manipur, India), Prof. Navdeep Shekhar (Faridkot, India) and Dr. M. M. Hoque (Chittagong, Bangladesh) for their commitment and dedication for bringing the final shape of this edited book. I am very much indebted to my professor, guide and philosopher, Dr. P. R. Mohana Rao, an eminent orchid embryologist for his invaluable support all throughout my academic journey.

We are also thankful to our colleagues and research scholars at the Department of Botany and Microbiology, Acharya Nagarjuna University, India, for the preparation of the manuscript. We profusely thank Ms. Aakanksha Tyagi, associate editor, Springer Science, India, and her staff for their unstinted support and very effective execution of this project.

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Shaik Mahammad Khasim is head of the Department of Botany and Microbiology, Acharya Nagarjuna University, India, and former chairman, Board of Studies in Biotechnology. He has made significant contribution in the field of orchid biology and conservation. He has been working on molecular characterization, biodiversity, micropropagation, ethnomedicine, and phytochemistry of orchids. He published good number of research papers in national and international journals. He is the recipient of the *Usha Vij Memorial Award-2018* for his outstanding contribution in orchid biology conferred by the Orchid Society of India (TOSI), Chandigarh. He is an associate fellow of A.P. Academy of Science (India); life member of TOSI, Indian Science Congress Association (Calcutta), and member of the American Orchid Society (Florida) and the International Society for Horticultural Science (ISHS), Belgium.

Sadanand N. Hegde, former Director of Orchid Research and Development Center, State Forest Research Institute, Arunachal Pradesh, India, has made significant contribution in orchid taxonomy and conservation. His initial works were on the cytotaxonomic studies on the orchids of Western Ghats of India at the University of Agricultural Sciences, Bangalore, and subsequently at the Karnataka University, Dharwad, on cyto- and chemotaxonomic studies in the tribe Epidendreae of Orchidaceae. He explored 600 orchid species from Arunachal Pradesh and registered 8 new species as well. During his tenure as Director, he developed orchids as a supplemental crop for the tribal farmers of Arunachal Pradesh and other Northeastern states of India. He registered 6 new hybrid orchids and bred 16 new hybrids. He is recipient of Dr. TN Khooshoo Memorial Environment Award (2004) conferred by the Orchid Society of India (TOSI). He is also the founder member of TOSI, Chandigarh.

María Teresa González-Arnao is a professor in Universidad Veracruzana, Veracruz, México, since 2004. She has been working on plant biotechnology especially in plant cryopreservation techniques for last several decades. She has published research papers in national and international journals such as *Acta Horticulturae*, *European Food Research and Technology*, *HortScience*, *Cryobiology*,

Revista Advances en Investigación Agropecuria (AIA), and *Journal of Plant Biochemistry and Biotechnology*. She also contributed several chapters in technical books published by Springer-Verlag. She is the member of the International Society for Horticultural Science (ISHS), Belgium.

Kanchit Thammasiri is an associate professor and head of the Department of Plant Science, Mahidol University, Bangkok (Thailand). He has made significant contribution in the field of cryopreservation of Thai orchid seeds and their micropropagation. His current research involves the orchid breeding and micropropagation of selected genera, such as cattleya alliance, dendrobiums, phalaenopsis and vandaceous-type orchids, and cryopreservation of Thai orchids. He published his research papers in national and international journals, such as *CryoLetters*, *Acta Horticulturae*, *ScienceAsia*, *Richardia*, *Lindleyana*, and *Plant Cell, Tissue and Organ Culture*. He is member of the American Orchid Society, Florida, and also International Society for Horticultural Science (ISHS), Belgium.

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Part I

Cryopreservation and Biotechnology

Cryopreservation Development of Some Endangered Thai Orchid Species

1

Kanchit Thammasiri

Abstract

Thailand is the origin of about 1, 300 tropical orchid species and 180–190 genera. Deforestation and over-collection of wild Thai orchids for trade has placed orchid species at a risk of extinction. Therefore, the conservation as well as sustainable use is urgently needed to conserve orchids by various means. The genera *Paphiopedilum* and *Dendrobium cruentum* are listed in Appendix I of CITES. At the Department of Plant Science, Faculty of Science, Mahidol University, various methods of cryopreservation and conservation of Thai orchid species have been carried out. For cryopreservation, recent methods were used, namely, vitrification (dehydration in PVS2 solution, consisted of 30% glycerol, 15% ethylene glycol and 15% dimethyl sulfoxide, prepared in modified Vacin and Went liquid medium), encapsulation-dehydration (encapsulation in calcium alginate beads followed by air-drying in a laminar air-flow cabinet), encapsulation-vitrification (encapsulation in calcium alginate beads followed by dehydration in PVS2 solution), droplet-vitrification (fast freezing from small drops of PVS2 solution on aluminium strip) and cryo-plate (a combination of encapsulation and droplet on very fast freezing aluminium plate) dehydrated with silica gel and drying beads. Application of these methods in seeds was successful in *Dendrobium chrysotoxum* (99%, vitrification), *D. cruentum* (32%, vitrification), *D. draconis* (95%, vitrification), *D. hercoglossum* (80%, encapsulation-vitrification), *Doritis pulcherrima* (62%, vitrification), *Rhynchostylis coelestis* (85%, vitrification), *Vanda coerulea* (67%, vitrification), as well as in protocorms of *D. cruentum* (33%, vitrification; 27%, encapsulation-dehydration), *D. cariniferum* (15%, encapsulation-vitrification), *Grammaytophyllum speciosum* (14%, encapsulation-vitrification), *Rhynchostylis gigantea* (19%, vitrification), *V. coerulea* (40%, encapsulation-dehydration), *Seidenfadenia mitrata* (67%, vitrification) and *Arundina graminifolia* (76% and 74%, cryo-plate dehydrated with drying beads

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and silica gel, respectively; 33% droplet-vitrification; 64% encapsulation-dehydration with drying beads or silica gel). Cryopreserved seeds and protocorms were able to develop into normal seedlings. These techniques appear to be promising for the cryopreservation of some Thai orchid germplasm.

Keywords
Vitrification · Encapsulation-dehydration · Encapsulation-vitrification · Droplet-vitrification · Cryo-plate · Drying beads · Silica gel

1.1 Introduction

Thailand is the origin of about 1, 300 tropical orchid species and 178 genera. Many Thai orchid species have good horticultural characteristics and are used as parents for breeding, making Thailand the No.1 orchid exporter. Climate change, deforestation (habitat destruction), and over-collection of wild Thai orchids for trade has placed Thai orchids at a risk of extinction. Therefore, conservation, social awareness and consciousness, as well as sustainable use are urgently needed to conserve orchids by various means (Thammasiri 2008). At the Department of Plant Science, Faculty of Science, Mahidol University, various methods of *ex situ* conservation of Thai orchid species have been carried out, namely, cryopreservation, seed stores under Orchid Seed Stores For Sustainable Use (OSSSU) project and micropropagation.

1.2 Cryopreservation Technology

After meeting and discussing with Professor Akira Sakai at the International Workshop on *In Vitro* Conservation of Plant Genetic Resources, 4–6 July 1995 in Kuala Lumpur, Malaysia, he came to demonstrate vitrification-based methods for plant cryopreservation at my department. A little later, I started doing research on cryopreservation of jackfruit embryonic axes which was very successful and novel (Thammasiri 1999). I then shifted my interest in cryopreservation research into orchids for which I am the pioneer. I published the first paper on *Doritis pulcherrima*, a wild Thai orchid, on seed cryopreservation by vitrification with 62% (Thammasiri 2000). It was also the first paper on seed cryopreservation by vitrification [dehydration in PVS2 solution, consisting of 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) dimethyl sulfoxide, prepared in modified Vacin and Went liquid medium].

Later my post-graduate and doctoral students studied recent methods, namely, vitrification (dehydration in PVS2 solution), encapsulation-dehydration (encapsulation in calcium alginate beads followed by air-drying in a laminar air-flow cabinet), encapsulation-vitrification (encapsulation in calcium alginate beads followed by dehydration in PVS2 solution) and droplet-vitrification (fast freezing from small

drops of PVS2 solution with plant materials inside on a 7 × 20 mm sterile aluminum foil strip). Application of these methods on seeds was successful in many Thai orchid species. Cryopreserved seeds and protocorms were able to develop into normal seedlings. These methods appear to be promising techniques for cryopreservation of many Thai orchid species.

Thammasiri (2002) presented "Preservation of Seeds of Some Thai Orchid Species by Vitrification" at the 16th World Orchid Conference. *Dendrobium chrysotoxum, D. draconis, Doritis pulcherrima and Rhynchostylis coelestis* had 99%, 95%, 62% and 85% germination, respectively, after seed cryopreservation by vitrification. Other Thai orchid seeds were later successfully cryopreserved, such as in *D. cruentum* (32% by vitrification) (Kagawa 2006), *Vanda coerulea* (67% by vitrification) (Thammasiri and Soamkul 2007), *D. hercoglossum* (80% by encapsulation-vitrification) as well as in protocorms of *D. cruentum* (33% by vitrification and 27% by encapsulation-dehydration) (Kagawa 2006), *D. cariniferum* (15% by encapsulation-vitrification) (Pornchuti and Thammasiri 2008), *Vanda coerulea* (40% by encapsulation-dehydration) (Jitsopakul et al. 2008) and *Seidenfadenia mitrata* (67% by vitrification).

Sopalun et al. (2010a, b) studied three vitrification-based methods, namely, droplet-vitrification, encapsulation-dehydration and encapsulation-vitrification, for cryopreservation of protocorms of *Grammatophyllum speciosum*, known as "Tiger orchid" or "Giant orchid". Protocorms, 0.1 cm in diameter, developed from 2-month-old germinating seeds were used. For droplet-vitrification (Fig. 1.1), protocorms were precultured on filter paper soaked in half strength Murashige and Skoog medium (½MS) containing 0.4 M sucrose at 25 ± 2 °C for 2 days, followed by soaking in loading solution (2 M glycerol and 0.4 M sucrose in ½MS liquid medium) for 20 min and then dehydrated with PVS2 solution in ½MS liquid medium containing 0.4 M sucrose at pH 5.7 for 30 min. For encapsulation-dehydration (Fig. 1.2), encapsulated protocorms were precultured in ½MS liquid medium containing 0.4M sucrose on a shaker (110 rpm) at 25 ± 2 °C for 2 days, followed by soaking in the same loading solution for 20 min and then exposed to a sterile air-flow at 2.5 inches/water column from the laminar air-flow cabinet for 8 h. For encapsulation-vitrification (Fig.1.3), encapsulated protocorms were precultured in ½MS liquid medium containing 0.4 M sucrose for 1 or 2 days, followed by soaking in the same loading solution for 20 min and then dehydrated with PVS2 solution for 60 min. For all three methods, preculturing with 0.4 M sucrose for 2 days resulted in a significant induction of dehydration and freezing tolerance. The cryopreservation results showed the highest protocorm regrowth after droplet-vitrification (38%), followed by encapsulation-dehydration (24%) and encapsulation-vitrification (14%). Plantlets developed from these three methods did not show any abnormal characteristics or ploidy level change when investigated by flow cytometry.

Cordova II and Thammasiri (2016) developed the cryo-plate method using silica gel or drying beads for dehydration (Fig. 1.4). Protocorms were placed in the preculture solution consisting of 0.7 M sucrose on a shaker (110 rpm) at 25 ± 3 °C for 1 day. After that, protocorms were placed one by one in the wells which filled before with the alginate solution containing 2% (w/v) sodium alginate in calcium-free 1/2 MS

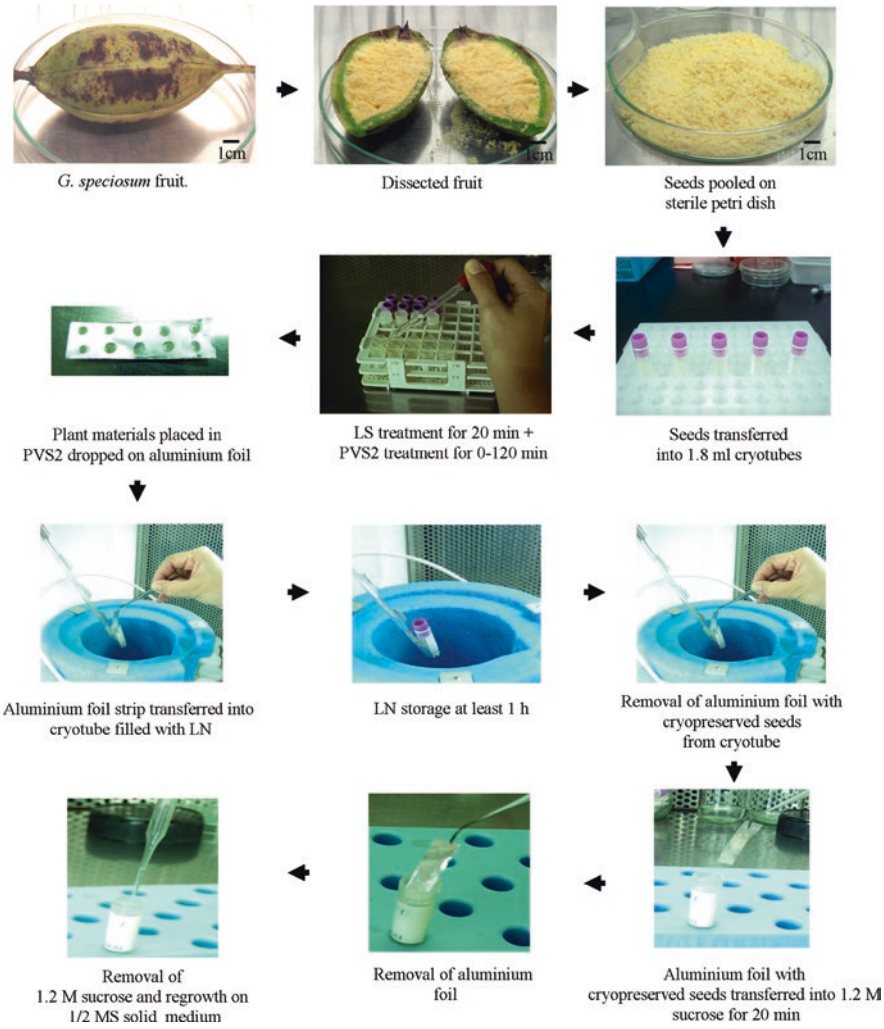

Fig. 1.1 Established protocol for cryopreservation of *G. speciosum* seeds by droplet-vitrification

basal medium with 0.4 M sucrose. The cryo-plates were hardened for 20 min by slowly dispensing the calcium chloride solution containing 0.1 M calcium chloride in 1/2 MS basal medium with 0.4 M sucrose. Then the cryo-plates were surface dried using sterile filter paper, placed in Petri dishes containing silica gel or drying beads in a laminar air-flow cabinet. Cryo-plates were dehydrated for 5 h until 25% moisture content was achieved. Dehydrated cryo-plates were placed in 2 ml cryotubes and plunged directly into liquid nitrogen for 1 day. Cryo-plates were removed from

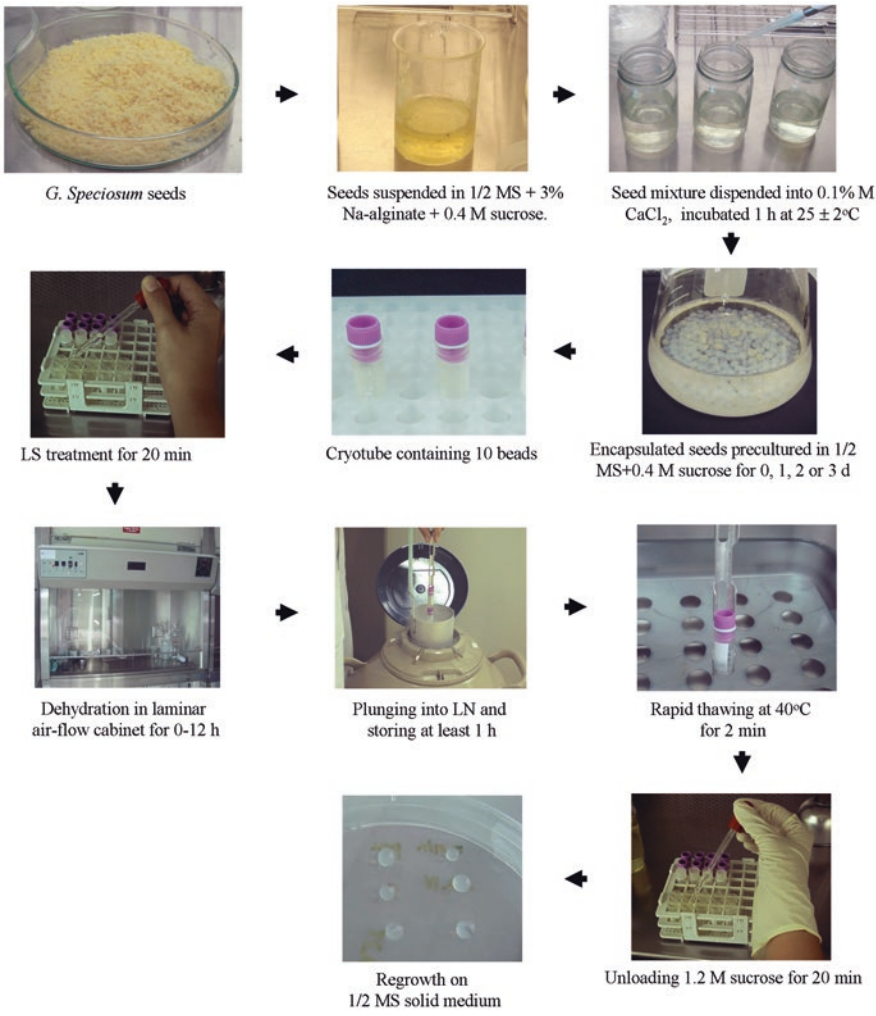

Fig. 1.2 Established protocol for cryopreservation of *G. speciosum* seeds by encapsulation-dehydration

cryotubes and warmed in unloading solution (1.2 M sucrose solution) for 20 min. Protocorms were then removed from the cryo-plate and placed on 1/2 MS agar medium for regrowth. Growth conditions were conducted using 16 h light at 25 ± 3 °C.

For effect of the cryo-plate method, regrowth of control treatments dehydrated using silica gel was observed to be 90%. Regrowth of control treatments dehydrated using drying beads was observed to be 92.1%. In all other treatments, regrowth was

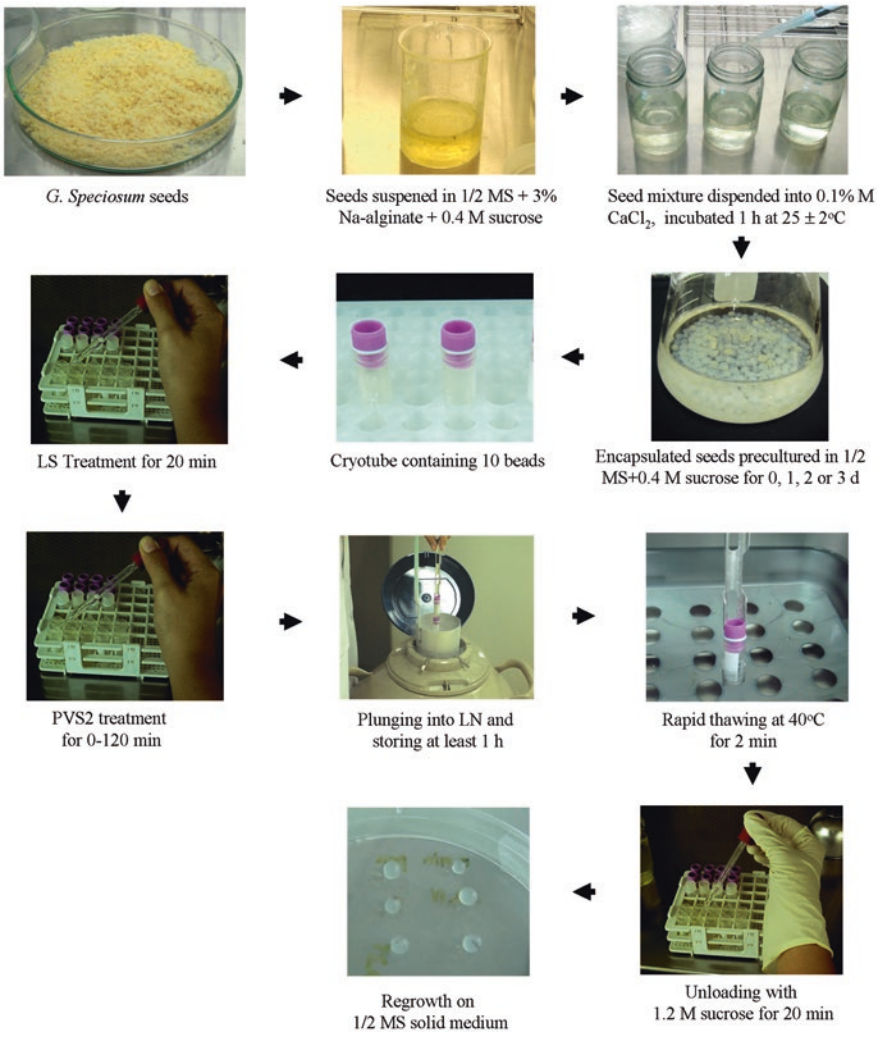

Fig. 1.3 Established protocol for cryopreservation of *G. speciosum* seeds by encapsulation-vitrification

observed to be 73.8% using silica gel for dehydration. Regrowth for all other treatments dehydrated using drying beads was observed to be 76.5%. Regrowth was observed in the second week of transfer to 1/2 MS media. Dehydration using silica gel or drying beads did not significantly affect regrowth rate. Protocorms dehydrated using silica gel or drying beads developed into normal plantlets.

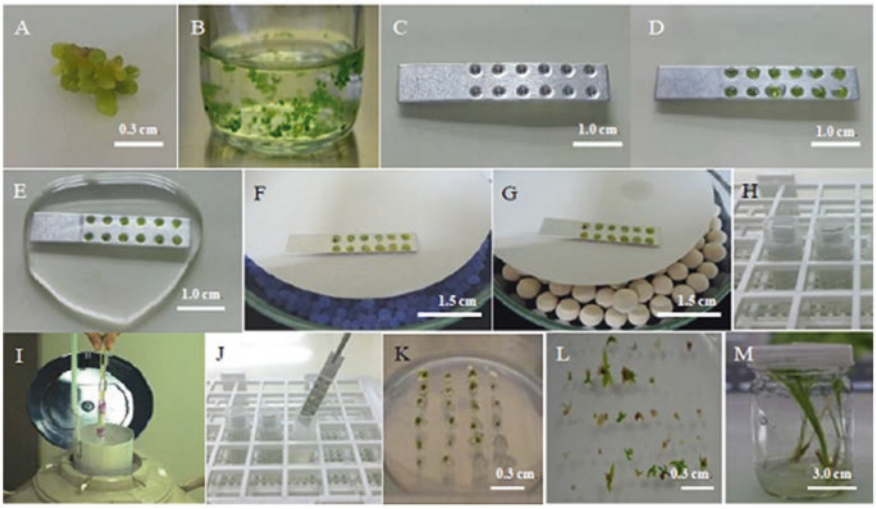

Fig. 1.4 Cryo-plate method dehydrated with silica gel or drying beads. (**a**) Protocorm development. (**b**) Preculture of protocorms in 1/2 MS liquid medium with 0.7 M sucrose for 1 day. (**c**) Pour the alginate solution containing 2% (w/v) sodium alginate in calcium-free 1/2 MS basal medium with 0.4 M sucrose in the wells. (**d**) Place the precultured protocorms in the wells one by one. (**e**) Pour the calcium chloride solution containing 0.1 M calcium chloride in 1/2 MS basal medium with 0.4 M sucrose. (**f**) Dehydration with 50 g silica gel. (**g**) Dehydration with 30 g drying beads. (**h**) Put each cryo-plate in a 2 ml cryotube. (**i**) Plunge 2 ml cryotubes into liquid nitrogen for 1 day. (**j**) Warming in 1.2 M sucrose solution for 20 min. (**k**) Plate on 1/2 MS agar medium. (**l**) Regrowth, (**m**) Regrowth after 60 days

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Status of Orchid Industry in India

2

Sadanand N. Hegde

Abstract

India is known for its rich biodiversity, and one of its dominant plant families is Orchidaceae consisting of about 1350 species in 185 genera occurring in diverse phytogeographical conditions ranging from tropical, subtropical and temperate conditions with varying microclimates. However, despite the rich natural occurrence and ideal agroclimatic conditions in India, orchid-based floriculture has not been systematically developed, and the people are yet to reap the benefit out of this natural resource having the least production area and minimum contribution in the overall turnover of floriculture products. In fact, India has lagged other countries in Orchid trade. In this paper, an attempt has been made to trace the history of sustainable development of orchids in India briefly. Distributions of some of the commercially important orchid species and hybrids in various agroclimatic conditions have been provided. The role of governments, non-government organizations and individuals in promoting the development of orchid industry in India has been discussed. An assessment and analysis of commercial activities based on import and export of various orchid products such as cut flowers, tissue culture plants and flasks from major ports of the country have been carried out. The need for a coordinated effort in focused R&D programme in developing new hybrid varieties suitable for various agroclimatic conditions of this country involving various Institutions of Excellence in developing climate-specific hybrids of temperate cymbidiums and paphiopedilums, tropical dendrobiums and vandas and intermediate cattleyas and phalaenopsis, besides other ornamental native species for both cut flowers and pot plants along with their cultivation practices and packages, has been suggested. Besides, potentials of medicinally important orchids and their R&D programme in boosting commercial production have also been suggested. A strong extension programme of the

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technology- and market-driven approach to reach the stakeholders, farmers and growers, in villages and urban clusters involving the communities in promoting cultivation and production of the commercial orchids, has been proposed for the development of a vibrant orchid industry. Involvement of corporate sector to produce quality planting materials in large quantities, distribution to the growers/farmers in village and urban clusters and marketing of their products has been emphasized for the benefit of the society and to boost orchid industry in India.

Keywords
Orchid industry · Orchid germination · Greenhouse technology · Export and import in India

2.1 Introduction

Over the last half a century, floriculture has emerged as an export-oriented multibillion-dollar business globally. It mainly involves cut flowers, pot plants, cut foliage, bulbs, tubers, seeds, rooted cuttings, tissue culture (TC) flasks, dry flowers and leaves. It has been observed that production and trade of floricultural crops have ever increasing trend. In the international trade, major crops involved are *Alstroemeria*, carnations, chrysanthemums, gerbera, gladiolus, gypsophila, anthuriums, lilies, roses, tulips and of course orchids (*Aranda*, *Cattleya*, *Cymbidium*, *Dendrobium*, *Oncidium*, *Paphiopedilum*, *Phalaenopsis*, *Vanda*, etc.). Orchids command high value and great in demand in the World Flower Trade. It is mainly because of attractive flower shape, size, colour and long shelf life of cut flowers and pot plants of orchids. Out of about US $21 billion floriculture trade, 8% is that of orchids and has an increasing trend of 15% annually. It is significant to note that Netherlands is the world's leading flower producer and exporter. The Dutch control the world export and auctioning of floricultural produces. Major consumers of floriculture products are Japan, European countries, South Korea, Thailand, Indonesia and Pacific countries with ever increasing demand. Major exporters of floriculture products are Holland, Columbia, Israel, Italy, Spain, Thailand, France, the USA, South America, New Zealand, Ecuador, etc. Orchids have gained importance as one of the highly priced floricultural crops across the world

2.2 Indian Scenario

India is one of the orchid-rich countries in the world with about 1350 species occurring in diverse phytogeographical conditions from warm coastal regions to the cool Himalayan ranges providing tropical, subtropical and temperate conditions with varying microclimates giving rise to rich biodiversity. Such a natural situation is congenial to grow commercial varieties of orchids to meet the world market demand. However, despite the rich natural occurrence and ideal agroclimatic conditions in

India, orchid-based floriculture has not been systematically developed, and the people are yet to reap the benefit out of this natural resource. Important floriculture crops in India are amaryllis, anthuriums, carnation, chrysanthemum, gladiolus, jasmine, marigold, petunias, roses and, of course, orchids. Total acreage of these crops is about 73,619 ha with 34,349 tons of loose flowers and 49,366 cut flowers. Orchids have the least production area and minimum contribution in the overall turnover of floriculture products. In fact, India has lagged other countries in orchid trade despite its rich natural resources, ideal agroclimate and technical know-how. Out of about Rs. 500 crore businesses in floriculture industry in India, orchids have the least contribution in our country.

2.3 Orchid Development in India

In India, for the first time, Hooker (1890) brought out "Flora of British India" describing 1200 species unravelling the richness of orchids in this country. Subsequent workers added number of species, and today we are a proud nation with about 1350 species in about 185 genera of orchids known in our country (Hegde 2014, Jain Mehrotra 1984; Misra 2007; Rao 2014). Out of them as many as 200 species are highly ornamental, about 55 species are medicinally important, and others are biological curiosities with ecological significance (Hegde 1997; Vij 2001).

Realizing the importance of orchids in floriculture especially that of the hybrid varieties developed from the native ornamental species from India and other parts of the world, the Government of India initiated developmental programmes through various government and non-government agencies to ensure conservation of native germplasm both in situ and ex situ and promoting sustainable development of orchid industry through research and development programmes through ICAR, universities and state and central government agencies (Hegde 1986, 2000, 2005, 2014). Many private players, viz. U.C. Pradhan Laboratories, Kalimpong; Indo-American Hybrid Seeds, Bangalore; and A.V. Thomas & Co, Kerala, are also involved in production of planting materials adopting tissue culture and modern biotechnological approaches and trade of planting materials. National Horticulture Board, NABARD and APEDA have been supporting entrepreneurs to undertake export-oriented orchid trade. Some NGO activities to promote research and development and creating awareness have also been undertaken by TOSI, OSA, TOSKAR and other floriculture societies in various parts of India.

Meanwhile, orchid growing as a hobby elsewhere in the world has transformed itself as a vibrant floriculture industry of commerce with multimillion-dollar business of plants and cut-flower trade. Discovery of aseptic culture of seeds by Knudson (1946) and meristem culture by Morel (1960) revolutionized orchid industry elsewhere in the world with an array of orchid hybrids and cut-flower varieties grown in modern climate-controlled polyhouses. Over the last 50 years, tissue culture technology has added dimension to the industry both in terms of quality and quantity. There are more than 1,25,000 man-made hybrids besides about 25,000 species worlds over making it highly specialized, competitive and vibrant industry of

commerce. With the modern biotechnological approaches, it has become possible to produce an array of hybrids and newer clones released to the market – specializing in flower decorations, corsages, bouquet making, pot plants with amazing colour of flowers of orchids, besides various social functions (Pathak et al. 2001; Hegde 2001, 2006, 2009; Vij 2001).

It is worthy to note India so far could produce and register hardly about 200 orchid hybrids (Pradhan 2017) which need to be accelerated to compete in the world market. Meanwhile, however, our expertises in biotechnological approach have contributed in producing tissue-cultured true-to-type hybrid clones which need to be commercially exploited.

2.4 Commercial Potentials

Commercial potentials of orchids in India or its strength in orchid development and trade lies in (1) rich orchid diversity/germplasm; (2) varying and ideal agroclimate from tropical to temperate regions to grow varieties of orchids; (3) technical know-how of orchid growing, propagation technique, biotechnological backing and green-house technology; (4) cheap labour; and (5) evergrowing high-end consumer market.

On the other hand, our weaknesses are (1) lack of quality planting materials in adequate quantity; (2) lack of market-driven approach in the production of plants and flowers adopting modern technologies; (3) lack of production of our own hybrids that can compete in world market; (4) lack of consistent R&D backup with new hybrid varieties and technical innovations; (5) lack of production of planting material; (6) lack of quality and quantity of cut flowers to feed the market; (7) lack of training and extension programmes; and (8) lack of involvement of communities both at rural and urban levels in suitable agroclimatic zones and developed hub of activities leading to market places.

2.5 Agroclimate and Orchid Germplasm

Agroclimatically, we have mainly three broad climatic zones, (i) tropical, (ii) sub-tropical and (iii) temperate, for growing orchids. Depending upon rain pattern, humidity, temperature and elevation, there exist varying types of microclimatic conditions. This has in fact given rise to orchid diversity with about 1350 species in 185 genera occurring in their natural habitats of our country. About 200 species of them are ornamental which could serve as germplasm for breeding, cultivation/farming and production of quality planting materials in the respective agroclimatic zones (Hegde 2001, Pathak et al. 2001). Besides, over the last 50 years, a large number of exotic species and hybrids have been imported and grown in various institutions and by individual hobbyists. This is indeed our strength and potential that should be sustainably utilized for developing orchid industry (Hegde 2014). It is important to note humidity ranging between 50% and 80% is a common requirement for all types

of orchids. Light regime of 3000–6000-foot candle (30000–60000 lux) is considered ideal.

It is worthy to note that a large number of Indian species have been used in developing modern-day hybrids elsewhere in the world. Hence, what we need now is intensive breeding programme on selected orchid genera making use of wild varieties and their modern hybrids. Germplasm of most of the modern-day hybrids are now available in India which could be utilized in breeding programme.

Thus, India with its varied agroclimatic zones from tropical conditions of peninsular India to subtropical and temperate areas of the Himalaya offers wonderful opportunity to grow varieties of orchids round the year. Gangtok in Sikkim, Kalimpong in West Bengal, N.E. States, Kerala, Goa, western parts of Karnataka and Maharashtra (Pune), eastern parts of Tamil Nadu (Nilgiri), Andhra and Orissa and some parts of North India are the ideal places for growing various types of orchids. Native ornamental species and exotic hybrids should be improved through intensive breeding adopting modern biotechnological approaches. Besides, cheap labour and evergrowing high-end consumer market make it highly profitable proposition to grow various orchids in India in a profitable manner (Hegde 2014).

At present in India, orchids are grown either as hobby or as a commercial enterprise. Hobbyists tend to grow varieties of orchid hybrids and species in the available space around the house or balcony. Commercial cultivation of orchids requires more space and perfection adopting the modern biotechnological approaches for producing quality plants and cut flowers in good quantity aiming at the market. The approach here should be market-driven.

Modern floriculture is an integrated technology based on biological sciences making use of hardware engineering mechanisms. Understanding of genetics and physiological requirements of a crop is essential to meet the stringent requirements in the quality of cut flowers to match the market demands. Hence, right from seed production, quality seedling production (through seeds or tissue culture), transplanting to farm houses, growing them to flower as per the requirement, harvesting up to transportation to the market, everything at every stage, biological principles are employed in achieving perfection. Accordingly, the tissue culture lab, hardening unit, farm houses, and postharvest packing and packaging require hardware engineering to achieve this perfection in floriculture industry. Breeding must be a continuous endeavour applying modern biotechnological tools coupled with micropropagation of selected clones to finally meet market demand, which is ever-changing with occasions and trend (Table 2.1).

2.6 Greenhouse Technology

Greenhouse technology is the latest trend and is most desired for export production with the required quality, quantity and regularity. In this regard, the Government of India's initiative to adopt plastics in agriculture has yielded considerable progress in augmenting floriculture. This is required to be modified and adopted to various agroclimatic zones, depending upon the crop (Singh and Dadlani 2000). There are

Table 2.1 Market specification and technological requirement in orchid trade

SL no.	Quality specification	Requirements of the plant	Technological makeup
1.	Preference of colour shape and size is very specific and is subject to change with time	These are genetic characters of the plant. Breeding new varieties should be continuous	Select the current varieties. Replace plants at every 4–5 years interval with upcoming varieties
2.	Strong straight spike	Plants should be healthy and must produce limited numbers of flowers	Proper nutritional management. Regulation of flowering beyond the optimum limit
3.	Flowers fully open, free from blemish, clear colour; broad strong petals; each flower facing the same side	Plants should be healthy and must produce only limited number of flowers. Distribution of sunlight should be uniform. Direct rainfall on plants and flower to be avoided	Proper nutritional management. Regulation of flowering, perfect layout in the north to south direction, appropriate shade, rain protection either with UVR film or Netlons
4.	No sign of insect damage or any diseases. At the same time insecticides and other chemicals should not adhere to the flowers	Prevent entry of insects. Maintain proper pH of the medium. Prevent growth of algae. Assure drainage. Ensure mild breeze 200M/H all the time	Greenhouse cultivation can prevent entry of insects. Follow integrated pest managements. Use proper size of the pot, repot timely. Use proper ratio in planting medium. Install proper ventilation device or adjust the layout with wind direction

specialized companies who manufacture modern environment-controlled greenhouses to suit the need of the crop. In India at present, most of the commercial labs have started tie up/joint venture programmes with leading companies like BV, Florist (Holland) and others from Southeast Asia and procure latest varieties to grow in India. In fact, these companies are specialized in the production of crop like cymbidiums from Australia and New Zealand; dendrobiums, vandas/mokaras, etc. from Singapore and Thailand; *Phalaenopsis* from Holland and Taiwan; and so on. In other words, we are totally dependent on other countries for planting materials. Till the time we become self-reliant, India will have to depend upon advanced countries for quality planting stock and distribute to the growers or farmers for production of plants and flowers.

To achieve quality production, it is essential to identify the suitable agroclimatic areas to establish "Flori-tech Village" *clusters* in each state of the region (Hegde 1999) to adopt low-cost greenhouse and rain shelters for small farmers and climate-controlled greenhouses for large export houses. In the Flori-tech Village Concept, cluster of villages will have a cooperative with central model farm to cater to the needs of planting materials, impart training to the farmers, set up low-cost small farm/polyhouses and to organize marketing of the produces.

In an export-oriented venture where quality, quantity and regularity of production and supply are to be ensured, greenhouse technology must be adopted. It requires investment and intensive management by trained managers devoted to the

profession. Clockwise timely action right from planting; watering; fertilizing; controlling humidity, temperature, ventilation, and light; pest and disease management; training of flower spikes; harvesting and postharvest handling; to transporting quickly and freshly up to the markets is of paramount importance for the success of orchid industry (Hegde 1999, 2001).

2.7 Medicinal and Aromatic Orchids

About 55 species have been reported to be used in various systems of medicines in India for treating various disorders and diseases (Kaushik 1983). Traditional practitioners mostly collect the plants in the wild, and as a result, most of them have become rare and endangered. Hence, there is a need to develop a package and practice for their cultivation and propagation commercially to help the pharma industry with authentic, quality of planting materials with sufficient quantity and help conserve the natural population to survive and proliferate.

Role of governments, non-government organizations and individuals is very crucial in promoting the development of orchid industry in India. There is a need for a coordinated effort in focused R&D programme in developing new hybrid strains suiting to various agroclimatic conditions of our country involving various Institutions of Excellence in developing climate-specific varieties of temperate cymbidiums and paphiopedilums, tropical dendrobiums and vandas and intermediate cattleyas and phalaenopsis, besides other ornamental native species for both cut flowers and pot plants along with their cultivation practices and packages.

2.8 Present Trend in Export and Import of Orchids

To assess the trend in export and import of orchids in India, a study was conducted to analyse the import and export data from important ports like Delhi, Mumbai, Kolkata, Chennai and Bangalore for the years 2013, 2014, 2015 and 2016. The EXIM reports consisting of month-wise import and export data for 4 years were collected from M/S Seair Exim Solutions, New Delhi, and the same was analysed and summarized as given below:

2.9 Summary of the Import and Export

- *Imports*:
  - Species most imported:
    Dendrobium ~85%
    Phalaenopsis ~8%
  - Type most imported: cut flowers ~ 66%
  - Most imported from: Thailand ~ 90%
  - Import port:

Largest import is to Delhi at ~ 30% and it is showing an increasing trend.
Bombay and Chennai are not far behind in imports.
In fact, all metros have a sizeable amount of imports.

- *Exports*:
  - Species most exported: *Phalaenopsis* ~ 98%
  - Type most exported: tissue culture ~ 97%
  - Country most exported to:
    United States ~ 98% (2013/14)
    Netherlands ~ 90% (2015/16)
  - Export port: almost all exports are from Bombay ~97%

From the analysis, the following two interesting trends in orchid industry could be noted:

(i) Most imported are the cut flowers (66%) of *Dendrobium* (85%) and *Phalaenopsis* (8%) from Thailand (90%).
(ii) Most exported are the tissue culture flask/seedlings (97%) of *Phalaenopsis* (98%) to Netherlands (90% in 2015–2016) and the United States (98% in 2013–2014).

It is interesting to note that from 2013 to 2016, there have been a decreasing trend in export of our orchid products and increasing trend in import from 2013 to 2016 (Fig 2.1). However, positive trend is noticed in 2016–2017. Further, it is encouraging to notice export of tissue-cultured seedlings/flasks (Fig. 2.2) pointing towards sustainable development of native species. And, of course, it is a positive trend in the conservation of native orchids/germplasm. Since large quantity of cut

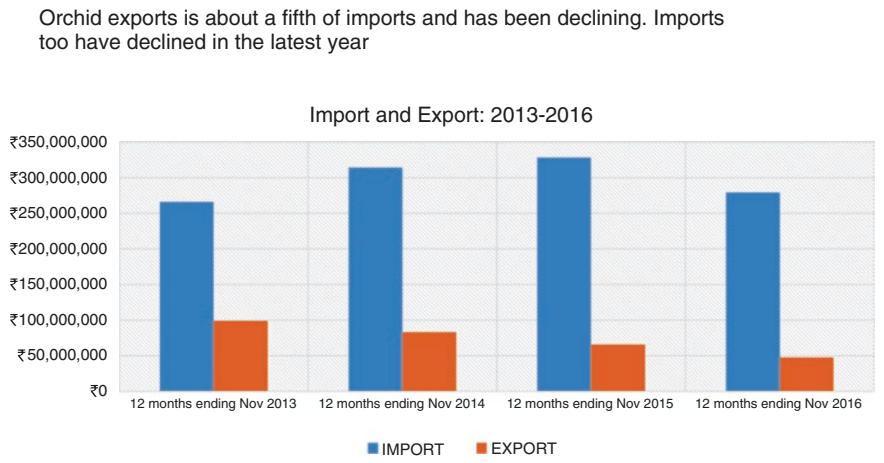

Fig. 2.1 Graphic presentation of orchid export and imports for the last 4 years

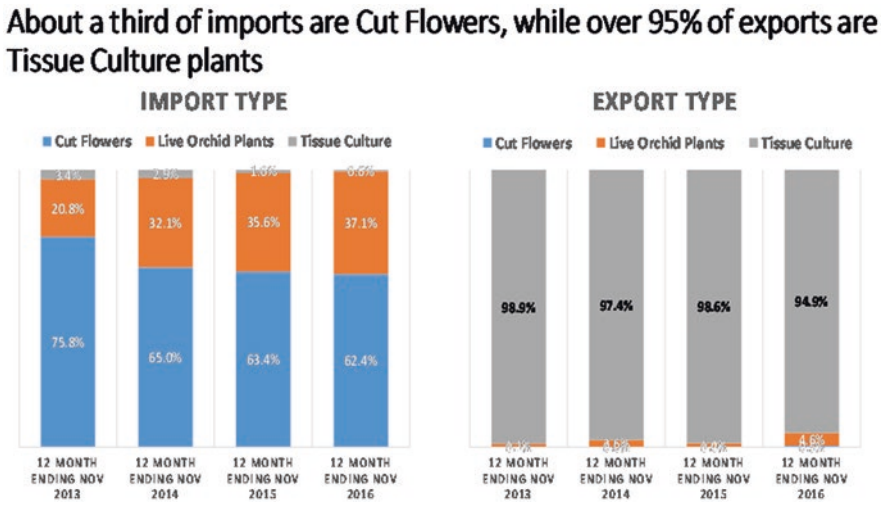

Fig. 2.2 Trend of import and export types of orchids – cut flowers, live plants and tissue culture plants

flowers and plants is imported from other countries, it is indicative that there exists domestic market with great demand for orchid cut flowers in our metros.

2.10 Conclusion

India is rich in orchid diversity and offers a good opportunity to grow orchids in various agroclimatic conditions of India in a sustainable manner adopting modern biotechnological approaches in the production of quality and quantity planting materials catering to the needs of the growers within and outside India. There is a need to carry out focused and intensive breeding of commercially important orchid genera suiting to various agroclimatic conditions of tropical, subtropical and temperate zones.

Further, based on the import and export analysis, our focus should be to produce quality cut flowers and pot plants in rural and urban areas adopting greenhouse technology to meet the domestic demand and, at the same time, target the export-oriented farming on demand basis from abroad.

Besides, there is a need to tap the potentials of medicinally important orchids through R&D programmes in boosting commercial production. A strong extension programme of the technology and market-driven approach to reach the stakeholders, farmers and growers, in villages and urban clusters involving the communities, is required in promoting cultivation and production of the commercial orchids with a market-driven approach for the development of a vibrant orchid industry. Involvement of corporate sector would help in the production of quality planting materials in large quantities, distribution to the growers/farmers in village and urban

clusters and marketing of their products for the benefit of the society and to boost orchid industry in India.

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Cryobiotechnological Studies in *Vanilla*: The Orchid of Multi-industrial Uses

3

María Teresa González-Arnao, Fabiola Hernández-Ramírez, Natalia Raquel Dolce, Martha P. Rascón-Díaz, and Carlos A. Cruz-Cruz

Abstract

In this work we provide new information about several cryobiotechnological studies conducted using *Vanilla planifolia* species as a case study. We evaluated the effect of three vitrification-based techniques, droplet-vitrification (D-V), V cryo-plate (V-Cp) and D cryo-plate (D-Cp), on the survival of two types of in vitro explants (shoot-tips and root-tips). Using differential scanning calorimetry (DSC), there were defined thermal characteristics of shoot-tips subjected to several desiccation times with SG following D-Cp procedure. It was calculated the removal of osmotically active water (OAW) by the effect of desiccation duration after the osmoprotective steps of preconditioning and loading. Using ISSR markers with six selected ISSR primers, we also evaluated the impact of tissue culture and of cryopreservation on genetic stability of vanilla shoot-tips dehydrated with the vitrification solution PVS2 and comparing D-V and V-Cp procedures. All these investigations are allowing the development and optimization of reliable cryopreservation protocols for long-term storage of *Vanilla* germplasm.

Keywords

Vanilla · Cryopreservation · Shoot-tips · Root-tips · DSC · ISSR

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3.1 Introduction

Vanilla is the only orchid with edible fruits which are used worldwide as spice. In contrast to other orchids which are cultivated with ornamental purposes, vanilla is the most famous orchid which is commercially cultivated to produce the important natural essence known as vanillin (Chugh et al. 2009).

The genus *Vanilla* belongs to the family Orchidaceae with more than 110 described species (Bory et al. 2008). *Vanilla planifolia* is considered the most economically relevant among cultivated vanilla species. It has been mostly used as a case study in numerous investigations with different biotechnological approaches, such as in vitro multiplication (Kalimuthu et al. 2006), somatic embryogenesis (Janarthanam and Seshadri 2008) and genetic transformation (Malabadi and Nataraja 2007), and development of in vitro conservation techniques (Divakaran et al. 2006; González-Arnao et al. 2009; Hernández-Ramírez et al. 2014).

Vanilla planifolia is native to Mexico, although it is now widely cultivated throughout the tropics (Soto-Arenas 2003). Unfortunately, its primary gene pool is profoundly threatened, implying an imperative need to protect its genetic resources (Grisoni et al. 2007). In addition, for the last 10 years some *V. planifolia* plantations in Mexico are also facing a critical problem associated with the premature fall of fruits, which is seriously affecting vanilla producers (Castro-Bobadilla et al. 2011). Currently, *V. planifolia* is considered a species subjected to special protection in Mexico (Semarnat 2010).

The long-term conservation of vanilla germplasm is usually conducted by collections of whole plants in the field. Different institutions around the world maintain preserving their germplasm under this ex situ condition; however, cryopreservation offers the safest, most efficient and cost-effective ex situ strategy among the alternatives for long-term storage of plants with vegetative propagation and for conservation of endangered species (Keller et al. 2008).

Prior to the first report of cryopreservation study with vanilla germplasm, Indian Institute of Spices Research already conserved 100 vanilla accessions in vitro and several self progenies under slow growth conditions. This strategy allowed maintaining germplasm for more than 7 years with yearly subcultures (Divakaran et al. 2006); however, only cryogenic storage could avoid the required yearly manipulation. Cryopreservation implies the total arrest of cellular division and metabolic processes by the effect of ultra-low temperature of storage, usually that of liquid nitrogen (–196 °C) (González-Arnao et al. 2008).

Based on the facts that in vitro-grown apices of several orchid species had been successfully cryopreserved using vitrification-based procedures (Thinh and Takagi 2000), our group started investigating different cryoprotective treatments associated to such a kind of protocols. Vanilla tissues were subjected to drastic dehydration conditions both by exposure to highly concentrated vitrification solutions or to the air current in a laminar air-flow cabinet or to silica gel before being rapidly or ultra-rapidly immersed in liquid nitrogen (González-Arnao et al. 2009; Hernández-Ramírez et al. 2014). As a result, it was detected that vanilla shoot-tips tolerated different cryoprotective conditions of several protocols but did not achieve

post-cryopreservation recovery. Following a droplet-vitrification approach, there were obtained a maximum of 30% survival and 10% regeneration of new shoots (González-Arnao et al. 2009), but even so, results are not always reproducible. Since then, further investigations have been performed to refine this first protocol and achieve survival more reliable and reproducible (Hernández-Ramírez et al. 2014). Other vitrification-based approaches (V and D cryo-plate) have also been evaluated and used different analytical techniques to complement and understand the results obtained empirically. We have studied the effect of different cryoprotective treatments on the accumulation of osmolytes using GCMS (Rosas et al. 2018); there have been conducted proteomic studies (González-Arnao et al. 2008), and we used the differential scanning calorimetry (DSC) and molecular markers (ISSR) to assess the impact of cryopreservation. We are presenting some of the cryobiotechnological studies carried out with vanilla (*V. planifolia*) shoot and root-tips to assist the development of effective alternatives for long-term conservation.

3.2 Cryopreservation of In Vitro-Derived *Vanilla* Explants

3.2.1 Comparison of Different Cryogenic Techniques with Two Types of In Vitro Vanilla Explants

The following cryopreservation experiments illustrate the research activities performed to optimise the dehydration conditions according to the individual behaviour of two in vitro explants of vanilla (*V. planifolia*). The protocols previously developed using shoot-tips (González-Arnao et al. 2009; Hernández-Ramírez et al. 2014) and root-tips (Dolce et al. 2019) were very helpful to continue refining the most critical steps of cryoprotection.

3.2.2 Plant Material

Shoot-tips (~3–5 mm in length) were dissected from in vitro multiplied plants of *Vanilla planifolia* species subcultured every 12 weeks in fresh MS (Murashige and Skoog 1962) semisolid medium supplemented with 1 mg L^{-1} 6-benzyl amino purine (BAP), 0.5 mg L^{-1} indole butyric acid (IBA), 20 g L^{-1} sucrose and 7 g L^{-1} agar (Sigma–Aldrich Ltd. Co.). Donor plantlets were maintained in a culture chamber at 24 ± 2 °C under a 16 h light/8 h dark photoperiod with a light intensity of 36 μmol $m^{-2}s^{-1}$ (González-Arnao et al. 2009).

Root-tips (~2 mm long) were excised from young roots of in vitro plantlets originating from microcuttings of *V. planifolia* species 30 days after their last subculture on MS semisolid medium and maintained in a growth chamber at 27 ± 2 °C under a 14 h light/10 h dark photoperiod with a photosynthetic flux of 116 μmol m^{-2} s^{-1} (Dolce et al. 2019).

Both in vitro explants (tips of shoots and roots) were subjected to three cryogenic procedures: (i) droplet-vitrification (D-V), (ii) V cryo-plate (V-Cp) and (iii) D

cryo-plate (D-Cp). In all cases, tissues were first preconditioned on MS semisolid medium supplemented with 0.3 M trehalose for 1d (Hernández-Ramírez et al. 2014).

When applied with D-V method, preconditioned samples were loaded with solution containing 0.4 M sucrose + 2 M glycerol for 30 min, exposed to vitrification solutions PVS2 [30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO and 13.7% (w/v)] (Sakai et al. 1990) and PVS3 [50% sucrose (w/v) + 50% glycerol (w/v)] (Nishizawa et al. 1993) for 30 min at room temperature, and then, they were transferred to droplets of PVS3 placed on aluminium foil strips (25 mm length × 6 mm width), which were immersed rapidly in liquid nitrogen. After 1 h storage at −196 °C, rewarming took place rapidly by plunging the aluminium foils in unloading solution containing 1.2 M sucrose for 15 min.

When applied with V-Cp and D-Cp methods, preconditioned tips were first attached with calcium alginate to the surface of aluminium cryo-plates with 37 mm length × 7 mm width and 0.5 mm thickness (Niino et al. 2014). For encapsulation, tissues were transferred onto a droplet (about 50 μL) of sodium alginate (2%, SIGMA, low viscosity) calcium-free solution, and then, calcium chloride (0.1M) solution (75 μL) was gently poured in the borders and over the droplet of alginate to provoke the polymerization during 15 min at room temperature. Encapsulated samples (gelled droplet of ~5 mm length) were treated with loading solutions containing 0.4 M sucrose + 2 M glycerol for 30 min at room temperature. Using V-Cp procedure, samples after loading were exposed to PVS2 or PVS3 for 30 min before being ultrarapidly immersed in liquid nitrogen. In the case of shoot-tips, it was also tested the exposure to PVS3 for 60 min. When applied with D-Cp method, cryo-plates with loaded samples were transferred to sealed containers containing 360 g of silica gel (SG) and desiccated for 90, 120, 150 or 180 min. After each desiccation period, the aluminium cryo-plates with samples were immersed directly in liquid nitrogen. After 1 h storage at −196 °C and following both cryogenic (V-Cp and D-Cp) protocols, rewarming of samples took place rapidly by plunging the cryo-plates in unloading solution containing 1.2 M sucrose for 15 min. Warmed tips of shoot or root were firstly transferred to filter papers for 1 to 2 min to drain off excess liquid and, then, recultured for recovery.

For regrowth, shoot-tips were cultured on the MS semisolid multiplication medium supplemented with 1 mg L^{-1} BAP and 0.5 mg L^{-1} IBA in dark until detecting any kind of recovery and, then, exposed to photoperiod on MS semisolid medium with 1 mg L^{-1} KIN. Root-tips were cultured on the MS semisolid medium with 1 mg L^{-1} KIN under permanent dark. After 45 days of culture, the explants were transferred to MS medium free of plant growth regulators and incubated under standard culture conditions for plant regeneration.

Survival of both in vitro explants of vanilla subjected to cryopreservation was recorded as the percentage of explants that showed any growth after 90 days of culture.

3.2.3 Outcome of Cryopreservation Experiments

The comparison of the three vitrification-based protocols demonstrated that both types of vanilla meristematic explants were very sensitive to both dehydration and thermal stress imposed by cryopreservation. Vanilla shoot-tips were less susceptible than root-tips because they could withstand both osmotic and physical dehydration and survival after immersion in liquid nitrogen using the three protocols. By contrast, root-tips only survived to cryopreservation when they were osmotically dehydrated with PVS3 by following D-V procedure (Table 3.1).

Nevertheless, post-cryopreservation recovery of vanilla shoot-tips resulted quite variable, except when used D-Cp protocol. The highest regeneration rate (33%) is still low, but it was reproducible. These results indicated that the replacement of osmotic dehydration with PVS by drying in SG allowed to improve the effectiveness of the protocol in terms of reproducibility. This could be explained by the size and the morphology of tissues used. According to Thinh and Takagi (2000), desiccation produces a more uniform drying than osmotic dehydration when dealing with complex structures of relatively large sizes. Shoot-tips with the apical dome partly covered had higher regrowth compared to fully covered ones (Niino et al. 2013). However, vanilla root-tips were smaller (2 mm in length maximum) than shoot-tips (up to 5 mm long), and this might explain why D-V procedure resulted more suitable to dehydrate naked tissues and cryopreserve smaller explants.

We also tried the triphenyl tetrazolium chloride (TTC) viability test complementarily to regrowth assessment (data not shown). TTC test was just conducted to have a quick preliminary diagnosis about survival (Fig. 3.1a); however, it did not provide a precise information of viability, because results after critical steps of dehydration are not always correlated to the real ability to regenerate new shoots and subsequently whole plants (Dolce, personal communication). Figure 3.1a shows two red-dyed shoot-tips, which according to TTC test, they survived osmoprotection with PVS3 applying D-V protocol; however, no regrowth was achieved as expected.

Table 3.1 Comparison of three vitrification-based procedures on shoot regeneration of vanilla (*V. planifolia*) shoot and root-tips before (-LN) and after (+LN) cryopreservation

Shoot regeneration (%)		Shoot-tips		Root-tips	
Technique	Dehydration	−LN	+LN	−LN	+ LN
D-V	PVS2 (30 min)	53.7 ± 3.5	10 ± 2.5	0	0
	PVS3 (30 min)	84.7 ± 4.5	10 ± 4.3	25	10
V-Cp	PVS2 (30 min)	57.0 ± 6.0	10 ± 2.5	0	0
	PVS3 (30 min)	65.7 ± 5.1	0	30	0
	PVS3 (60 min)	66.3 ± 5.5	0	–	–
D-Cp	SG90 min	10.0 ± 7.0	0	0	0
	SG120 min	16.0 ± 2.6	0	0	0
	SG150 min	45.0 ± 2.5	33 ± 3.2	0	0
	SG180 min	20.0 ± 8.6	0	0	0

D-V Droplet-vitrification, *V-Cp* V cryo-plate, *D-Cp* D cryo-plate, *PVS* Plant vitrification solution, *SG* silica gel, - non-tested

On the other hand, using D-Cp protocol, it was observed indirect regrowth of vanilla shoot-tips during post-cryopreservation reculture (Fig. 3.1b). Shoot-tips that survived first formed a callus-like mass on the multiplication medium, from which multiple shoots were progressively generated when exposed to photoperiod on the MS semisolid medium with 1 mg L^{-1} KIN, and then, they developed new plantlets (Fig. 3.1c). The embryogenic character of generated callus mass was also histologically analysed in further investigations (Hernández-Ramírez et al. in preparation). By contrast, surviving root-tips showed a direct organogenesis with bud initiation at the apical end of the explants (Fig. 3.2) within 30–45 days of reculture (Dolce et al. 2019).

To analyse factors affecting post-cryopreservation survival of vanilla shoot-tips, new assessments were conducted using differential scanning calorimetry (DSC).

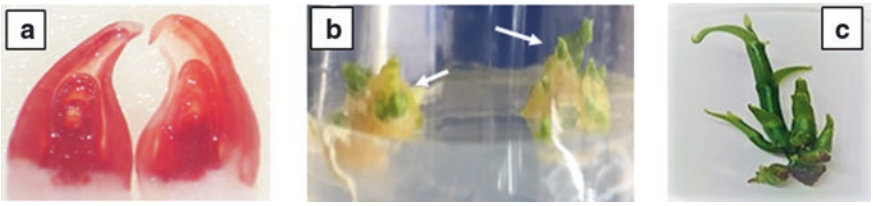

Fig. 3.1 Evaluation of viability and shoot regeneration of vanilla shoot-tips prior (**a**) and after (**b** and **c**) cryopreservation following different cryogenic protocols. (**a**) Results of TTC test by using shoot-tips isolated from in vitro-grown plants, subjected to 1d preconditioning on semisolid medium supplemented with 0.3M trehalose, loaded in 0.4 M sucrose + 2 M glycerol solution (30 min) and exposed to PVS3 for 30 min according to droplet-vitrification. (**b**) Regeneration of multiple shoots after cryopreservation by indirect organogenesis. Shoot-tips were isolated from in vitro-grown plants, subjected to 1d preconditioning on MS semisolid medium supplemented with 0.3M trehalose, loaded in 0.4 M sucrose + 2 M glycerol solution (30 min) and desiccated and exposed to SG for 150 min according to D cryo-plate procedure. (**c**) In vitro plants regenerated by indirect organogenesis of a cryopreserved shoot-tip after 10 months of reculture following the D cryo-plate protocol previously described

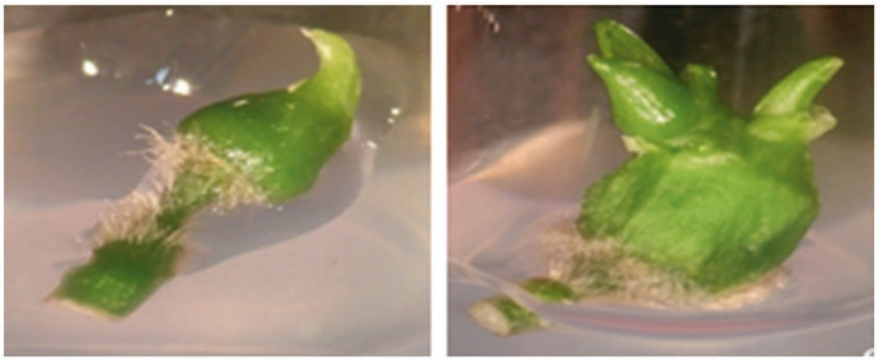

Fig. 3.2 Regeneration process of cryopreserved vanilla (*V. planifolia*) root-tip (Dolce et al. 2019)

3.3 Thermal Analysis of Vanilla Shoot-Tips by DSC

Development and optimization of an effective protocol for cryopreservation of tropical plant species is a challenge and difficult task, since they are not usually exposed to cold or extreme desiccation during their normal cycles of life (Nadarajan et al. 2008). Because these species do not have a natural acclimation response, the tolerance needs to be artificially induced by manipulating different stress conditions to achieve a successful cryopreservation procedure.

The effects of physicochemical processes that alter water status in samples after each cryoprotective step before the liquid nitrogen immersion modulate the thermal behaviour of materials. This can be analysed by differential scanning calorimetry (DSC) which allows to identify the phase transitions and the removal of the osmotically active water responsible for producing irreversible damages in tissues and, consequently, the lost of viability (Gamez-Pastrana et al. 2011).

In order to elucidate the impact of critical cryoprotective treatments on the physical thermal events during cryopreservation of vanilla shoot-tips, samples of about six shoot-tips dissected of in vitro-grown plantlets were subjected to D-Cp protocol previously described, because these conditions allowed obtaining low but reproducible results of survival after exposure to liquid nitrogen. For DSC analysis, samples were desiccated by the exposure to SG (360 g) in sealed containers during 90, 120, 150, 180, 240 and 300 min. Shoot-tips after each control step (dissection, loading treatment and the different desiccation times) were weighted (5 mg) using a Sartorius CPA225D microbalance precision class E2 and placed in aluminium Tzero™ pans to be sealed with Tzero™ Hermetic Lids. For analysing samples after both loading and desiccation treatments, shoot-tips were taken out of cryo-plates, and the calcium alginate gel was eliminated.

Thermal analyses were performed using a DSC Q2000 V 23.4 apparatus with a refrigerant cooling system (RCS, −90 °C), both from TA Instruments, Inc., New Castle, Delaware, USA. The DSC program comprised a cooling ramp of 10 °Cmin^{-1} from +40° to −70 °C and heating of 3 °Cmin^{-1} from −70 °C to +40 °C. It was calculated the osmotically active water (OAW) of shoot-tips after each control step by dividing the change of entropy during melting by the water enthalpy of fusion at 0 °C (ΔH = 333.55 J/g). OAW was expressed as a fraction of fresh weight of the sample.

3.3.1 DSC Analysis Results

The recorded DSC thermograms during heating phase showed the progressive depression of melting temperatures from 1.19°C in non-treated shoot-tips to −30.13°C in samples preconditioned, loaded and desiccated for 300 min (Table 3.2).

Table 3.2 DSC analysis of vanilla (*V. planifolia*) shoot-tips subjected to different desiccation times using D cryo-plate technique. Evaluations were performed during heating phase

Desiccation (min)	Midpoint T_m (°C)	ΔH (J/g)	Osmotically active water (OAW)	
			(g g^{-1} FW)	(Relative % FW)
Non-treated	1.19	259.1	0.78	100
0	−3.96	163.3	0.49	62.8
90	−8.29	102.3	0.30	38.4
120	−17.78	75.3	0.23	29.4
150	−18.87	68.6	0.21	26.9
180	−18.27	63.2	0.19	24.3
240	−20.89	41.1	0.12	15.3
300	−30.13	7.7	0.02	2.5

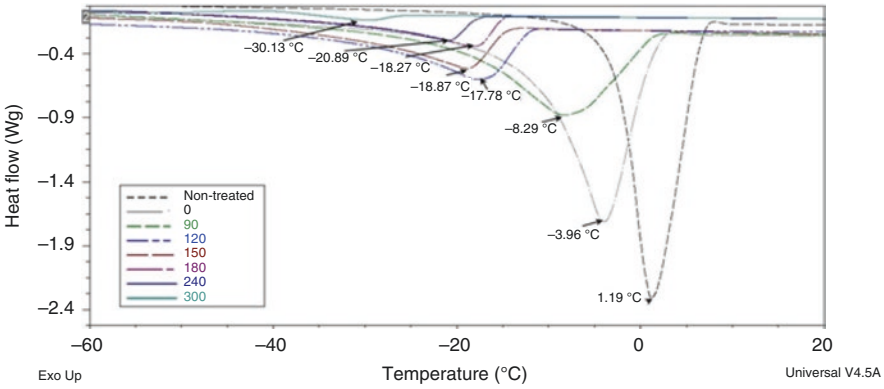

Fig. 3.3 Effect of desiccation time on thermal properties of *Vanilla planifolia* shoot-tips dissected from in vitro-grown plants and subjected to 1d preconditioning on MS semisolid medium supplemented with 0.3 M trehalose, a loading treatment in a solution with sucrose 0.4 M + glycerol 2 M and exposed to SG

Representative thermograms of shoot-tips subjected to the different cryoprotective stages (after dissection, gelification over the cryo-plates + loading treatment and gelification + loading treatment + different desiccation times) showed thermal profiles with endothermic peaks until the longest desiccation time, indicating that ice melting still occurred at this condition (Fig. 3.3).

The DSC thermal analysis revealed that the relative proportion of osmotically active water, compared to the initial water content of non-treated shoot-tips (78% OAW FW, considered as 100%), progressively declined, and consequently, melting enthalpies also changed proportionally.

However, vanilla shoot-tips only were able to withstand cryopreservation when OAW content remained around 26.9%FW (150 min of desiccation). Shorter desiccation periods (90 and 120 min) did not allow to achieve survival apparently due to insufficient removal of water, and longer exposure times (from 180 to 300 min), probably because of the over dehydration.

We used a relatively slow heating rate (3 °Cmin^{-1}) for DSC analysis; this might explain why there were detected melt enthalpies even at very low water contents and/or indicate the instability of the glasses which could have potentially formed during cooling.

The results of calorimetric studies suggest that to achieve glass transition and/or improve the stability of glasses formed during cryopreservation of vanilla shoot-tips, it would be useful to remove a greater quantity of OAW. However, this has proved to be a very difficult process and would represent a higher level of stress. Therefore, the most recommended strategy could be to optimise the dissection method and reduce the size of vanilla shoot-tips.

3.4 Assessment of Genetic Stability During In Vitro Culture and Cryopreservation of Vanilla Shoot-Tips Using ISSR Markers

Molecular markers provide a direct and reliable method to examine genetic variation at the DNA level (Yi et al. 2015).The inter-simple sequence repeat (ISSR) markers are very useful and mostly used for detecting genetic variability (Martínez-Estrada et al. 2017; Atul et al. 2018). In addition, ISSR markers are highly polymorphic; the technique is simple, quick and reproducible, and it does not require prior information about the genome (Peng et al. 2015).

In this study, we used six selected ISSR primers (Table 3.3) to evaluate genetic stability during cryopreservation of vanilla (*V. planifolia*) shoot-tips. Shoot-tips were subjected to droplet-vitrification (D-V) and V cryo-plate (V-Cp) procedures using PVS2 for dehydration.

We studied the impact of tissue culture before (in vitro newly introduced plant and in vitro plant with multiple subcultures) and after (plants derived of cryopreserved shoot-tips) cryopreservation, since the application of tissue culture techniques is required first for the multiplication of the donor plants and, then, for the recovery of biological material subjected to cryopreservation. We also evaluated the effect of different cryoprotective steps (preconditioning, loading, dehydration with PVS2 and liquid nitrogen), according to D-V and V-Cp protocols, and we previously selected those specific conditions which allowed plantlet regeneration of cryopreserved vanilla shoot-tips. Following D-V protocol, shoot-tips were subjected to preconditioning on MS semisolid medium supplemented with 0.3 M trehalose for 1d, loaded with 0.4M sucrose + 2M glycerol and exposed to PVS2 for 30 min at room temperature prior to ultrarapid immersion in liquid nitrogen. Following V-Cp protocol, shoot-tips were subjected to preconditioning on MS semisolid medium for 7 days and, then, on MS medium supplemented with 0.3 M sucrose for additional 7 days, loaded with 0.4M sucrose + 2M glycerol and exposed to PVS2 for 30 min at room temperature prior to ultrarapid immersion in liquid nitrogen.

Table 3.3 List of ISSR primers used to verify the genetic stability of vanilla germplasm during in vitro culture and cryopreservation

Primer	Sequence	Range of amplicons (bp)
T05	5′ CGTTGTGTGTGTTGTTGT 3′	342–1290
T06	5′ AGAGAGAGAGAGAGAGT 3′	306–2091
C07	5′ GAGAGAGAGAGAGAGAC 3′	330–1353
UBC823	5′ TCTCTCTCTCTCTCTCC 3′	900–1848
UBC825	5′ ACACACACACACACACT 3′	565–2020
UBC848	5′ CACACACACACACACARG 3′	370–1107

DNA extractions were performed according to Haque et al. (2008) using young leaves isolated from in vivo plants (control), in vitro plantlets with one subculturing, plantlets with multiple subculturings (> 20), shoot-tips at the different cryoprotective steps and in vitro plantlets regenerated of cryopreserved shoot-tips.

ISSR-PCR analysis was conducted according to Martínez-Estrada et al. (2017), and data was processed using the statistical software Popgene, 1.32 version. Results of Nei's genetic distance were schematised by dendrograms, based on the unweighted pair-group method with arithmetic mean (UPGMA). The calculation of the polymorphic information content (PIC) of each primer was carried out using the statistical software Info-Gen, 2016 version.

3.4.1 Preliminary Results of Genetic Stability Assessment Effect of Tissue Culture

The analysis of electrophoretic profiles according to the six ISSR primers used detected a total of 153 bands of which 142 were monomorphic and 11 polymorphic, representing a total percentage of polymorphism of 7.2%. The range of the bands varied between 306 and 2091 bp. UPGMA dendrogram based on Nei's genetic distance is presented in Fig. 3.4.

3.4.2 Effect of Cryoprotective Steps of D-V Procedure

The analysis of electrophoretic profiles according to the six ISSR primers detected a total of 199 bands of which 182 were monomorphic and 17 polymorphic, representing a total percentage of polymorphism of 8.5%. The range of the bands varied between 240 and 2091 bp.

UPGMA dendrogram based on Nei's genetic distance is presented in Fig. 3.5.

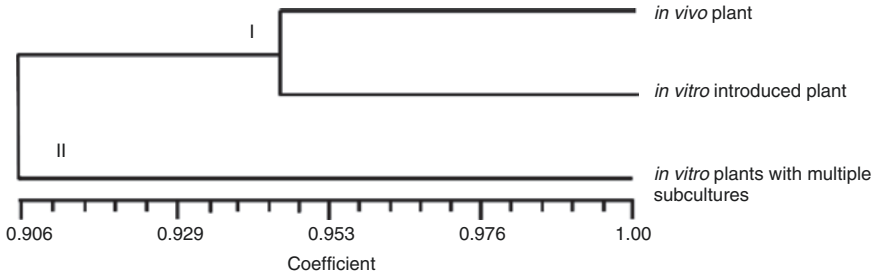

Fig. 3.4 Dendrogram of clustering of in vivo and in vitro plants analysed by ISSR

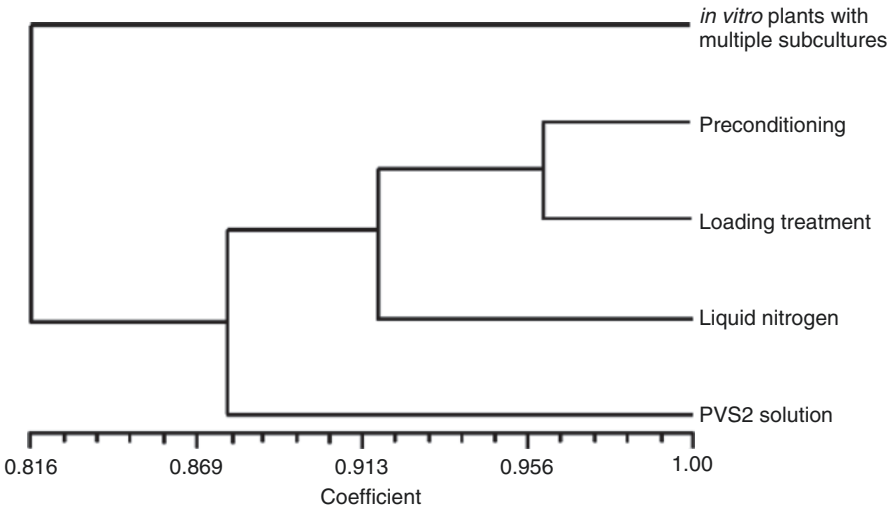

Fig. 3.5 Dendrogram of clustering of in vitro multiplied plants and shoot-tips subjected to droplet-vitrification (D-V) technique

3.4.3 Effect of Cryoprotective Steps of V-Cp Procedure

The analysis of electrophoretic profiles according to the six ISSR primers used detected a total of 194 bands of which 188 were monomorphic and 6 polymorphic, representing a total percentage of polymorphism of 3%. The range of the bands varied between 240 and 2091 bp. UPGMA dendrogram based on Nei's genetic distance is presented in Fig. 3.6.

The general information provided by genetic stability studies demonstrated that plants with multiple in vitro subcultures showed greater variation (similarity index of 0.906) than newly introduced with respect to in vivo plant. The exposure of shoot-tips to vitrification solution PVS2 was the osmoprotective step which induced the greatest molecular variability after both cryogenic protocols (average of similarity index: 0.083) compared to control plant. After cryopreservation, D-V protocol

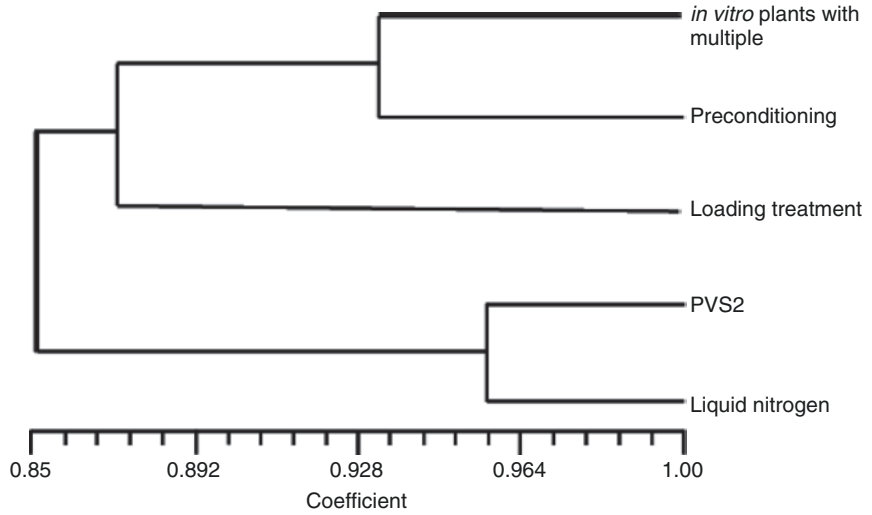

Fig. 3.6 Dendrogram of clustering of in vitro multiplied plants and shoot-tips subjected to V cryo-plate (V-Cp) technique

generated greater variation compared with V-Cp (similarity indexes of 0.08195 and 0.9048 for D-V and V-Cp, respectively). Nevertheless, despite these results, it was noted that both cryopreservation processes maintained relatively high (over 80%) molecular genetic similarity levels, regardless of the methodology used.

According to ISSR markers, these results also indicated that neither the thermal stress which produces the cryogenic storage nor the recovery culture after cryopreservation produced significant additional variations to those already detected, although cryopreserved shoot-tips regenerated new plants by indirect organogenesis.

3.5 Conclusions

This chapter presents different technological aspects related to the development and optimization of cryopreservation techniques, as well as their impact on genetic stability of plant material. In our studies we used different in vitro cultures of *V. planifolia* as a case study. We evaluated the effect of three vitrification-based cryogenic techniques, droplet-vitrification (D-V), V cryo-plate (V-Cp) and D cryo-plate (D-Cp), on the survival of shoot-tips and root-tips. According to these procedures, we analysed the impact of osmotic dehydration with or without encapsulation of the explants over the cryo-plates and of desiccation in SG. DSC analysis allowed to illustrate the thermal behaviour of shoot-tips after combined cryoprotective steps during the application of D-Cp. The effect of tissue culture and of cryopreservation on vanilla shoot-tips dehydrated with PVS2 was assessed with ISSR markers.

Our experiments showed that desiccation with SG according to D-Cp protocol allowed better dehydration of large vanilla shoot-tips than osmotic dehydration using PVS with the same size of explant. This allowed improvements after cryopreservation in the recovery rate and in its reproducibility. The best cryoprotective conditions comprised dissection of shoot-tips from in vitro-grown plants, 1d preconditioning on MS semisolid medium supplemented with 0.3 M trehalose and gelification of shoot-tips with calcium alginate over the cryo-plates to be loaded in a solution of 0.4 M sucrose and 2 M glycerol for 30 min and desiccated by exposure to SG for 150 min before cooling. D-Cp procedure also allowed enhancing survival (33% vs 10%) in comparison with the other protocols tested. By contrast, vanilla root-tips subjected to same cryogenic approaches only tolerated the osmotic dehydration with the less toxic vitrification solution (PVS3) using D-V procedure. These results corroborate that the size and morphology of explants influence the optimization of cryoprotective conditions.

DSC analysis of shoot-tips, subjected to different desiccation times following D-Cp protocol, only detected melt enthalpies regardless the desiccation duration. This indicated that the water content (26% FW) was still high to prevent the crystallisation of residual OAW both during cooling and warming. To extend the exposure periods to SG was detrimental for survival.

Considering that cryopreservation involves different stressing conditions including tissue culture, cryoprotection, freezing, thawing and recovery culture, which are manipulated to enhance survival and regeneration, these stages will have a potential influence on genetic stability. Our studies revealed that both, subculturing number associated to multiplication of donor-plantlets and the composition of vitrification solution (PVS2) used for osmoprotection of vanilla shoot-tips, influenced genetic variability with respect to control; however, the genetic similarity index after the cryopreservation process was over 80% regardless the cryogenic methodology used.

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4

In Vitro Propagation and Germplasm Conservation of Wild Orchids from South America

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Abstract

Orchids are an important part of plant biodiversity on this planet due to their high variability among species and their habitats. South America represents more than thirty percent of all known orchid species, Colombia, Ecuador, Brazil, Peru, and Bolivia being among the richest countries in the world in terms of orchid biodiversity. Nevertheless, concerning the orchid conservation status, in Colombia precisely orchids occupy the unlucky first place as the plant family with the highest number of threatened species. There is a similar situation in the rest of the South American countries. The two main threats to orchid survival are both anthropogenic: the first one is deforestation, and the second largest threat to orchids is collection from the wild. One desirable action to safeguard these endangered species is to develop procedures that make possible their massive propagation, which would provide material for both environmental restoration and commercial purposes avoiding extractions from nature. Likewise, the development of systems that allow the *ex situ* conservation of orchid germplasm is imperative. This chapter reviews the progresses of different *in vitro* approaches for orchid propagation and germplasm conservation, safeguarding the genetic biodiversity of these species. Several study cases are presented and described to exemplify the protocols developed in the Botanical Institute of Northeast (UNNE-CONICET) for propagating and long-term storing the germplasm of

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wild orchids from Argentina (*Cattleya lundii*, *Cohniella cepula*, *C. jonesiana*, *Gomesa bifolia*, *Aa achalensis*, *Cyrtopodium brandonianum*, *C. hatschbachii*, *Habenaria bractescens*). Moreover, it has been attempted to put together most of the available literature on *in vitro* propagation and germplasm conservation for South American orchids using different explants and procedures. There are researches of good scientific quality that even cover critical insights into the physiology and factors affecting growth and development as well as storage of several orchid materials. Moreover, studies are still necessary to cover a major number of South American species as well as the use of selected material (clonal) for both propagation and conservation approaches.

Keywords
Orchid biodiversity · Seed germination · Somatic embryogenesis · Shoot organogenesis · Plant regeneration · Seeds storage · Pollen storage · Cryopreservation

4.1 Introduction

South America is a megadiverse continent in terms of orchid species. Colombia and Ecuador alone, the two richest countries in the world in orchids, add 9000 species (which represent the 30% of all known orchid species). However, in both countries the number of orchid species threatened with extinction may well add 3000 species, a figure quite worrying when we consider that the main cause of this extinction is deforestation of the Andean forests. Precisely, the forests of these mountains provide most of the water resources for the large cities and their agriculture and industries (Orejuela Gartner 2010).

Orchids fascinate people more than any other plants do. They were and are the reason for journeys to remote corners of the planet in order to discover new species (Vásquez et al. 2003). Even more, there is a rich history between orchids and people in many cultures across the world. Orchids have had many practical uses, but they have also had a unique allure based on their aesthetic appeal. The first known documentation of the appreciation and use of orchids dates back to Confucius (551–479 B.C.). Since then, these plants have been used as a source of food, medicines, ornamentals, flavoring (vanilla), teas, charms, aphrodisiacs, ingredients in magic, to promote or retard fertility, for clothing, art, poisons, narcotics, and religious ceremonies (Cuoco and Cronan 2009; Koopowitz 2001). Hence, the significance of orchids in human life cannot be underestimated.

Due to the high specificity for insect pollinators, minute seeds without endosperm and a unique life cycle requiring an association with specific mycorrhizal fungi during the early stages of development, orchids are vulnerable to minor biotic and abiotic changes (Popova et al. 2016). Thus, widespread degradation of ecosystems (e.g., as a result of an increased use of weed killers and artificial fertilizers, deforestation, and land clearance) has imperiled orchids in their natural habitat (Farrell and Fitzgerald 1989; Kandavel et al. 2004; Swarts and Dixon 2009; Wood

1989). Moreover, global warming is predicted to produce irreversible changes in orchid communities (Seaton et al. 2010).

Internationally, the importance of conserving orchids has been recognized since the 1970s when orchids were listed in CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora). While CITES originally focused on gathering data on the international trade of animals, the design shifted to include the preservation of both flora and fauna, partly in reaction to a perceived increase in the illicit trade of plants (Koopowitz 2001). At present, orchids figure prominently in the Red Data Book prepared by International Union of Conservation of Nature and Natural Resources (IUCN). In fact, the entire family is now listed under Appendix I or II of the CITES (http://www.cites.org). As palliative action, governments from many countries have established biosphere reserves, national parks, and sanctuaries in the orchid-rich regions besides banning the export of orchids collected in wild. Unfortunately, *in situ* conservation is not always a viable option because of reasons like fragmented habitats, absence of pollinators due to indiscriminate use of pesticides, or other modifications of the biome etc. (Chugh et al. 2009).

Despite having a large number of orchid species and diversified agroclimatic conditions, the orchid industry has not been developed in South America although it has great potential. Hence, an efficient strategy needs to be designed not only to save these beautiful members of the plant kingdom but also to harness the economic potential by scientific and judicious management. This requires a complex integration of preserving natural habitats (*in situ* conservation), developing and applying *ex situ* conservation methodologies, it being necessary to standardize commercial-scale micropropagation techniques for the production of quality planting material of important, rare, endangered, threatened, as well as exotic hybrid orchids. Moreover, deeper insights of orchid biology, evolution, and ecology are needed, as well as a better understanding of the orchid trade and horticultural practices (Popova et al. 2016).

The aim of this chapter is to review and provide information about the available methods for the *in vitro* propagation and germplasm conservation of wild orchids from South America. Several study cases are presenting to illustrate the development of these biotechnological approaches in the Botanical Institute of Northeast (IBONE), UNNE-CONICET (Argentina).

4.2 Biodiversity of South American Orchids

Orchids are an important part of plant biodiversity on this planet due to their high variability among species and their habitats. The highest diversity of orchid species has been found in the Andes of Colombia and Ecuador, tropical rainforests of Borneo, Sumatra, New Guinea, and Madagascar (Cribb et al. 2003; Swarts and Dixon 2009). Every year, botanists discover over a hundred new orchid species (e.g., Carnevali et al. 2014; Kolanowska 2015; Noguera-Savelli et al. 2008; Vale et al. 2014). Clearly, our knowledge of orchid genetic diversity is fairly incomplete, and there is the prospect that many orchid species may be lost before their discovery.

Concerning South America, it presents more than 30% of all known orchid species. Colombia and Ecuador are the richest countries in the world in terms of orchid biodiversity. In Colombia, it has been cited about 3500 to 4270 species, of which 1572 (37%) are endemic (Jørgensen et al. 2011; Ministerio de Ambiente y Desarrollo Sostenible y Universidad Nacional de Colombia 2015), while Ecuador has identified 4032 orchid species, of which 1714 (43%) are endemic (Cerna et al. 2016). Likewise, Brazil with 2548 recorded orchid species (Zappi et al. 2015) and Peru with 2020–3500 orchid species (Perú Ministerio del Ambiente 2015; Roque and León 2006) are known among the countries with more orchid megadiversity in the world. Concerning Bolivia, until very recent times it was rather underestimated and neglected with regard to orchid diversity; however, according to current estimates (considering the currently known species number, the discovery rates and the dimensions of the unsampled areas) there are about 2000–3000 orchid species in the country (Vásquez et al. 2003). Thus, Bolivia's orchid diversity could become comparable to more northern Andean countries like Peru and Ecuador where much more effort has been spent on orchid inventory. Similarly, in Venezuela the Orchidaceae is one of the best represented plant families with 1506 species. Detailed floristics and inventories, however, are needed for different unexplored and underexplored areas of the national parks of Venezuela (Noguera-Savelli et al. 2015). On the other hand, for countries of the Southern Cone of South America (Argentina, Chile, Paraguay, Uruguay, and Southern Brazil) 1042 orchid species have been identified, of which 294 are endemic (Zuloaga and Belgrano 2015), while 760 species were recorded for Guyana, Suriname, and French Guiana (Funk et al. 2007) and approximately 200 species have been reported from Trinidad and Tobago (Kenny 2008).

Regarding the orchid conservation status, unfortunately Colombia occupies the first position as the plant family with the highest number of threatened species (Calderón-Sáenz 2007). Colombia contains two recognized biodiversity hotspots, the Northern Andes and the Tumbes-Chocó-Magdalena (Myers et al. 2000), of which the Chocó region on the Pacific coast harbors unparalleled plant biodiversity (Gentry 1986). All of these habitats are under considerable threat of deforestation, due to the continued direct impact of habitat conversion for agriculture and mining (both legal and illegal) as well as the increasingly common forest fires (González et al. 2011). In 2014, the Colombian national deforestation rate registered an increase of 14% compared with the previous year, with a total of 140,356 ha lost. Only 4% of the original cover of tropical dry forest remains in Colombia, and most deforestation are occurring in the Amazon and Andean regions (Cavelier and Etter 1995; Fandiño and Wyngaarden 2005; Pizano and García 2014). In the Chocó biogeographic region along the Pacific littoral, which still retains a high proportion of natural forest habitat, the annual average of deforested area moved from 2874 ha per year 2012–2013 to 24,025 ha in 2015–2016 (IDEAM 2017). In addition to habitat degradation, orchids, being of commercial interest, are subject to the pressure of collection from wild populations (Calderón-Sáenz 2007). Something similar is happening in Ecuador, the second megadiverse country in orchids, which has a great variety of climatic regions that potentiate biodiversity. In Ecuador, the incredible orchid array is also being threatened by land-clearing practices and illicit collection.

This situation is the result of (a) insufficient resources to address conservation needs, (b) weak funding and inter-agency coordination at the government level, (c) poor historic coordination among the large regional network of conservation groups, and (d) a disconnect between conservation agendas and local communities (Cuoco and Cronan 2009). Similarly, this situation is repeated in most of the South American countries.

Thus, the two main threats to orchid survival are both anthropogenic. The first threat is deforestation. Many species of orchids in their natural state require large areas of undisturbed forests to be reproductively successful (Dodson and Escobar 1993). Unfortunately, orchid habitat in most of South America has been destroyed to clear lands for cattle ranching, agriculture, and road building. Collection from the wild is the second largest threat to orchids (Dodson and Escobar 1993). Given the lack of regulation of wild orchid collection and the illicit nature of most collections, it is nearly impossible to determine the volume of orchids that are removed from forests every year (Cuoco and Cronan 2009).

One desirable action to safeguard these endangered species is to develop procedures that make possible their massive propagation, which should provide material for the reinsertion of specimens into their natural habitats, exchange with other entities, supply to orchid merchants to avoid extractions of nature, and the availability of material for future research. Likewise, the development of systems that allow the *ex situ* conservation of orchid germplasm is imperative. *Ex situ* conservation refers to the preservation of plant germplasm outside its natural habitat (Heywood and Iriondo 2003). This involves different methods, some of which are classified as dynamic, like botanic gardens and field genebanks, because both provide the opportunity of monitoring the evolutionary trajectory of samples during the storage. Other *ex situ* methods are classified as static, because they safeguard the genes outside of the evolutionary context (Shands 1991). They are considered safest, uninfluenced by the climate conditions, and more cost-effective than dynamic methods. Some alternatives for *ex situ* conservation are the seed banks and the storage of different plant materials in liquid nitrogen (LN, −196 °C).

4.3 *In Vitro* Propagation Approaches in Orchids

4.3.1 *In Vitro* Seed Germination

One of the most significant applications of biotechnology to orchid diversity conservation and sustainable use is *in vitro* germination (Popova et al. 2016). Orchid seeds (which are produced in large numbers in each capsule) are very small and lack food reserves (Heywood et al. 2007; Mitra 1971; Paudel et al. 2012). In nature, orchid seeds germinate in association with specific mycorrhizal fungi (symbiotic germination) that induce germination, promote embryo growth, and supply the required nutrients to the embryo (Rasmussen et al. 2015; Valadares et al. 2012). Thus, propagating orchids through seeds may require the presence of specific fungi or specific culture media which can replace the nutrient supply given by such fungi (Otero Ospina and Bayman 2009).

Development of asymbiotic germination methods (i.e., without fungal inoculation) of orchid seeds took place following the formulation of Knudson B and C medium (Knudson 1922, 1946). Thereafter, other culture media with varied carbon sources have been tried for orchid germination such as Vacin and Went medium (VW; Vacin and Went 1949), Murashige and Skoog medium (MS; Murashige and Skoog 1962), Mitra medium (M; Mitra et al. 1976), Malmgren ModifiedTerrestrial Orchid Medium (MM; Malmgren 1996), banana culture medium (CMB; Barbery and Molares 2011), tomato culture medium (CMT; Barbery and Molares 2011), and PDA medium (potato dextrose agar), among others (Hossain et al. 2010; Paul et al. 2011; Pedroso-de-Moraes et al. 2012; Pedroza-Manrique and Mican-Gutiérrez 2006; Pereira et al. 2015, 2017; Piri et al. 2013; Roy et al. 2011; Wida Utami et al. 2017). These media can have different results depending on the orchid species, suggesting intrinsic differences in nutrient requirements for different species. It has also been suggested that particular species may have different limiting factors for germination and seedling early growth (Rasmussen et al. 2015). For example, mature seeds of some orchid species require several months of cold stratification before germination (Nikishina et al. 2007), and the whole process of embryo development to plantlet formation can take months to years, particularly for temperate species (Nikishina et al. 2001; Oliva and Arditti 1984). Likewise, mature seeds of *Vanilla* species require chemical scarification before culture for asymbiotic germination, since they have hard coats (Pedroso-de-Moraes et al. 2012). For such species, immature seeds that readily germinate after sowing are considered as primary material for the initiation of tissue cultures (Hirano et al. 2005a, b; Nikishina et al. 2007; Palama et al. 2010; Ramírez-Mosqueda and Iglesias-Andreu 2015). However, based on an understanding of dry seed storage of other species, such immature seeds may not be optimal for long-term storage (Popova et al. 2016).

Asymbiotic germination can be more effective than symbiotic germination for several reasons: (1) it does not require the isolation and identification of the mycobiont of the target orchid species; (2) it allows for more controlled, aseptic conditions, resulting in less overall contamination (Abraham et al. 2012; Aggarwal and Zettler 2010; Johnson et al. 2007); (3) in symbiotic germination, the seeds inoculated with the mycorrhiza fungi can be completely covered with the fungal hyphae, making difficult the evaluation of germination and embryo development, sometimes requiring a change in the culture media, increasing the risk of contamination (Pereira et al. 2017). However, it is likely that further plant development and/or reintroduction of plants in the field could require fungal association to enhance survival (Otero Ospina and Bayman 2009; Pereira et al. 2015; Thakur and Dongarwar 2013). Anyway, both symbiotic and asymbiotic seed germination can be very helpful for the conservation of rare or endangered species, since orchids produce a large amount of seeds and high genetic variation is preserved, compared to cloning techniques.

During the last 20 years, tissue culture techniques have been extensively used for rapid and large-scale propagation of several South American orchids by both asymbiotic and symbiotic seed germination (see Table 4.1).

Table 4.1 Representative examples of *in vitro* propagation for South American orchids

Species	Tissues	Measured parameters	References
Aa achalensis[a]	Seeds	Symbiotic seed germination.	Fracchia et al. (2014a)
Anathallis adenochila[b]	Seedlings[d]	*In vitro* seedling survival and growth.	Endres Júnior et al. (2014)
Barbosella sp.[b]	Seeds	Asymbiotic seed germination and *in vitro* seedling development.	Roberts et al. (2007)
Bipinnula fimbriata[a]	Seeds	Symbiotic seed germination.	Steinfort et al. (2010)
	Seeds	Seed viability and asymbiotic germination, seedling development.	Pereira et al. (2015)
Brachionidium muscosum[b]	Seeds	Asymbiotic seed germination and *in vitro* seedling development.	Roberts et al. (2007)
Brasilidium forbesii (=*Oncidium forbesii*)[b]	Thin cell layers of protocorms[d]	Protocorm Like Bodies (PLBs) regeneration.	Pereira Gomes et al. (2015)
Brassavola perrinii[b]	Seedlings[d]	Multiplication rate and seedling growth.	Pasqual et al. (2011)
Brassavola, *Cattleya* and *Laelia* species double hybrid ("BCL Pastoral Innocence")[b,c]	Seedlings[d]	Seedling growth.	Prizão et al. (2012)
Brassia bidens[a]	Seeds	Asymbiotic seed germination and *in vitro* seedling development.	Monteiro do Rêgo et al. (2009)
Brassocattleya "Pastoral"[b]	Seedlings[d]	Multiplication rate, height increase, and sugar content reduction.	Cardoso and Ono (2011)
Cattleya bicolor[b,c]	Seedlings[d]	Seedling growth.	Prizão et al. (2012)
C. cinnabarina (=*Hoffmannseggella cinnabarina*)[c]	Seeds	Asymbiotic seed germination and *in vitro* seedling development.	Suzuki et al. (2012)
C. flava (=*Laelia flava*)[b]	Seeds	Seedling survival and growth.	Moraes et al. (2005)
C. intermedia[b]	Seedlings[d]	Seedling growth during *ex vitro* acclimation.	Schnitzer et al. (2010)
	Seedlings[d]	Seedling survival and growth during *ex vitro* acclimation.	Dorneles and Trevelin (2011)
	Seeds	*In vitro* seedling survival and growth.	Sasamori et al. (2015)
C. intermedia x *C. purpurata* (=*Hadrolaelia purpurata*)[b]	Seedlings[d]	Seedling growth during *ex vitro* acclimation.	Lone et al. (2010)

(continued)

Table 4.1 (continued)

Species	Tissues	Measured parameters	References
C. jenmanii[b]	Seedlings[d]	Anatomical characters in response to culture condition.	Torres and Sanabria (2011)
C. loddigesii[b,c]	Seeds	*In vitro* seedling growth, plant survival, and growth during *ex vitro* acclimation.	Galdiano Júnior et al. (2012a)
	Seedlings[d]	Seedling growth.	Gomes de Araújo et al. (2009)
C. longipes (=*Laelia longipes*)[b,c]	Seedlings[d]	Seedling growth.	Stancato et al. (2008)
C. lueddemanniana[b]	Seedlings[d]	Anatomical characters in response to culture condition.	Torres and Sanabria (2011)
C. lundii (=*Microlaelia lundii*)[b,c]	Seedlings[d]	*In vitro* seedling growth and chlorophyll content. Seedling growth during *ex vitro* acclimation.	Favetta et al. (2017)
C. maxima[b]	Seedlings[d]	Somatic embryogenesis induction.	Cueva-Agila et al. (2013)
	Leaves of *in vitro* seedlings[d]	Somatic embryos induction, genetic expression analysis.	Cueva-Agila et al. (2015)
C. mendelii[b,c]	Seeds	Asymbiotic seed germination and *in vitro* seedling development.	Díaz-Álvarez et al. (2015)
C. purpurata (=*Hadrolaelia purpurata*)[b]	Seeds	Asymbiotic seed germination and *in vitro* seedling development.	De Menezes Gonçalves et al. (2012)
C. purpurata (=*Laelia purpurata*) *var. carnea*[b]	Seedlings[d]	Anatomical analysis of leaves and roots.	Da Silva Júnior et al. (2012)
	Seedlings[d]	*In vitro* seedling growth, root anatomical changes, and chlorophyll content.	Da Silva Júnior et al. (2013)
C. quadricolor[b]	Seeds	Asymbiotic seed germination and *in vitro* seedling development.	Díaz-Álvarez et al. (2015)
C. tigrina[b]	Leaves of *in vitro* seedlings[d]	DNA methylation and endogenous polyamine levels during PLBs induction.	Almeida et al. (2017)
C. trianae[b]	Seedlings[d]	*Ex vitro* seedling survival and growth.	Franco et al. (2007)
	Seedlings[d]	Seedling growth.	Galdiano Júnior et al. (2012b)

(continued)

Table 4.1 (continued)

Species	Tissues	Measured parameters	References
C. violacea[b]	Seeds	Asymbiotic seed germination and *in vitro* seedling development.	Galdiano Júnior et al. (2013)
C. walkeriana[b]	Seedlings[d]	Seedling growth.	Dignart et al. (2009)
	Seedlings[d]	Seedling survival and growth during *ex vitro* acclimation.	Galdiano Júnior et al. (2011)
	Seeds	Seed germination morphologic analysis and seedling growth.	Galdiano Júnior et al. (2014)
Chloraea crispa[a]	Seeds	Asymbiotic seed germination and *in vitro* seedling development.	Pereira et al. (2017)
	Seeds	Asymbiotic seed germination and embryogenic calli formation.	Quiroz et al. (2017)
C. gavilu[a]	Seeds	Asymbiotic seed germination and *in vitro* seedling development.	Pereira et al. (2017)
	Seeds, protocorms, thin cell layers from protocorms and seedling leaves[d]	Asymbiotic seed germination, *in vitro* seedling development, PLB induction.	Romero et al. (2018)
C. riojana[a]	Seeds	Symbiotic seed germination.	Fracchia et al. (2016)
C. virescens[a]	Seeds	Asymbiotic seed germination and *in vitro* seedling development.	Pereira et al. (2017)
Comparettia falcata[b]	Seeds	Asymbiotic seed germination and *in vitro* seedling development.	Pedroza-Manrique et al. (2005)
	Seeds	Symbiotic and asymbiotic seed germination.	Chávez et al. (2014)
Cyrtochilum aureum (=*Odontoglossum aureum*)[b]	Seeds	Asymbiotic seed germination and *in vitro* seedling development.	Roberts et al. (2007)
C. caespitosum (=*Rusbyella caespitose*)[b]	Seeds	Asymbiotic seed germination and *in vitro* seedling development.	Roberts et al. (2007)
C. loxense[b]	Seedlings[d]	Somatic embryogenesis receptor-like kinase (SERK) expression analysis.	Cueva-Agila et al. (2012)

(continued)

Table 4.1 (continued)

Species	Tissues	Measured parameters	References
Cyrtopodium brandonianum[a]	Root-tips from *in vitro* seedlings[d]	Adventitious shoot organogenesis.	Flachsland et al. (2011)
C. glutiniferum[a,c]	Seeds	Asymbiotic seed germination and *in vitro* seedling development.	Vogel and Macedo (2011)
	Seeds	Symbiotic seed germination and *in vitro* seedling development, *ex vitro* growth.	Rodrigues Guimarães et al. (2013)
	Seeds	Symbiotic seed germination, *in vitro* seedling development, fungal colonization.	Corrêa Pereira et al. (2015)
C. paludicolum[a]	Seeds	Symbiotic and asymbiotic seed germination.	De Carvalho et al. (2018)
C. saintlegerianum[b]	Seeds and seedlings[d]	Asymbiotic seed germination and *in vitro* seedling development, plant growth during *ex vitro* acclimation.	Rodrigues et al. (2015)
Encyclia cordigera[b]	Seedlings[d]	*In vitro* seedling growth.	Mantovani and Pivetta (2016)
E. microtos[b]	Seedlings[d]	*In vitro* seedling development and multiplication.	Condemarín-Montealegre et al. (2007)
E. randii[b]	Seeds	Asymbiotic seed germination and *in vitro* seedling development.	Gonçalves et al. (2012)
Epidendrum cardenasii[b]	Seeds	Asymbiotic seed germination and *in vitro* seedling development.	Roberts et al. (2007)
E. ibaguense[b]	Nodal segments of field-grown plants	Contamination control and shoot growth.	Rodrigues et al. (2013)
E. nocturnum[b]	Seeds and seedlings[d]	*In vitro* seedling growth with and without michorrization.	Sousa Silva et al. (2016)
E. secundum[b]	Seeds	Symbiotic seed germination and *in vitro* seedling development.	Corrêa Pereira et al. (2011)
	Seeds	Seedling growth.	Massaro et al. (2012)
Gavilea australis[a]	Seeds	Symbiotic seed germination.	Fracchia et al. (2014b)
Gomesa flexuosa (=*Oncidium flexuosum*)[b]	Seedlings[d]	*In vitro* seedling growth.	Caovila et al. (2016)

(continued)

Table 4.1 (continued)

Species	Tissues	Measured parameters	References
Gongora quinquenervis[a]	Seeds	*In vitro* seedling development.	Cavalcante Martini et al. (2001)
Habenaria bractescens[a]	Multinodal segment from *in vitro* seedlings[d]	Upright leafy shoots, swollen buds, and root tubers.	Medina et al. (2009)
Laeliocattleya x *Brassolaeliocattleya* hibryd[b]	Seedlings[d]	Multiplication rate and seedling growth.	Pasqual et al. (2011)
Laeliocattleya hybrid (*Hadrolaelia purpurata* x *Cattleya intermedia*)[b]	Seedlings[d]	*In vitro* seedling growth	De Menezes Gonçalves et al. (2016)
Masdevallia yungasensis[b]	Seeds	Asymbiotic seed germination and *in vitro* seedling development.	Roberts et al. (2007)
Miltonia clowesii[b]	Seedlings[d]	Seedling growth during *ex vitro* acclimation.	Schnitzer et al. (2010)
M. flavescens[b]	Seeds	Seedling survival and growth.	Moraes et al. (2005)
M. spectabilis[b]	Seedlings[d]	Seedling growth.	Stancato et al. (2008)
Odontoglossum gloriosum[b]	Seeds	Asymbiotic seed germination.	Pedroza-Manrique and Mican-Gutiérrez (2006)
Oncidesa (=*Oncidium*) "Gower Ramsey"[b]	Floral buds of inflorescence from *ex vitro* plant	PLB formation, multiplication, and plantlet regeneration.	Santana and Chaparro (1999)
Oncidium baueri[b]	Seedlings[d]	Seedling growth.	Sorace et al. (2008)
O. leucochilum[b]	Shoots[d]	*In vitro* multiplication, elongation, and rooting; *ex vitro* acclimation.	Da Silva et al. (2014)
O. trulliferum[b]	Seeds	Seedling survival and growth.	Moraes et al. (2005)
Schomburgkia crispa[b,c]	Nodal segments from *in vitro* plants	*In vitro* multiplication and rooting.	Pereira et al. (2018)
S. gloriosa[b]	Seeds	Seedling growth.	Dezan et al. (2012)
Sophronitis tenebrosa (=*Laelia tenebrosa*)[b]	Seedlings[d]	Seedling growth.	Stancato et al. (2008)
Telipogon sp.[b]	Seeds	Asymbiotic seed germination and *in vitro* seedling development.	Roberts et al. (2007)
Vanilla planifolia[a,b]	Seeds	Effect of scarification on asymbiotic seed germination and *in vitro* seedling development.	Pedroso-de-Moraes et al. (2012)

[a]Terrestrial, [b]Epiphyte, [c]Lithophyte, [d]Seed-derived material

4.3.2 Clonal Propagation Using Various Explants

In vitro propagation using seedlings is less desirable especially for horticultural uses due to the long juvenile period before flowering (Decruse et al. 2003). Moreover, as orchids are outbreeders, their propagation using seeds leads to the production of heterozygous plants. Hence, protocols providing regeneration from various vegetative parts of mature plants are essential.

In 1949, Rotor at Cornell University demonstrated that plantlets could be induced by aseptic culturing of the dormant buds on the basal nodes of *Phalaenopsis* inflorescence (Arditti and Krikorian 1996). Thereafter, Morel (1960) cultured shoot tips for obtaining virus-free *Cymbidium* clones *in vitro*. Many efforts have been made since then for rapid clonal propagation using somatic explants from several orchid species, including shoot tips (Geetha and Shetty 2000; Kalimuthu et al. 2006; Ket et al. 2004; Seeni and Latha 2000; Sharma and Tandon 1992;Sheela et al. 2004), inflorescence explants, shoot apex, flower stalk nodes, floret tissues (Chen and Chang 2000; Chen et al. 2002; Goh and Wong 1990; Intuwong and Sagawa 1973; Mitsukuri et al. 2009; Shimasaki and Uemoto 1991; Vendrame et al. 2007a), leaf explants (Goh and Tan 1979; Janarthanam and Seshadri 2008; Sharma and Vij 1997; Teng et al. 1997; Vij and Kaur 1999), and rhizome segments (Shimasaki and Uemoto 1990). The major advantage of clonal propagation is that the plantlets produced are usually identical to their parents (clones). This is of great advantage to the cut-flower industry in production of uniform blossoms during predictable periods to meet market demands (Chugh et al. 2009).

Concerning South American orchids, protocols for clonal large-scale propagation have been reported for *Epidendrum ibaguense* and *Oncidesa* "Gower Ramsey" (the most cultivated orchid hybrid for cut flower) through *in vitro* culture of nodal segments and inflorescence explants of field-grown adult plants, respectively (Rodrigues et al. 2013; Santana and Chaparro 1999; Table 4.1).

4.4 *In vitro* Conservation Approaches in Orchids

All types of *in vitro*-cultured materials that have been used for mass rapid propagation of orchids may be also utilized for conservation purposes (Popova et al. 2016). For example, over 90% of seed-derived *in vitro* seedlings of *Dendrobium officinale* tolerated 12 months of storage at 4 °C in darkness without subculture (Shi et al. 2000). Likewise, *in vitro* plantlets of *Dendrobium draconis* and *Ipsea malabarica* maintained high viability during storage at 25 °C for 6 and 27 months, respectively (Martin and Pradeep 2003; Rangsayatorn et al. 2009). More recently, *in vitro* slow growth techniques and storage at low positive temperatures (from 0 to 16 °C) have proved to be effective for some *Dendrobium* species (Teixeira da Silva et al. 2014). However, short- to medium-term *in vitro* conservation is relatively labor intensive and costly; moreover, phenotypical and genetic variations in the course of repeated subcultures have been documented for orchid materials (Arditti 2008; Khoddamzadeh et al. 2010; Teixeira da Silva et al. 2014; Tokuhara and Mii 1998).

These limitations have promoted the development of less expensive and more reliable conservation methods such as cryopreservation, which allows safe and long-term storage of orchid germplasm once an appropriate protocol is designed and validated for each genotype (Popova et al. 2016).

Cryopreservation, that is, storage of samples at ultralow temperature of LN (–196 °C), has become the most important tool to modern science for the long-term storage of plant materials since it provides the possibility of significantly extending the storage period with the maximum genetic stability. At this temperature, all cellular divisions and metabolic processes are stopped. The plant material can thus be stored without alteration or modification for a theoretically unlimited period of time (Ashmore 1997; Engelmann 2011). However, cryopreservation presents a series of problems mainly associated with the initial moisture content (MC) of sample and the alterations to which the material is subjected during the process of cooling/rewarming. Both factors should be evaluated for each biological material before using any cryopreservation method. The MC of tissues is the most critical factor for successful cryopreservation (Vertucci and Roos 1993). Optimal survival is generally obtained when samples are frozen with an MC comprised between 10% and 20% (fresh weight basis) (Engelmann 2011). Likewise, the use of proper cryoprotectants [glycerol, ethylene glycol, dimethyl sulfoxide (DMSO), and plant vitrification solutions (PVS)] can increase the success of cryopreservation protocols by suppressing ice crystallization during cooling to and warming from LN (Sakai et al. 1990).

Current work in cryopreservation has permitted the storage of virtually all explant types for many plant species (Ashmore et al. 2011; Engelmann 2004, 2011; González-Arnao and Engelmann 2006; González-Arnao et al. 2008, 2017; Panis and Lambardi 2005; Uragami 1993). In orchids, cryopreservation has been an efficient means of conserving seeds and pollen (e.g., Dolce and González-Arnao 2019; Dolce et al. 2016; Surenciski et al. 2012; Hay et al. 2010; Vendrame et al. 2007b, 2008; Mweetwa et al. 2007; Flachsland et al. 2006; Popov et al. 2004; Koopowitz and Thornhill, 1994; Koopowitz 1986; Pritchard 1984). By contrast, attempts to cryopreserve somatic explants (i.e., clonal material) are scarce and have resulted in variable regrowth (Dolce et al. 2018; González-Arnao et al. 2009; Hernández-Ramírez et al. 2014; Kondo et al. 2001; Lurswijidjarus and Thammasiri 2004; Na and Kondo 1996; Thinh and Takagi 2000; Tsukazaki et al. 2000).

Concerning to South American orchids, till date researches has been reported for the *ex situ* germplasm conservation for 30 species (Table 4.2). This is quite worrying when we consider the orchid megadiversity that occurs in South America (more than 10,000 species) and the orchid conservation status in the continent.

Modern propagation and production technology has made orchids accessible to a much broader section of the society. Cost-efficient protocols for mass propagation of rare, threatened, and endangered orchids, as well as new orchid hybrids, have to be developed further in order to commercialize and conserve them. For this, critical insights into the physiology and factors affecting growth and development of orchids are essential. The floriculture industry as well as conservation efforts would get a huge boost if the protocols developed in the laboratories and these could be

Table 4.2 Conservation methods for South American orchids

Species	Tissues	Conservation method	References
Acianthera glumacea (=*Pleurothallis glumacea*)[b]	Seeds	Storage at 5 °C with 6% MC.	Alvarez-Pardo and Ferreira (2006)
Bifrenaria inodora[b]	Seeds	Storage at 5 °C with 6% MC.	Alvarez-Pardo and Ferreira (2006)
Catasetum atratum[b]	Seeds	Cryostorage by vitrification technique with PVS2 solution.	Suzuki et al. (2018)
Cattleya bicolor[b,c]	Seeds	Storage at 5 °C with 6% MC.	Alvarez-Pardo and Ferreira (2006)
	Seeds	Storage at 10 and 25 °C over silica gel.	Mora et al. (2008)
C. grandis (=*Hadrolaelia grandis*)[b]	Seeds	Storage at −20 and −80 °C.	Vudala and Ribas (2017)
C. granulosa[b]	Seeds	Storage at −18 °C.	Hosomi et al. (2012)
C. hegeriana[c]	Seeds	Storage at −18 °C.	Hosomi et al. (2012)
C. intermedia[b]	Seeds	Storage at −18, 5, and 25 °C, seeds with 6% MC.	Alvarez-Pardo et al. (2006)
	Seeds	Storage at 5 °C with 6% MC.	Alvarez-Pardo and Ferreira (2006)
	Seeds	Storage at −18 °C.	Hosomi et al. (2012)
C. intermedia var. *pallida*[b]	Seeds	Storage at 5 °C with 6% MC.	Alvarez-Pardo and Ferreira (2006)
C. labiata[b]	Seeds	Storage at 5 °C with 6% MC.	Alvarez-Pardo and Ferreira (2006)
C. mossiae[b]	Seeds	Storage at −18 °C.	Hosomi et al. (2012)
C. purpurata	Seeds	Storage at −18 °C.	Hosomi et al. (2012)
(=*Hadrolaelia purpurata* and *Laelia purpurata*)[b]	Seeds	Storage at 5 °C with 6% MC.	Alvarez-Pardo and Ferreira (2006)
C. sanguiloba (=*Laelia sanguiloba*)[c]	Seeds	Storage at −18 °C.	Hosomi et al. (2012)
C. tenuis[b]	Seeds	Storage at −18 °C.	Hosomi et al. (2012)
C. tigrina[b]	Seeds	Storage at −18 °C.	Hosomi et al. (2012)
C. walkeriana[b]	Seeds	Storage at −18 °C.	Hosomi et al. (2012)

(continued)

Table 4.2 (continued)

Species	Tissues	Conservation method	References
	Seeds	Cryostorage by vitrification method with PVS2 solution.	Galdiano Júnior et al. (2017)
Cohniella cepula[b]	Pollinia	Storage at −70 and −196 °C without pre-treatment.	Dolce et al. (2016)
	Seeds	Cryostorage without seed pre-treatment.	Dolce and González-Arnao (2019)
Cyrtopodium hatschbachii[b,c]	Immature seeds	Cryostorage by encapsulation-dehydration technique.	Surenciski et al. (2012)
Encyclia odoratissima[b]	Seeds	Storage at 5 °C with 6% MC.	Alvarez-Pardo and Ferreira (2006)
Encyclia pygmaea[b]	Seeds	Storage at 5 °C with 6% MC.	Alvarez-Pardo and Ferreira (2006)
Epidendrum anderssonii[b]	Seeds	Cryostorage by vitrification method.	Cerna et al. (2018)
E. fulgens[a,b]	Seeds	Storage at 5 °C with 6% MC.	Alvarez-Pardo and Ferreira (2006)
E. quitensium[b]	Seeds	Cryostorage by vitrification method.	Cerna et al. (2018)
Gomesa bifolia (=*Oncidium bifolium*)[b]	Seeds and protocorms	Cryostorage by encapsulation-dehydration technique.	Flachsland et al. (2006)
G. flexuosa (=*Oncidium flexuosum*)[b]	Seeds	Storage at 5 °C with 6% MC.	Alvarez-Pardo and Ferreira (2006)
Grobya sp.[b]	Seeds	Storage at 5 °C with 6% MC.	Alvarez-Pardo and Ferreira (2006)
Laeliocattleya natural hybrid[b]	Seeds	Storage at 5 °C with 6% MC.	Alvarez-Pardo and Ferreira (2006)
Maxillaria picta[b]	Seeds	Storage at 5 °C with 6% MC.	Alvarez-Pardo and Ferreira (2006)
Oncidium enderianum[b]	Seeds	Storage at 5 °C with 6% MC.	Alvarez-Pardo and Ferreira (2006)
Sobralia rosea[a]	Seeds	Cryostorage by vitrification method.	Cerna et al. (2018)
Trichocentrum pumilum (=*Oncidium pumilum*)[b]	Seeds	Storage at 5 °C with 6% MC.	Alvarez-Pardo and Ferreira (2006)

[a]Terrestrial, [b]Epiphyte, [c]Lithophyte

further standardized and transferred to industries and organizations involved with *ex situ* conservation of this alluring flower (Chugh et al. 2009).

Table 4.3 summarizes information about scientific works recorded so far for different South American countries regarding *in vitro* propagation and germplasm conservation of native orchids from this continent, according to the Scopus database (key words: "propagation AND orchid," "conservation AND orchid," "cryopreservation AND orchid"). It could be noted that Brazil has the highest number of published works both on propagation and germplasm conservation, while for some countries only researches referring to propagation of native orchid species are reported and for other countries (Guyana, Paraguay, Trinidad and Tobago, Suriname, Uruguay) there were no registered works in any of the topics.

Table 4.3 Scientific articles published by South American countries (according to Scopus database, at 11-01-2018)

Country	Topic	Number of scientific articles	References
Argentina	Propagation	5	Flachsland et al. (2011), Fracchia et al. (2014a,b, 2016), Medina et al. (2009)
	Conservation	5	Dolce et al. (2016), Duarte et al. (2017), Flachsland et al. (2006), Surenciski et al. (2007, 2012)
Bolivia	Propagation	1	Roberts et al. (2007)
	Conservation	0	
Brazil	Propagation	48	Almeida et al. (2017), Caovila et al. (2016), Cardoso and Ono (2011), Cavalcante Martini et al. (2001), Corrêa Pereira et al. (2011, 2015), Da Silva et al. (2014), Da Silva Júnior et al. (2012), 2013, De Carvalho et al. (2018), De Conti et al. (2018), De Melo Ferreira et al. (2017), De Menezes Gonçalves et al. (2012, 2016), Dezan et al. (2012), Dignart et al. (2009), Dorneles and Trevelin (2011), Endres Júnior et al. (2014), Favetta et al. (2017), Galdiano Júnior et al. (2011, 2012a,b, 2013, 2014), Gomes de Araújo et al. (2009), Hosomi et al. (2017), Lando et al. (2016), Lone et al. (2010), Mantovani and Pivetta (2016), Massaro et al. (2012), Monteiro do Rêgo et al. (2009), Moraes et al. (2005), Pasqual et al. (2011), Pedroso-de-Moraes et al. (2012), Pereira et al. (2018), Pereira Gomes et al. (2015), Prizão et al. (2012), Rodrigues et al. (2013), Rodrigues et al. (2015), Rodrigues Guimarães et al. (2013), Sasamori et al. (2015), Schnitzer et al. (2010), Sorace et al. (2008), Sousa Silva et al. (2016), Stancato et al. (2008), Suzuki et al. (2012), Villa et al. (2014), Vogel and Macedo (2011)

(continued)

Table 4.3 (continued)

Country	Topic	Number of scientific articles	References
	Conservation	7	Alvarez-Pardo and Ferreira (2006), Galdiano Júnior et al. (2017), Hosomi et al. (2011, 2012), Mora et al. (2008), Suzuki et al. (2018), Vudala and Ribas (2017)
Chile	Propagation	5	Pereira et al. (2015, 2017), Quiroz et al. (2017), Romero et al. (2018), Steinfort et al. (2010)
	Conservation	0	–
Colombia	Propagation	6	Chávez et al. (2014), Díaz-Álvarez et al. (2015), Franco et al. (2007), Pedroza-Manrique and Mican-Gutiérrez (2006), Pedroza-Manrique et al. (2005), Santana and Chaparro (1999)
	Conservation	1	Flanagan and Mosquera Espinosa (2016)
Ecuador	Propagation	3	Cueva-Agila et al. (2012), (2015)
	Conservation	1	Cerna et al. (2018)
French Guiana, Guyana, and Suriname	Propagation	0	–
	Conservation	0	–
Paraguay	Propagation	0	–
	Conservation	0	–
Peru	Propagation	1	Condemarín-Montealegre et al. (2007)
	Conservation	0	–
Trinidad and Tobago	Propagation	0	–
	Conservation	0	–
Uruguay	Propagation	0	–
	Conservation	0	–
Venezuela	Propagation	1	Torres and Sanabria (2011)
	Conservation	0	–

4.5 *In vitro* Propagation and Germplasm Conservation of Wild Orchids from Argentina, Case Studies

In Argentina, 281 orchid species (18 of them are endemics) were identified and distributed mainly in northern tropical and subtropical regions of the country (Schinini 2008; Zuloaga and Belgrano 2015). The highest diversity of orchid species has been found in Misiones (129 species) and Corrientes (76 species) (Zuloaga et al. 1999). To date, there are no records of extinct orchid species for Argentina; however, 14 species are threatened or in danger of extinction according to the database of plants from Argentina PlanEAr (http://www.lista-planear.org). Likewise, many orchid species have ornamental value both for their colorful flowers and their

vegetative aspect. These species are subject to extractive action by local people, this being the reason why they should be also considered for their conservation.

For the last 20 years, great progress has been made by the IBONE (Corrientes, Argentina) staff toward the development of efficient *in vitro* propagation and germplasm conservation systems for several wild orchids from Argentina. Some of these studies are presented below to illustrate the development of these biotechnological approaches in the IBONE.

4.5.1 Epiphytic Orchids

4.5.1.1 Genus: *Cattleya*

The genus *Cattleya* Lindl. (Subfamily Epidendroideae, Tribe Epidendreae, Subtribe Laeliinae) is one of the largest in the family Orchidaceae. It is a Neotropical genus which comprises 114 species of outstanding horticultural importance (van den Berg 2005, 2008, 2014). *Cattleya* species occur mainly in two distinct regions, such as forests throughout the Brazilian east coast and in the lower part of the humid declivities of the Andean Mountains in Peru, Colombia, Ecuador, and Venezuela, reaching the south of Mexico. These species occupy predominantly epiphytic habitats and most of them show crassulacean acid metabolism (Andrade-Souza et al. 2009). They are of high ornamental value due to the size of their flowers and many species are used for hybridization (van den Berg and Martins 1998; van den Berg et al. 2000). Lately, new combinations and names were proposed in *Cattleya* to accommodate species previously assigned to *Laelia* and *Sophronitis*. These were needed in order to maintain the monophyly of the genus in light of recent phylogenetic hypotheses (van den Berg et al. 2000, 2009). In Argentina *Cattleya* spp such as *C. cernua*, *C. coccinea*, *C. loddigesii*, and *C. Lundii* were recorded.

Cattleya lundii (Rchb. F. & Warm.) Van den Berg is native from Brazil, Bolivia, and Argentina, growing at an altitude of 740 to 1000 m in the coastal mountains or in the Yungas forests. In Argentina, it was found in the province of Misiones, where it grows in flooded regions. It was also cited for the province of Salta (Johnson 2001). *C. lundii* includes epiphytic and lithophytic plants of medium size, with small pseudobulbs that bear two leaves (9–15 cm long). It blooms during the winter and has individual flowers or 2-flower inflorescences. Flowers (38 × 31 mm) are fragrant, erect, resupinate, and pedicelled, with labellum trilobulate, stretched, fleshy, white with violet nerves, and a yellowish base (Johnson 2001). Their flowers remain open for 10–15 days and have good size as well as interesting colors from a commercial point of view, so they can serve as a source for crosses with other species to obtain plants with better appearance and more abundant flowering (Cardoso 2014; Cardoso and Israel 2005).

In the last years, staff of the IBONE has conducted studies aimed at developing efficient plant propagation systems for *C. lundii* through *in vitro* seed germination (unpublished data). Results from these researches are briefly presenting below.

1. *Seed germination*: Fruits (capsules) of 6–9 months after hand-pollination were used for this study, which were surface sterilized and seeds were aseptically removed. A total of 24 culture media were assessed, which were constituted by full- or half-strength MS, EFp (Eduardo Flachsland peptone medium: modified MS according to Eduardo Flachsland, with changes in the macronutrient composition and supplemented with soy peptone), or Hyponex®2 $g.L^{-1}$(a commercial fertilizer formulation 6.5−6−19), alone or supplemented with of 25 $g.L^{-1}$ green banana puree and/or 250 $mg.L^{-1}$ activated charcoal. All media were supplemented with 3% sucrose. Cultures were incubated in a growth room at 27 ± 2 °C with 14-h light/10-h dark photoperiod (116 $\mu mol.m^{-2}.s^{-1}$ Photosynthetic Photon Flux Density (PPFD)).

Asymbiotic seed germination was significantly affected by the different fruit development stages assessed in this study. Seeds from 6- and 7-month-old capsules showed fast oxidation and subsequent death, without allowing plant regeneration. Seeds from 8-month-old fruits displayed high oxidation rate and very low germination percentages (<10%), regardless of the culture media. On the other hand, seeds from 9-month-old capsules showed scarce oxidation and germinated in all the culture media evaluated with percentages varying between 25% and 65% according to the media composition. The onset of germination was observed 2 months after seed sowing. Germinating seeds showed enlargement, changed to a green color, and developed into protocorms. Germination percentage (= seeds that developed protocorms) was determined 5 months after seed sowing. Full-strength MS, EFp, and Hyponex® supplemented with both green banana puree and activated charcoal allowed the significantly highest germination percentages (55–65%). These results demonstrate the promotory effect of the natural additives added to culture media on seed germination of *C. lundii*. A large number of complex additives like peptone, carrot juice, tomato juice, beef extract, potato extract, and especially coconut water, banana extract, etc., are commonly added to orchid media (Chen et al. 2015, Chugh et al. 2009, Vijayakumar et al. 2012). As early as the 1950s, Steward and Simmonds (1954) reported that substances stimulating cell divisions in carrot cells are present in the formative layers of banana fruit. Banana pulp is a rich source of natural cytokinins as well as auxin and gibberellins (Arditti and Ernst 1993; Khalifah 1966; Lahav and Gottreich 1984). On the other hand, the addition of activated charcoal to the medium can help overcome inhibitory effects of phenolics released into the medium and have often been used in orchid media (Chugh et al. 2009). Activated charcoal seems to adsorb the toxic substances that may form in the medium as a result of autoclaving or be released by the explant. It may also stimulate rooting by absorbing the toxins and excluding light from the medium (Paek and Murthy 1977; Yam et al. 1989). Eymar et al. (2000) observed that the addition of activated charcoal increased and maintained pH levels during culture, increased the nitrogen uptake, improved growth and visual aspects of the explants, and reduced the inhibitory effect of exogenous cytokinin on root growth. However, activated carbon is likely to interfere with other additives as well. Therefore, its use should be evaluated for each case.

2. *Seedling growth*: Protocorms were subsequently transferred to fresh media of the same composition to promote seedling growth and well-developed plantlet formation, which is desirable for *ex vitro* acclimation. After additional 14 months of culture, protocorms developed into healthy plantlets with well-formed pseudobulbs, leaves, and roots in all the culture media evaluated. However, the number of shoots, pseudobulbs, leaves, and roots as well as the dry weight of shoots and roots were significantly affected by the media composition. Full-strength MS supplemented with activated charcoal allowed the significantly highest growth rate of seedling, displaying a mean number of 9 shoots, 5 pseudobulbs, 26 leaves, and 24 roots per plant, with a mean dry weight of 117 mg and 103 mg per plant for shoots and roots, respectively. Results from this assay corroborate that activated charcoal is useful for seedling growth of *C. lundii*. On the other hand, banana puree added to the culture media demonstrated an inhibitory effect of on *C. lundii* seedling growth, contrarily what taken place during the germination phase. In other *Cattleya* species was previously reported a promotory effect of banana puree on increase the seedling growth and root number as well as in other orchid genus (Arditti 1968; Lo et al. 2004; Vyas et al. 2009).

Finally, 28 months after the start of the experiment (9 months from hand-pollination to fruit maturation + 5 months for seed germination + 14 months for seedling growth), well-developed plantlets were transplanted to moss and tree bark in plastic pots and successfully transferred to a greenhouse for hardening. This protocol of plant regeneration by asymbiotic seed germination should permit massive propagation and conservation of this species with ornamental value.

4.5.1.2 Genus: *Cohniella*

The genus *Cohniella* Pfitzer (Subfamily Epidendroideae, Tribe Cymbidieae, Subtribe Oncidiinae) is a Neotropical genus of 13 species, which are known in horticulture as the "rat-tail oncidiums." The genus is distributed widely from northern Mexico into southern Brazil and northern Argentina, mostly in the lowlands. It is characterized by medium to large plants with inconspicuous to small pseudobulbs that bear a single, succulent, terete leaf (Carnevali et al. 2010). Two species were cited for Argentina: *C. cepula* and *C. jonesiana*.

- *C. cepula* (Hoffmanns.) Carnevali and G. Romero was found in Argentina, Bolivia, Brazil, Paraguay, and Peru. In Argentina, it is known from the "Norte Grande" region, where it grows in riparian forests at 0–900 m over the sea level. This species includes epiphytic erect herbs, shortly creeping to cespitose, with short, thin rhizome. It blooms from January to May (summer in the southern hemisphere) and has the smallest flowers of the genus (20–24 mm diameter), with yellow labellum, in racemose or paniculate inflorescences (with 6–26 flowers) longer than the leaves (Carnevali et al. 2010; Cetzal-Ix et al. 2012; Insaurralde and Radins 2007).

- *C. jonesiana* (Rchb. f.) Christenson is known from Argentina, Bolivia, Brazil, and Paraguay. Most of the *C. jonesiana* collections are concentrated on the oriental portion of Paraguay, growing epiphytically in gallery forest along the Paraguay River. Similarly, it grows in the northeast portion of the province of Corrientes and Misiones in Argentina, bordering Paraguay. This species includes epiphytic pendent herbs, shortly creeping to cespitose, with short, thin rhizome. It is easily recognized from other *Cohniella* taxa by the combination of a large flower (43–60 mm diameter) with a white central lobe of the labellum that has red spots toward its base, in racemose inflorescences (with 2–10 flowers) which bloom during the summer, shorter than the leaves (Carnevali et al. 2010; Cetzal-Ix et al. 2012; Insaurralde and Radins 2007).

Both species have ornamental value and their natural populations are on decline due to their wild over-collection for marketing and widespread disturbance of their ecosystems. Consequently, it is imperative to develop effective propagation and *ex situ* preservation strategies for these orchids to safeguard the threatened diversity of the genus *Cohniella*, mainly due to the anthropogenic impact. In recent years, staff of the IBONE has made important advances on *in vitro* plant propagation and germplasm conservation of *C. cepula* and *C. jonesiana*. Results from these researches are briefly presented below.

1. *Seed germination*: Asymbiotic seed germination of *C. cepula* and *C. jonesiana* was assessed with the aim of developing efficient propagation systems for these species (unpublished data). The effects of fruit maturity (12–14 weeks after hand-pollination for *C. cepula* and 19–22 weeks after hand-pollination for *C. jonesiana*), the nutritive media composition (full-, half-, or quarter-strength MS medium with 3% sucrose, alone or supplemented with 500 $mg.L^{-1}$ soy peptone, banana powder, and/or activated charcoal; assessing a total of 24 culture media), and light condition during culture incubation (14-h light/10-h dark photoperiod with 116 $\mu mol.m^{-2}.s^{-1}$ PPFD or permanent darkness) on seed germination were evaluated. In all cases, fruits were surface sterilized and seeds aseptically removed previous to sowing on the different culture media.

The onset of germination was observed 15–20 days after seed sowing. Germinating seeds showed enlargement, change to green/white color, and development into protocorms. Germination percentage (= seeds that developed protocorms) was determined 8 weeks after sowing. Seeds of both species germinated in all evaluated conditions; however, the germination percentage was significantly affected by the nutritive media composition and light condition during the incubation. The highest germination percentages were obtained on full-strength MS alone or supplemented with activated charcoal (~80% seed germination for *C. cepula*) and full-strength MS supplemented with soy peptone alone or combined with activated charcoal and/or banana powder (60–65% seed germination for *C. jonesiana*), and

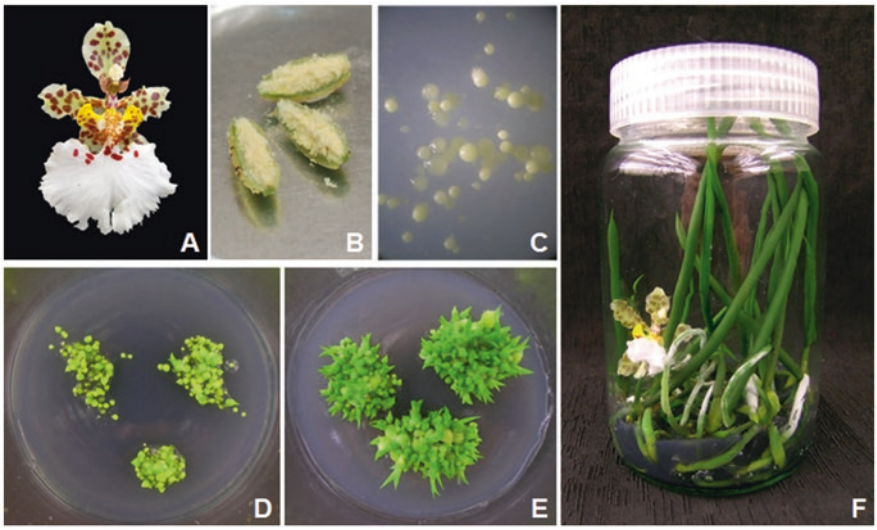

Fig. 4.1 *Cohniella jonesiana* plant propagation through asymbiotic seed germination. (**a**) Flower from the working collection maintained in the greenhouse. (**b**) Fruit at 22 weeks after hand-pollination aseptically opened for removing seed samples. (**c–e**) Protocorms and plantlets obtained by asymbiotic germination 75 days after seed sowing on MS supplemented with activated charcoal (**c**, **d**) and soy peptone (**e**), and incubating the cultures in permanent darkness (**c**) or under 14-h light/10-h dark photoperiod (**d, e**). (**f**) *In vitro* plants of *C. jonesiana* in suitable conditions to be transferred to a greenhouse for hardening

incubating the cultures under 14-h light/10-h dark photoperiod (Fig. 4.1). These results demonstrate again the promotory effect of the natural additives added to culture media (such as activated charcoal and soy peptone) on seed germination of orchid species. The potential effects of activated charcoal were discussed above. Regarding peptone, its promotory effect may be because peptone contains amino acid, protein (Nhut et al. 2008), and vitamin: biotin, pyridoxine, thiamin, and nitrogen (Arditti 1992), and can increase the growth and the development of explants (Dutra et al. 2008). The result from this study is in agreement with Hossain and Dey (2013) who reported that MS, Phytamax, and P723 media containing peptone supported seed germination in *Spathoglotis plicata* better than devoid of peptone. Likewise, the supplementation of peptone in Knudson C and VW basal media promoted seed germination and seedling development in *Vanda belvola* (David et al. 2015) and *D. lasianthera* (Wida Utami et al. 2017), respectively.

It is worth mentioning that seed germination was not significantly affected by the different fruit development stages assessed in this study, demonstrating that seeds from all fruits of both species were properly developed at the moment of fruit collection. Seeds from immature capsules are suitable for *in vitro* germination as embryos become viable and develop normally prior to the capsule ripening, which makes possible their easy surface sterilization (Arditti 1967; Mitchell 1989; Yam and Weatherhead 1988). Also, stringent surface sterilization of seeds after fruit

dehiscence may affect their viability and reduce the germination percentage in orchids (Van Waes and Debergh 1986).

Protocorms developed leaf primordia and rhizoids and successfully developed into seedlings, which then showed well-formed leaves and roots. Seedlings transferred to the same medium devoid of any plant growth regulator displayed continuous growth and after 22–26 weeks of seed sowing, whole plantlets (6–10 cm long) were developed, which were successfully transferred to pots and acclimatized to greenhouse conditions. Plants attained maturity and developed normal flowers and capsules after about 2 years of the culture establishment. This protocol of successful plant regeneration by asymbiotic seed germination should permit rapid propagation and conservation of these threatened *Cohniella* species with ornamental value.

2. *Direct embryogenesis from somatic explants*: Somatic embryogenesis from vegetative explants was assessed aimed to develop an effective tissue culture system for clonal propagation of *C. cepula* and *C. jonesiana* (unpublished data). *In vitro* plants of both species grown on MS devoid of plant growth regulator and incubated under a 14-h light/10-h dark photoperiod (116 μmol.m^{-2}.s^{-1} PPFD) were used in this experiment. The effects of six types of explants (basal, middle, and apical segments of young leaves and roots), combinations and concentrations of six plant growth regulators [cytokinins: N6-benzylaminopurine (BAP), thidiazuron (TDZ), kinetin (KIN); auxins: naphthalene acetic acid (NAA), indole-3 butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D)], and light condition during culture incubation (14-h light/10-h dark photoperiod or permanent darkness) on direct embryogenesis were studied.

After 60 days of culture, the basal segment of young leaves were the only explants that showed PLB formation in all the conditions assayed. However, embryogenesis was significantly affected by the culture media and light condition during incubation. KIN, BAP, or TDZ (3 mg.L^{-1}) alone or combined with either IBA or 2,4-D (0.1 mg.L^{-1}) and incubation on permanent darkness provided the highest percentages of PLB induction (80–95%). After transference of PLBs to fresh MS devoid of any plant growth regulator, they developed leaf primordia and rhizoids and successfully grew into plantlets with normal vegetative morphology (Fig. 4.2). The highest percentages of plant regeneration were obtained from leaf explants induced with KIN or BAP (3 mg L^{-1}) alone or combined with IBA (0.1 mg. L^{-1}). It was previously reported that TDZ is more effective than other cytokinins (the adenine-type cytokinins like BAP and KIN) in inducing shoot bud differentiation from various explants (Ernst 1994; Nayak et al. 1997a, b). However, the drawback of using TDZ in regeneration studies includes difficulty in elongation and rooting of regenerated shoots. This may be due to the high cytokinin activity and persistence of TDZ in the tissue compared to adenine-type cytokinins (Huetteman and Preece 1993). Nayak et al. (1997a, b) overcame the problem of shoot elongation in *Acampe praemorsa*, *Cymbidium aloifolium*, *Dendrobium aphyllum*, and *Dendrobium moschatum* by incorporating an auxin (NAA) at lower concentrations along with TDZ or by transferring the shoot clumps to a medium containing

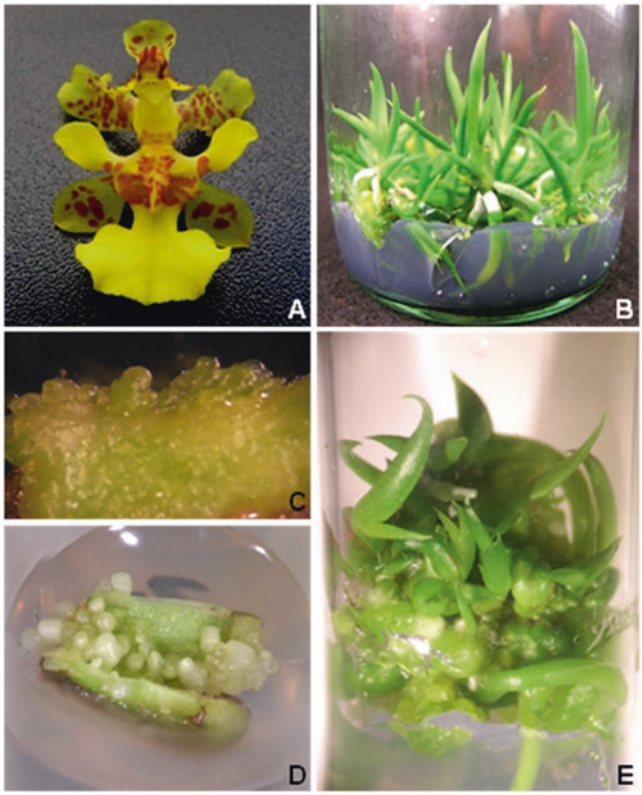

Fig. 4.2 C*ohniella cepula* plant regeneration through somatic embryogenesis. (**a**) Flower from the working collection maintained in the greenhouse. (**b**) *In vitro* plants of *C. cepula* grown on MS devoid of plant growth regulator, used as a source of explants in somatic embryogenesis experiment. (**c, d**) Development of PLBs from basal segments of young leaves after 30 (**c**) and 60 (**d**) days induction on MS supplemented with 3 mg L^{-1} KIN. (**e**) Plantlets obtained 60 days after transference of PLBs to MS devoid of plant growth regulator

different phytohormones (BAP and NAA). Results from this research showed that incorporation of auxins (NAA, IBA, or 2,4-D) in combination with TDZ was not efficient for shoot elongation even after 60 days of transference to MS devoid of plant growth regulator. Therefore, the adenine-type cytokinins alone or combined with low concentration of IBA were more competent for plant regeneration of *C. cepula* and *C. jonesiana* from pieces of young leaves. Regenerated plantlets were successfully transferred to pots and acclimatized to greenhouse conditions. Thus, these results offer efficient means for mass clonal propagation of these and possibly other related *Cohniella* species.

3. *Pollinia storage*: The behavior of *C. cepula* pollinia (without any preconditioning) stored at different temperatures was examined, as a means for preservation of haploid gene pool of its genetic resources (Dolce et al. 2016). Pollinia were

collected from 1-day opened flowers of *C. cepula* and immediately transferred to cryovials and stored at four temperatures: +4 °C (refrigerator), −20 °C (conventional freezer), –70 °C (ultra-freezer), and –196 °C (by direct immersion in LN). Pollen viability was evaluated through the fertilizing ability of fresh and stored (30–360 days) pollinia, which was determined by the fruit formation for each treatment as well as by the seed viability and *in vitro* seed germination. Results from this study revealed that *C. cepula* pollinia have "partially dehydrated pollen" (~12% MC) at anthesis, suggesting that this orchid pollen would be not recalcitrant. Regarding the storage assay, when pollinia were stored at +4 or –20 °C their longevity was reduced (60–120 days). This indicates that deleterious physical and chemical changes proceed gradually in refrigerator-stored pollen and they are not fully detained by the colder temperature attained in a conventional freezer. On the other hand, pollinia stored at −70 and −196 °C showed high fertilizing ability (94–100%) even 1 year after collection, revealing no significant differences with fresh pollinia. Additionally, seeds showed high viability (91–94%) through the 2,3,5-triphenyltetrazolium chloride (TTC) reduction assay and high germination percentages (92–97%). No significant differences were found when seed viability and germination from all treatments (flowers pollinated with fresh and stored pollinia) were compared.

It is interesting to consider here that ultralow storage of pollinia was feasible without any desiccation, cryoprotection, or precooling treatment before placing into an ultra-freezer (−70 °C) or immersing in LN (−196 °C). This is probably due to the low initial MC shown in the fresh pollinia for this species. Moreover, into the highly organized waxy pollinia the pollen is tightly packed in the pollen sac and embedded in a highly viscous fluid, that is, elastoviscin (Pacini and Hesse 2002). The pollen cytoplasm and elastoviscin in pollinia are assumed to contain sucrose or other chemicals enough to protect the pollen from freezing injury. Sucrose allows pollen to be stored at low temperatures by protecting membrane integrity and through intracellular glass formation, thus preventing the formation of ice crystals (Firon et al. 2012; Speranza et al. 1997). It is assumed that the formation of highly viscous intracellular glasses decreases molecular mobility and impedes diffusion within the cytoplasm, thus slowing the deleterious reactions (Firon et al. 2012). Increased viscosity contributed by solutes concentrated in cells inhibits the coming together of water molecules to form ice and is described as the mechanism of glass transition (Benson 2008).

Results from this study showed that successful ultracold storage of *C. cepula* pollinia is feasible without any desiccation, cryoprotection, or precooling treatment before placing them into an ultra-freezer (−70 °C) or immersing in LN (−196 °C). Both fresh and stored pollinia of *C. cepula* allowed successful crosses generating fruits and viable seeds which germinated and developed into healthy and normal seedlings. Additional work in our laboratory proved the possibility of extending this ultracold storage procedure to other wild relative species such as *C. jonesiana* and *Gomesa bifolia*. Such information allows better planning of controlled breeding programs and the potential production of more diverse crosses.

4. *Seed storage*: The behavior of *C. cepula* and *C. jonesiana* seeds removed from fruits at different ripening stages (next to the date of their natural dehiscence) and maintained at different temperatures was examined (Dolce and González-Arnao 2019), since there was no antecedent reporting about seed conservation of *Cohniella* species. Fruits were collected at 12–14 weeks after hand-pollination for *C. cepula* and 19–22 weeks after hand-pollination for *C. jonesiana*. Immediately after collection, fruits were surface sterilized and seeds were aseptically removed. Seed samples from fruits at each ripening stage were transferred to cryovials and stored (up to 36 months) at four temperatures: +27 °C (growth room), +4 °C (refrigerator), –20 °C (conventional freezer), and –196 °C (by direct immersion in LN). Moisture content (MC) and *in vitro* germination were determined for fresh seeds from each fruits.

The MC of seeds from fruits at the different ripening stage ranged between 5% and12%. Seeds from all fruits stored at −196 °C showed high germinability (~90%) even 36 months after collection, revealing no significant differences with fresh seed germination. This result agrees with those reported in the literature, since high post-cryopreservation germination has been achieved in most studies suggesting that the majority of orchid seeds at less than ca. 13% MC can be successfully cryopreserved by the simple method of direct cryopreservation (Popova et al. 2016). On the other hand, seeds stored at −20 °C retained high germinability (~60%) after 12 months, but germination markedly decreased to 10–20% after 18 months and fell to 0% at 24 months after storage. Likewise, seed germination of *Coelogyne foerstermannii* Rchb.f., *C. rumphii* Lindl., and *Dendrobium stratiotes* Rchb.f. fell to 1–5% from initial values of 65–96% after being equilibrated to 15% RH and stored at −20 °C for 9–12months. In contrast, *Xylobium undulatum* (Ruiz & Pav.) Rolfe seeds lost only 13% germinability during the same interval (Seaton et al. 2013). Based on these responses, we can conclude that orchid seeds conservation is a relatively underdeveloped area that demands further research. For the time being, seed storage at temperatures above freezing or under conventional banking conditions (−18 °C and 5% MC) does not get an acceptable result in keeping high viability of certain orchids for a long period (Chang et al. 2006; Hay et al. 2010; Hirano et al. 2009; Pritchard and Dickie 2003). This fact has reinforced the need to investigate the cryostorage behavior of orchid seeds (Merritt et al. 2014; Pritchard et al. 1999) as well as the seeds of other species (Li and Pritchard 2009). Finally, when seeds were stored at +27 and +4 °C, germination rapidly decreased to 0% within 1–6 months' storage. These results agree with those reported by other authors (Koopowitz and Thornhill 1994; Pritchard and Seaton 1993; Pritchard et al. 1999), who informed that orchid seeds stored under room temperature and warm conditions (e.g., 20–40 °C) may lose viability in weeks or days.

Results from this research showed that the key factor to extend the longevity of *C. cepula* and *C. jonesiana* seeds is the storage temperature. Only those seeds maintained at −196 °C retained germination percentages similar to fresh seeds, even after being stored for 3 years. Thus, the establishment of cryobanks present great potential for the long-term storage of seeds from these species. Further work will

determine the applicability of this procedure to a wider range of *Cohniella* species, so that this approach could be used for the establishment of cryogenic collection of germplasm for different orchid species.

4.5.1.3 Genus: *Gomesa*

The genus *Gomesa* R. Br. (Subfamily Epidendroideae, Tribe Cymbidieae, Subtribe Oncidiinae) is a Neotropical genus with about 130 species (Govaerts 2018). The genus is distributed in tropical and subtropical regions from South America, with southern limit in northern Argentina and eastern Uruguay. It is characterized from small to big, generally epiphytic plants, with racemose or paniculate multifloral inflorescences (Valebella 2017).

Gomesa bifolia (Sims) M.W. Chase & N.H. Williams, commonly called the duckling orchid, was found in Bolivia, Brazil, Paraguay, Uruguay, and Argentina in the warm lowlands in the shade along streams and in the coolest mountain forests at an altitude of about 2000 m. Its southern limit of natural distribution is the riparian forests of the Río de la Plata, in the province of Buenos Aires (Argentina), which probably makes it the most austral epiphytic orchid on the planet (Cellini et al. 2009). It is a perennial epiphytic orchid that presents ovoid or ovoid-oblong pseudobulbs, deeply furrowed, with 1 or 2 apical oblong-linear and acuminate leaves. It has showy yellow flowers (20–30 mm × 40–55 mm diameter) with brown markings on sepals and petals, fragranceless and with oil as reward, in racemose or paniculate inflorescences (20–50 cm long, with 7–15 flowers). *G. bifolia* is a mainly self-incompatible, non-autogamous, and pollinator-dependent species (Torretta et al. 2011). Traditionally, *G. bifolia* was considered within the genus *Oncidium*, one of the most conspicuous and systematically controversial genera of Neotropical orchids. On the basis of molecular phylogenetic studies, Chase et al. (2009) transferred *O. bifolium* Sims to the genus *Gomesa* R. Br. with the purpose of redefining the taxonomic limits of *Oncidium* to a monophyletic group of species (Torretta et al. 2011; Valebella 2017).

In the last years, staff of the IBONE has conducted researches aimed at *in vitro* plant regeneration as well as at long-term preservation of *G. bifolia* germplasm. Results from these studies are briefly presenting below.

1. *Seed germination*: Asymbiotic germination of *G. bifolia* seeds was assessed aiming at developing an efficient propagation method for this species (unpublished data). The effect of fruit maturity (18–20 weeks after hand-pollination), the nutritive media composition (full-, half-, or quarter-strength MS medium with 3% sucrose, alone or supplemented with 500 $mg.L^{-1}$ soy peptone, banana powder and/or activated charcoal, assessing a total of 24 culture media), and light condition during culture incubation (14-h light/10-h dark photoperiod with 116 $\mu mol.m^{-2}.s^{-1}$ PPFD or permanent darkness) on seed germination was evaluated.

The onset of germination was observed 15–20 days after seed sowing. Germinating seeds showed enlargement, change to green/white color, and development into protocorms. Germination percentage (= seeds that developed protocorms)

was determined 8 weeks after sowing. Seed germination took place in all the conditions tested; however, the germination percentage was significantly affected by the nutritive media composition and light condition during the incubation. The highest germination percentage (82%) was obtained on half-strength MS supplemented with soy peptone and incubating the cultures under 14-h light/10-h dark photoperiod. Here again, as occurred with *C. jonesiana*, the promotory effect of soy peptone added to culture media on seed germination of orchid species was demonstrated.

On the other hand, as was observed with *C. cepula* and *C. jonesiana*, seed germination was not affected by the different fruit development stages assessed in this study, indicating that seeds were adequately developed at the moment of fruit collection. Protocorms developed leaf primordia and rhizoids and successfully developed into seedlings, which then showed well-formed leaves and roots. Seedlings transferred to the same medium devoid of any plant growth regulator showed continuous growth and after additional 14–18 weeks, whole plantlets with conspicuous pseudobulbs and normal vegetative morphology were developed (Fig. 4.3), which were successfully transferred to pots and acclimatized to greenhouse conditions. This successful plant regeneration protocol through asymbiotic seed germination allows rapid propagation and conservation of this orchid species with ornamental value.

2. *Seed and protocorm cryopreservation*: The first report for germplasm preservation of *G. bifolia* (Flachsland et al. 2006) was performed with seeds and protocorms following the encapsulation-dehydration technique. Fresh seeds from green capsules (120 days after hand-pollination) and protocorms (derived from seeds germinated on liquid half-strength MS with 3% sucrose after 60 days of culture) were used in this experiment. Both seeds and protocorms were encapsulated in 3% sodium alginate polymerized with calcium chloride ($CaCl_2$) at 0.1 M. Encapsulated seeds were then pre-treated in liquid half-strength MS enriched with a progressively increasing sucrose concentration, using the following sequence: 0.15 M (24 h); 0.25 M (48 h); 0.5 M (24 h); and 0.75 M (24 h). Pre-treatment was performed by placing samples at 27 °C on an orbital shaker at 80 rpm. After pre-treatment, the beads were surface-dried on filter paper and dehydrated using silica gel. Encapsulated seeds were dehydrated for 0–6 h while encapsulated protocorms were dehydrated for 0–10 h. Samples were then placed into cryotubes and rapidly immersed in LN. After 1 h cryostorage, samples were rewarmed by immersing the cryotubes in a water bath at 30 °C for 2 min. Encapsulated seeds and protocorms were then post-treated in liquid half-strength MS enriched with a progressively decreasing sucrose concentration: 0.75 M (24 h); 0.5 M (24 h); 0.25 M (48 h); and 0.15 M (24 h). The beads were then transferred to liquid half-strength MS with 3% sucrose for recovery. Survival was evaluated at various periods: after 30 days, by counting the number of seeds that turned green; after 90 days, by counting the number of seeds which formed protocorms; and after 180 days, by counting the number of protocorms that developed into plantlets.

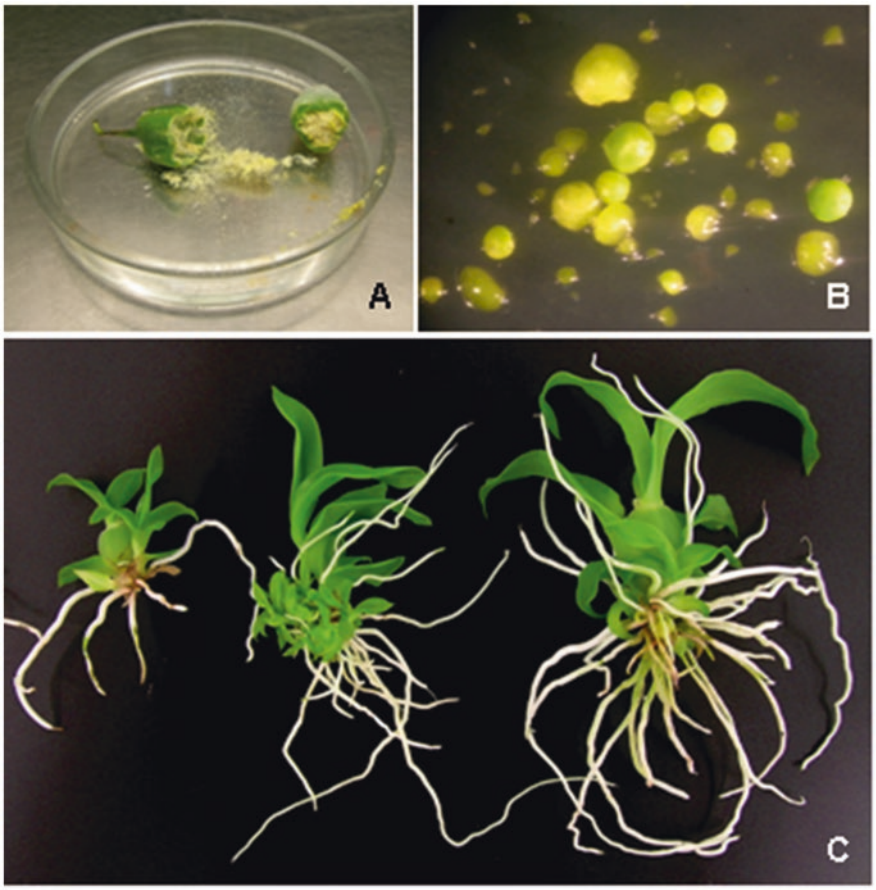

Fig. 4.3 *Gomesa bifolia* plant propagation through asymbiotic seed germination. (**a**) Fruit at 19 weeks after hand-pollination aseptically opened for removing seed samples used for germination experiments. (**b**) Protocorms obtained by asymbiotic germination 60 days after seed sowing on MS supplemented with soy peptone and activated charcoal. (**c**) Plantlets obtained after 6–7 months from seed sowing, which have suitable conditions to be transferred to a greenhouse for hardening

For encapsulated seeds, bead MC varied from an initial 72–13% (fresh weight basis) after 6 h dehydration. Survival of control seeds (−LN) was not affected by dehydration and remained above 88% in all cases. After cooling (+LN), seed survival was nil for up to 2 h dehydration; it increased progressively to reach 89% after 5 h (19% bead MC) and decreased again to 67% after 6 h (13% bead MC). However, despite the high survival after 5 h dehydration, the percentage of protocorm development was only 5%. For encapsulated protocorms, bead MC varied from 83% to 6% after 10 h desiccation. Survival of dehydration controls remained high (±80%) up to 7 h dehydration and then dropped rapidly to reach 20% after 10 h dehydration. Survival of cryopreserved protocorms was nil for 0 to 3 h dehydration and it increased progressively afterward to reach 80% after 7 h dehydration (21% bead

MC). Thereafter, survival decreased progressively and reached 0% after 10 h dehydration. Regarding to the plantlets formation, when encapsulated protocorms were pre-treated with progressively increasing sucrose concentration and then dehydrated for 7 h, 11% of the cryostored protocorms were able to continue growth and developed into whole plants with normal vegetative morphology.

Although it is necessary to optimize plant regeneration through cryopreserved seeds and protocorms, this research describes for the first time the recovery of whole plants after cryopreservation of *G. bifolia* seeds and protocorms following the encapsulation-dehydration technique. This cryogenic procedure does not require any special equipment and is simpler than other vitrification techniques with the material studied, that is, minute explants. This report also opens up the possibility of recovering plants from cryopreserved seeds and protocorms of other *Gomesa* species.

More recently, the possibility of storing *G. bifolia* seeds (without any preconditioning) was examined (unpublished data). Fruits were collected at 19 weeks after hand-pollination. Immediately after collection, fruits were surface sterilized and seeds were aseptically removed. Seed samples were transferred to cryovials and stored (up to 36 months) at four temperatures: +27 °C (growth room), +4 °C (refrigerator), –20 °C (conventional freezer), and –196 °C (by direct immersion in LN). Moisture content (MC) and *in vitro* germination were determined for fresh seeds.

Fresh seeds showed ~5% MC and high germination percentages (~90%). Seeds stored at −196 °C displayed high germinability (~90 %) even 36 months after collection, revealing no significant differences with fresh seed germination. On the other hand, seeds stored at −20 °C maintained high germinability (~65%) after 12 months, but germination fell to 0% after 24 months of storage. Finally, when seeds were stored at +27 and +4 °C, germination rapidly decreased to 0% within 1–6 months of storage. As was observed for *C. cepula* and *C. jonesiana*, this research showed that *G. bifolia* seeds may be stored without any preconditioning if they are removed from mature fruits with low MC. Moreover, the key factor to extend the longevity of *G. bifolia* seeds is the storage temperature. Only those seeds maintained at −196 °C retained germination percentages similar to fresh seeds, even after being stored for three years. Thus, the establishment of cryobanks presents great potential for long-term storage of seeds from this species.

4.5.2 Terrestrial Orchids

4.5.2.1 Genus: *Aa*

The genus *Aa* (Subfamily Orchidoideae, Tribe Cranichideae, Subtribe Prescottiinae) has 25 described species endemic from mountain environments of South America. Five species were cited for Argentina: *A. achalensis*, *A. fiebrigii*, *A. hieronymi*, *A. paludosa*, and *A. Weddelliana* (Schinini et al. 2008).

Aa achalensis Schltr. is 20–30 cm high and its small white flowers bloom in raceme from September to December (spring in the southern hemisphere). The habitats of this species include the Chaco Serrano woodlands and the highland grasses up

to 3000 m with relative low temperatures and rocky soils in West and Central Argentina (Bianco and Cantero 1985; Sérsic et al. 2006; Sobral and Fracchia 2010). *A. achalensis* was previously categorized as vulnerable and included in the red list of the International Union for Conservation of Nature (Vischi et al. 2004). However, new populations of this species were recently found in the slopes of the Velasco Mountains in the Province of La Rioja (Argentina), near 500 km from the previously known populations (Sobral and Fracchia 2010). Although these new findings require a modification in the conservation status of the species, the former and new populations are not included in a national protected area and are thus subjected to grazing, forest fires, illegal extractions, land conversions to agriculture, and the invasion of exotic species among others (Cagnolo et al. 2006; Marco and Páez 2000).

Since scarce information about the orchid mycorrhizal status in Argentina is available (Fracchia et al. 2008; Urcelay et al. 2005) and literature reporting successful symbiotic germination was absent, researchers from several scientific institutions from Argentina carried out a collaborative work with the aim of isolate root-associated fungi from the species *A. achalensis* and to determine their role in seed germination and protocorm development (Fracchia et al. 2014a). Results from this study are briefly presenting below.

1. *Fungal isolation and culture*: Healthy roots were detached from plants of *A. achalensis* at various developmental stages, which were collected from natural habitat. After their surface-sterilization, transversal root slices were transferred to PDA medium supplemented with antibiotics and incubated at 22 °C in the dark. They were observed periodically until fungal colonies were observed emerging from the root disks. Myceliums from these colonies were subcultured onto fresh PDA for purification. Purified fungal strains were stored at 5 °C and included with a strain number in the fungal collection at the Centro Regional de Investigaciones Científicas (La Rioja, Argentina).
2. *Fungal morphological characterization*: Fungal isolates were grown in PDA at 22 °C for 7–21 days and colony color and growth rate were measured. Replicates of each strain were left for at least 7 weeks to allow the development of sclerotia and monilioid cells (Rhizoctonia-like) and sporulation (dark septate endophytes - DSE). The soil-agar method of Stretton et al. (1964) was used to induce teleomorph formation of Rhizoctonia-like isolates.

Pelotons and hyphal coils of Rhizoctonia-like mycorrhizal fungi were observed in all *A. achalensis* sampling individuals, with a mean percentage of 32%. Melanized hyphae were also observed in root samples from all individuals (9%). These fungi colonized the first cell layers of the root parenchyma without any necrotic tissue symptom. Globose to sub-globose microsclerotia were detected in 40% of the sampled individuals. Five endophytic fungal isolates (two DSE, two Rhizoctonia-like, one septate hyaline endophyte) were recovered from the roots: CC8, CC10, CC26, CC28, and CC29. However, the colonial appearances, morphological features, and growth rates of isolates were ineffective at allowing us to determine the taxonomic

identity of any fungal strains. No sporulation or teleomorphic stages were observed. Sclerotial masses were developed in both Rhizoctonia-like fungi and septate hyaline endophyte.

3. *Fungal molecular characterization*: Total genomic DNA was extracted and used as template for the PCR amplification of the intergenic spacer region from the nuclear ribosomal DNA (ITS hereafter). Amplification and sequencing were carried out using the primers ITS4 and ITS5 (White et al. 1990). All sequences were submitted to a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Alignments were performed using MAFFT program version 6 (Katoh and Toh 2008) available online (http://mafft.cbrc.jp/alignment/server/). Phylogenetic analyses were performed to assign isolates to a specific fungal group using ITS representative sequences available in GenBank. Sequences with at least 97% similarity were chosen. Isolates BLAST analyses revealed that the sequences from isolates CC8 and CC10 were similar (99%) to *Gaeumannomyces cylindrica*/*Phialophora graminicola*. Isolate CC26 resulted similar (98%) to uncultured *Pezizaceae* sequences. Isolates CC28 and CC29 were similar (98 and 99%, respectively) to *Thanatephorus cucumeris*/*Rhizoctonia solani*.
4. *Symbiotic seed germination*: *A. achalensis* seeds were surface sterilized and platted on oat meal agar medium. The plates were inoculated with a plug of each fungal inoculum taken from the hyphal edge after culturing on PDA. Uninoculated plates served as a control treatment. Seed germination and protocorm development were monitored weekly and scored on a scale of 0–5: 0) ungerminated seed, testa intact; (1) enlarged embryo, testa ruptured; (2) appearance of rhizoids (=germination); (3) appearance of protomeristem; (4) emergence of first leaf; (5) presence of second leaf (=seedling). Visualization of the mycobiont structures inside protocorms was evaluated at week 4, after staining them with Trypan Blue overnight and observed under the microscope. Moreover, seed viability was determined using the tetrazolium reduction assay (Singh 1981).

The tetrazolium test revealed a viability of 43% for the harvesting *A. achalensis* seeds. Regarding seed germination, in all treatments the embryos swelled breaking the testa within 25 days after sowing. At 5 weeks, careful examination of protocorms after Trypan Blue staining revealed typical pelotons in the treatments inoculated with the Rhizoctonia-like fungi (strains CC28, CC29) and the sterile hyaline strain (CC26). The DSE fungi colonized the seeds with coiling hyphae inside the protocorm cells but no compact pelotons were observed. Total seed germination was significantly higher in all inoculated treatments, the Rhizoctonia-like fungi (CC28, CC29) and the sterile hyaline strain (CC26) both being the most effective. In the asymbiotic treatment (control), the seeds swelled but we did not observe rhizoids along the assay.

Symbiotic orchid propagation had been previously achieved in some South American native species (epiphytes and terrestrial) from Colombia (Otero Ospina and Bayman 2009), Brazil (Pereira et al. 2005), and Chile (Steinfort et al. 2010).

Nonetheless, there was no literature reporting symbiotic propagation assays for any Argentine species. Thereby, this work was the first report of a successful *in vitro* symbiotic germination protocol for a native orchid species from Argentina. Data obtained from this study will help not only with the propagation and conservation of this species but also to collect information for future research on eight other terrestrial orchid species sympatric with *A. achalensis* in Central and West Argentina. Seedling acclimation, time required to further plant growth, and an evaluation of the survival rate in nature are the next steps toward a better knowledge of the species and to improve success in future conservation programs of this and other native orchid species.

4.5.2.2 Genus: *Cyrtopodium*

The genus *Cyrtopodium* (Subfamily Epidendroideae, Tribu Cymbidieae, Subtribu Cyrtopodiinae) is widely distributed in tropical and subtropical countries of Central and South America, which comprises about 30 species. It is representative from the central-west region of Brazil (Planalto Central) and extends throughout the South American continent reaching the north of Argentina (Menezes 2000). In Argentina, 5 species of *Cyrtopodium* are known: *C. brandonianum*, *C. hatschbachii*, *C. palmifrons*, *C. pflanzii*, and *C. punctatum* (Schinini et al. 2008).

- *Cyrtopodium brandonianum* Barb. Rodr. is a terrestrial orchid that was cited for Argentina, Bolivia, Brazil, and Uruguay. In Argentina, it grows spontaneously in sunny grasslands of Corrientes and Misiones (Sánchez 1986, Schinini et al. 2008). It has semi-buried pseudobulbs that bear lanceolate leaves of about 30 cm. Flowers (3–3.5 cm in diameter) pinkish-brown with spots and a purple labellum, in racemose inflorescences (up to 15 flowers) bloom in the new pseudobulbs in late spring or early summer. This orchid is popular among collectors due to its high ornamental value because of the beauty of its flowers (Menezes 2000), but its populations have decreased drastically over the last years due to the depredation of many populations as well as the destruction of their habitats.
- *Cyrtopodium hatschbachii* Pabst. is a terrestrial orchid which was discovered in Jataí, State of Goiás (Brazil) at 400 m above sea level (Menezes 2000), reaching north-eastern Argentina and Paraguay. In Argentina it was found in the south of the province of Misiones (Sánchez and Valebella 2012; Schinini et al. 2008). This species grows spontaneously in flood-prone areas, totally exposed to the sun. It has prolonged and fusiform pseudobulbs (6–8 cm long). Their flowers (3–3.5 cm in diameter) are reddish, pink, or pinkish with a yellowish labellum, in racemose inflorescences (with 8–15 flowers) which bloom in late winter. The callus labellum is used to identify the species of this genus (Surenciski et al. 2012). Due to the restricted distribution of *C. hatschbachii* natural populations, its conservation acquires ecological significance. Our previous studies have demonstrated that immature seeds of this species exhibit a higher *in vitro* germination than the mature ones (unpublished data). Since immature seeds are kept under sterile conditions, they represent a suitable material for cryopreservation.

As mentioned above, it is imperative to develop effective propagation methods and *ex situ* preservation strategies for these orchids to safeguard the threatened diversity of the genus *Cyrtopodium*, mainly due to the anthropogenic impact. In recent years, staff of the IBONE has made important advances on *in vitro* plant regeneration and germplasm conservation of *C. brandonianum* and *C. hatschbachii*. Results from these researches are briefly presenting below.

1. *Seed germination*: Asymbiotic seed germination of *C. brandonianum* and *C. hatschbachii* was assessed with the aim of developping efficient propagation systems for these species (unpublished data). Fruits of 17 weeks after hand-pollination were used for this study, which were surface sterilized and seeds were aseptically removed (Fig. 4.4). A total of 16 culture media were assessed, which were constituted by full- or half-strength MS or EFp, alone or supplemented with 25 $g.L^{-1}$ green banana puree and/or 2 $g.L^{-1}$ activated charcoal. All media were supplemented with 3% sucrose. Cultures were incubated in a growth room at 27 ± 2 °C with 14-h light/10-h dark photoperiod (116 $\mu mol.m^{-2}.s^{-1}$PPFD).

The onset of germination was observed 15–20 days after seed sowing. Moreover, in some culture media, oxidation and subsequent death of *C. brandonianum* seeds was observed. This fact specially occurred in the media lacking banana puree and

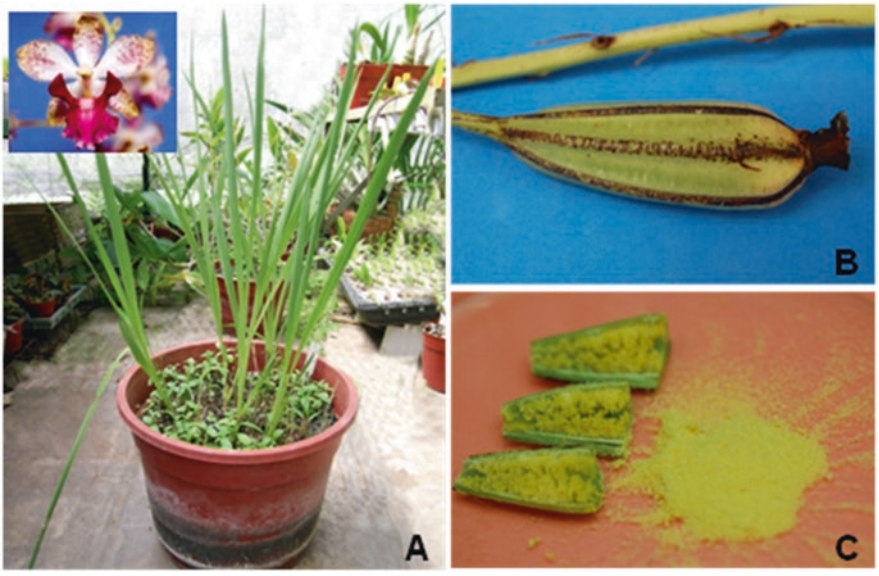

Fig. 4.4 (**a**) *Cyrtopodium brandonianum* plants of the working collection maintained in the greenhouse; a detail of a flower is shown in the upper left. (**b, c**) Fruit at 17 weeks after hand-pollination aseptically opened for removing seed samples for asymbiotic germination experiments

activated charcoal. Germinating seeds showed enlargement, change to green color, and development into protocorms. Germination percentage (= seeds that developed protocorms) was determined 8 weeks after seed sowing. Asymbiotic seed germination occurred in all the culture media assessed in this study with percentages varying between 24% and 50% according to the media composition. Half-strength MS or EFp as well as full-strength EFp supplemented with green banana puree and activated charcoal allowed the significantly highest germination percentages (47–50%) for *C. brandonianum*, while half-strength MS supplemented with only activated charcoal allowed the highest germination percentage (49%) for *C. hatschbachii*. These results demonstrate once again the promotory effect of the natural additives added to culture media (such as banana puree and activated charcoal) on seed germination of orchid species.

Protocorms developed leaf primordia and rhizoids, and successfully developed into seedlings. In the case of *C. brandonianum*, seedlings transferred to the same culture medium devoid of any plant growth regulator showed continuous growth and developed into whole plantlets with normal vegetative morphology. On the other hand, seedlings of *C. hatschbachii* were transferred to half-strength MS + 2 g. L^{-1} activated charcoal (basal medium) supplemented with 0.1–0.5 mg.L^{-1} NAA, IBA, or indole-3-aceticacid (IAA) alone or combined with 1 mg.L^{-1} BAP. Seedling developed into healthy plantlets with well-formed pseudobulbs, leaves, and roots in all the culture media evaluated. However, the number and length of leaves and roots as well as the dry weight per plant were significantly affected by the media composition. Basal medium supplemented with 0.5 mg.L^{-1} NAA allowed the significantly highest growth rate of seedling.

In both species, 200 day-old *in vitro* regenerated plants were transplanted into a mixture of *Sphagnum* moss, peat, and perlite (1:1:1) in plastic containers for *ex vitro* acclimation. Ninety days after plants transference to *ex vitro* growth conditions, 60% of plants survived and showed normal vegetative morphology. This plant regeneration protocol by asymbiotic seed germination should permit rapid propagation and conservation of these threatened *Cyrtopodium* species with ornamental value.

2. *Shoot regeneration from root explants*: The use of root-tip culture for orchid propagation has attracted the attention of several researchers because it is a non-destructive technique in which the donor plants regenerate new roots in natural form. Also the availability of roots during the whole year makes them suitable explants for the *in vitro* cultivation (Kerbauy 1991; Park et al. 2003). In this work, a protocol for *in vitro* plant multiplication of *C. brandonianum* from root-tip culture was developed (Flachsland et al. 2011; Figs. 4.5 and 4.6).

Root-tips isolated from *in vitro* plants of 150 days after germination and growth on half-strength MS supplemented with green banana puree and activated charcoal were used as explants. The effect of the type [BAP, TDZ, KIN, 6-(4-hydroxy-3-methylbut-2-enylamino)purine (ZEA), and N6-(2-isopentenyl)adenine (2iP)] and concentration (0, 0.1, 0.5 and 1 mg.L^{-1}) of cytokinins added to half-strength MS

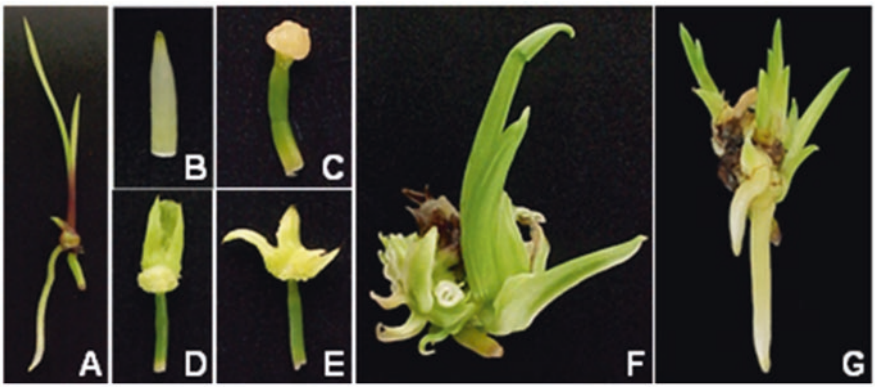

Fig. 4.5 *Cyrtopodium brandonianum* plant regeneration from root-tips. (**a**) *In vitro* plant used as a source of explants. (**b–f**) Callus and adventitious bud and shoot differentiation from root-tips after 0 (**b**), 30 (**c**), 90 (**d, e**), and 150 (**f**) days induction on half-strength MS supplemented with 0.5 mg.L^{-1} TDZ. (**g**) Plantlets obtained 60 days after shoot transference to rooting medium

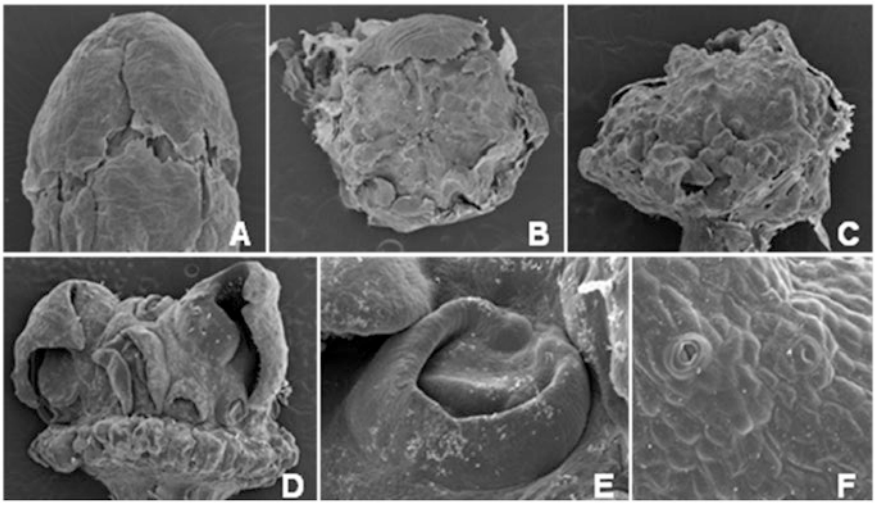

Fig. 4.6 Photomicrographs of *Cyrtopodium brandonianum* plant regeneration from root-tips. (**a–d**) Callus and adventitious bud and shoot differentiation from root-tips after 0 (**a**), 30 (**b**), 60 (**c**), and 90 (**d**) days induction on half-strength MS supplemented with 0.5 mg.L^{-1} TDZ. (**e**) Detail of an adventitious bud. (**f**) Surface view of a leaf fragment with stomata

medium on adventitious bud and shoot induction was evaluated. All cultures were incubated in a growth room at 27 ± 2 °C with 14-h light/10-h dark photoperiod (116 μmol.m^{-2}.s^{-1} PPFD).

After 30–45 root-tips culture, the earliest visible signs of callus growth were noticeable. In some treatments, root-tips gradually enlarged and small, compact, white yellowish or green calluses were observed. On longer incubation (60–70 days), adventitious bud and shoot differentiation taken place in 10 out of 16 variants

of the medium were investigated. Half-strength MS supplemented with 0.5 $mg.L^{-1}$ TDZ provided the highest percentages of shoot induction (43%). On the contrary, there was no shoot regeneration when root-tips were cultured on media without any cytokinin or those containing KIN (any of the concentrations evaluated) as well as the lowest concentration of ZEA or the highest level of 2iP used in this study.

The adventitious shoots were then transferred to half-strength MS with sucrose at 87.6, 175.2, and 262.8 mM alone or supplemented with IBA, NAA, or IAA at 1 or 3 $mg.L^{-1}$ for root induction. The percentage of shoots that formed roots and the mean number of roots per shoot varied significantly with the different concentrations of sucrose and auxins. The highest percentage of rooting (~30%) and the highest root number (~4 roots per explants), with no intervening callus, was observed in half-strength MS supplemented with 6% sucrose and 1 $mg.L^{-1}$ NAA. Media without auxins did not promote root induction regardless of the sucrose concentration.

Results from this study demonstrate the potential of *in vitro* shoot regeneration from root-tips of *C. brandonianum*. As was previously reported for species of *Cattleya* and *Oncidium*, this process took place through indirect organogenesis (Kerbauy 1991, 1993). The requirements of exogenous plant growth regulators are similar to the ones of other orchid species in which shoot regeneration from root-tips was obtained by the addition of one cytokinin alone to the culture medium (Colli and Kerbauy 1993; Park et al. 2003; Peres et al. 1999). On the other hand, Sánchez (1988) increased the direct plant regeneration from *Cyrtpodium punctatum* root-tips adding coconut milk, a substance rich in cytokinins, to the basal medium. The beneficial effects of TDZ, a potent cytokinin for plant tissue culture (Huetteman and Preece 1993), on *in vitro* plant propagation found in this work are in agreement with the results obtained in other orchid genera such as *Phalaenopsis* (Chen and Piluek 1995; Chen et al. 2000; Ernst 1994), *Doritaenopsis* (Ernst 1994), *Cymbidium* (Chang and Chang 1998; Nayak et al. 1997b, 1998), *Dendrobium* (Nayak et al. 1997b), and *Oncidium* (Chen and Chang 2000; Chen et al. 1999).

In spite of the recognized limited morphogenetic competence of root-tip of higher plants, the utility of root explants for orchid micropropagation purposes is being increasingly realized due to their year-round availability, low oxidation rate, and the ease with which they can be explanted (Chugh et al. 2009).

3. *Seed cryopreservation*: The aim of this study was to adjust a cryopreservation protocol for immature seeds of *C. hatschbachii* using the encapsulation-dehydration technique (Surenciski et al. 2012). Sterile immature seeds were encapsulated in 3% sodium alginate polymerized with calcium chloride ($CaCl_2$) at 0.1 M. Encapsulated seeds were then pre-treated in liquid half-strength MS enriched with a progressively increasing sucrose concentration, using the following sequence: 0.08 M (24 h); 0.15 M (24 h); 0.25 M (48 h); 0.5 M (24 h); and 0.75 M (24 h). Pre-treatment was performed by placing samples at 27 °C on an orbital shaker at 60 rpm. After pre-treatment, the beads were surface-dried on filter paper and dehydrated using silica gel for 5 h (equivalent to capsule MC of 18%, fresh weight basis). Samples were then placed into cryotubes and rapidly immersed in LN. After 12 h cryostorage, samples were rewarmed by immersing

the cryotubes in a water-bath at 30 °C for 1 min. Encapsulated seeds were then post-treated in liquid half-strength MS enriched with a progressively decreasing sucrose concentration: 0.75 M (24 h); 0.5 M (24 h); 0.25 M (48 h); 0.15 M (24 h); and 0.08 M (24 h), on an orbital shaker at 60 rpm. The beads were then transferred to semisolid germination medium (MS + 3% sucrose + 2 $g.L^{-1}$ activated charcoal + 0.7% agar) for recovery. Seed survival was registered 45 days after culture by counting the seeds with hypertrophied embryos and those which developed protocorms.

Using the encapsulation-dehydration technique, cryopreservation of *C. hatschbachii* immature seeds was achieved with high survival rates (64%). Following this protocol, the seed germination percentage was significantly higher than means obtained in other treatments, even compared with control treatments (−LN). This phenomenon could be attributed to seed coat damage during cooling-rewarming cycles (Tikhonova et al. 1997) that enhances seed permeability, allowing the uptake of nutrients from the culture medium. Results from this study agree with those obtained by Popova et al. (2003) and Popov et al. (2004) who also observed a rapid growth of cryopreserved seeds and the subsequent protocorm develop in the orchid hybrid *Bratonia*.

A high percentage of protocorms from both cryopreserved and non-cryopreserved seeds showed continuous growth (in the same medium devoid of any plant growth regulator) and developed whole plantlets with normal vegetative morphology, which were successfully transferred to pots and acclimatized to greenhouse conditions. There were no phenotypic differences when compared with plants derived from cryopreserved and non-cryopreserved seeds, 10 months after plants transference to *ex vitro* growth conditions. In addition, the cytogenetic stability was confirmed for plants derived from cryopreserved seeds (Surenciski et al. 2007).

In this work, the encapsulation-dehydration technique was applied for the first time in the *Cyrtopodium* genus in order to achieve the long-term conservation of immature seeds of *C. hatschbachii*. This technique does not require toxic cryoprotectors like dimethyl-sulfoxide (DMSO) and ethylene glycol, used in other vitrification techniques. This work opens the possibility of using the encapsulation-dehydration technique in other *Cyrtopodium* species and other members of Orchidaceae.

4.5.2.3 Genus: *Habenaria*

The genus *Habenaria* (Subfamily Orchidoideae, Tribe Orchideae, Subtribe Habenariinae) has a pantropical distribution, with about 600 species (Hoehne 1940), mainly from perennial, terrestrial, and wetland habits. In Argentina, 21 species of *Habenaria* are known; four of these inhabit the Ibera macrosystem. The Ibera macrosystem, a protected natural area, is the second-largest wetland ecosystem in South America, and supports 57% of the 2640 plant species documented in the Province of Corrientes, Argentina (Tressens and Arbo 2002).

Habenaria bractescens Lindl. inhabits river bank and wetland environments in Brazil, Uruguay, Paraguay, and Argentina and is rare even in its natural habitat (Johnson 2001). It is characterized by the production of resupinate white flowers

with a fringed lip and long slender spur, inserted in a terminal, pauciflorous inflorescence. *H. bractescens* has three types of underground organs: absorbing roots, droppers, and root tubers. Absorbing roots are those that possess a typical structure consisting of a radical meristem tip with a root cap, followed by cell division and elongation zones, a root hair zone, and a maturation zone where lateral roots originate. Droppers are organs that grow downward, either vertically or obliquely, and terminate in a root tuber. Root tubers are swollen storage roots that bear shoot buds (Bell 1991; Pridgeon and Chase 1995). The formation of this storage organ is related to a phenomenon known as tuberization.

Many terrestrial and wetland orchids form storage organs, such as root tubers and rhizomes, which are important in their propagation (Pridgeon et al. 1999, 2001, 2003). Root tubers are typical vegetative plant propagules of Orchidoideae subfamily, which can survive in dry or cold conditions as dormant organs. These storage organs are very common in Orchidinae subtribe group 2 (Habenariinae sen. auct.) in the Orchideae tribe (Pridgeon et al. 2001).

Staff of the IBONE has made important advances on *in vitro* plant regeneration and root tuber differentiation of *H. bractescens* (Medina et al. 2009). Results from this research are briefly presented below.

1. *In vitro plant regeneration and ex vitro acclimation*: *In vitro* plants of *H. bractescens* derived by seed germination and grown on full-strength MS devoid of plant growth regulator and incubated under a 14-h light/10-h dark photoperiod (116 μmol.m^{-2}.s^{-1} PPFD) were used as a source of explants in this experiment (Fig. 4.7). Multinodal stem segments (at least five nodes and 2 cm long) were dissected from *in vitro* plants and used as explants for the micropropagation assay. A total of 12 culture media were assessed, which were constituted by full-strength MS supplemented with different concentrations and combinations of BAP (0, 4.4, 22.2, 44.4 μM) and sucrose (87.6, 146.7, and 292.1 mM). After 45 days of culture, multinodal segments regenerated shoots (upright leafy shoots), swollen buds (similar to pseudobulbs), and/or root tubers (Fig. 4.8) depending on the culture media. Shoot differentiation decreased with increasing BAP concentration regardless of the sucrose concentration. BAP-free media did not promote the growth of swollen buds, regardless of sucrose concentration. BAP supplementation stimulated the differentiation of swollen buds; however, in media with high sucrose concentration (292.2 mM) this capacity decreased. High sucrose concentration also had a depressive effect on the number of shoots and swollen buds regenerated per explant.

Shoots, swollen buds, and root tubers developed on the different culture media were transferred separately to MS basal medium (87.6 mM sucrose) to complete plant regeneration. After 60 days of culture, upright leafy shoots produced on BAP-free media or media supplemented with 4.4 or 22.2 μM BAP and sucrose at the three concentrations evaluated were able to develop complete plants when transferred to MS basal medium; however, none of the shoots obtained with 44.4 μM BAP regenerated plants regardless of the sucrose concentration. On the other hand, swollen buds differentiated in all the media were able to regenerate plants.

Fig. 4.7 (**a**) *Habenaria bractescens* plants of the working collection maintained in the greenhouse. (**b**) *In vivo* underground organs of *H. bractescens*. (**c**) *In vitro* plants used as a source of explants for the organogenesis and plant regeneration experiments

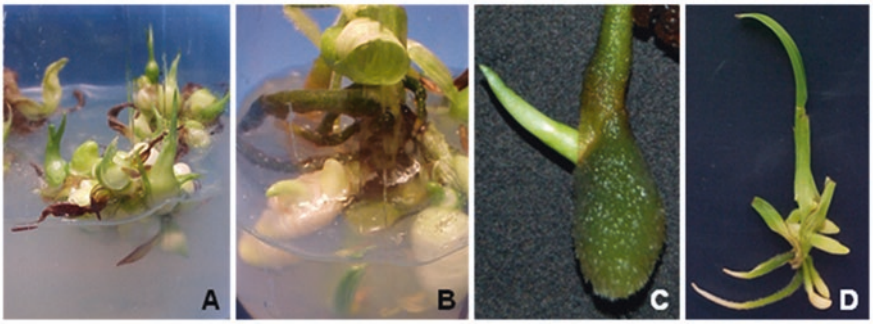

Fig. 4.8 *Habenaria bractescens* morphogenesis from multinodal segments after 45 days of culture on MS supplemented with different concentrations and combinations of BAP and sucrose. (**a–b**) *In vitro* regenerated swollen buds (**a**) and root tubers (**b**). (**c**) Sprouted *in vitro* root tuber.(**d**) *In vitro* regenerated plant showing different underground organs

Plant regeneration percentage from swollen buds developed on MS supplemented with 146.7 mM sucrose and 4.4 μM BAP was statistically different to the plant regeneration through swollen buds produced on MS with the same sucrose level but with 44.4 μM BAP as well as on MS with 292 mM sucrose regardless of the BAP concentration. Root tubers differentiated in all the responsive media were able to regenerate plants in percentages between 93% and 98%.

In vitro plants derived from upright leafy shoots, swollen buds, and root tubers were removed from glass flasks, soaked in tap water to remove the remaining culture medium, and rinsed carefully. They were submerged in fungicide solution for 30 min and then transplanted into a mixture of *Sphagnum* moss, humus, and perlite in plastic containers for *ex vitro* acclimation. Plants derived from *in vitro* upright leafy shoots and swollen buds were incubated in a humidity chamber at 90% relative humidity (RH) for 7 days and an irradiance of 160 μmol m^{-2} s^{-1}. The RH was then gradually decreased to 70%. After 30 days, the pre-acclimatized plants were transferred to a greenhouse under 80% sunlight. On the other hand, plants derived from *in vitro* root tubers were directly transferred to greenhouse conditions without previous acclimation. After 60 days of transplantation, plants derived from either upright leafy shoots, swollen buds, or root tubers showed similar vegetative morphology to plants grown under wild conditions; however, the *ex vitro* survival ratio was significantly dependent from the explant origin. Plants derived by root tubers had the highest survival rate (95%) and in all cases they sprouted and rooted, allowing the regeneration of vigorous and healthy plants regardless of origin media.

2. *In vitro root tuber differentiation*: On the other hand, 45 days after culture of multinodal segments on MS with different concentrations and combinations of BAP and sucrose, typical *H. bractescens* underground organs were regenerated *in vitro* (i.e., absorbing roots with abundant root hairs, thin root-like structures called droppers, and root tubers in different maturation stages) in a relative proportion per explant depending on culture media. Immature root tubers derived from the swollen apical portion of the dropper and mature root tubers with an emerging bud were observed. These root tubers originated from a slender dropper or from a very short dropper that emerged from the stem. Two weeks after root tuber formation, root tubers sprouted from the expanding bud. In all cases, 2–9 absorbing roots were observed on each explant, and in some media, absorbing roots were the only type of underground organ formed. The addition of BAP increased the number of droppers that differentiated per explant and the number of droppers that developed root tubers, except at the highest sucrose concentrations with 22.2 and 44.4 μM BAP. In BAP-free medium containing 146.7 or 292.1 mM sucrose, the number of droppers per explant was reduced to 50% of that in media with 87.6 mM sucrose. In the culture media with 87.6 mM sucrose combined with the different BAP concentrations, the number of droppers per explant remained constant (2 per explant). However, the presence of BAP in combination with 146.7 mM sucrose increased the number of droppers per explant to three per explant, regardless of the BAP concentration. Media with 292.1 mM sucrose and 4.4 μM BAP produced the highest number of droppers

per explant; however, higher BAP concentrations inhibited dropper regeneration. Root tuber formation was influenced by media composition. At 45 days, root tuber differentiation was promoted in 8 of the 12 culture media assayed, earlier than for container-grown specimens from the Ibera macrosystem (ca. 3–4 months after planting). The root tuber originated from the distal end of a dropper between the bud and the root apical meristem. Tuberization took place when the apical portion of the dropper underwent radial expansion, maintaining the typical radical structure, and the cortical parenchyma had starch granules. One of the most effective media for root tuber regeneration was the MS with 87.6 mM sucrose plus 4.4 μM BAP. In all the cases, each explant produced one root tuber.

Terrestrial orchids can be propagated through several *in vitro* procedures to produce a large number of healthy plants. However, plants derived from symbiotic or asymbiotic seed germination or through vegetative explant multiplication are generally fragile and poorly survive after transplantation in comparison to plants derived by the sprouting of robust or field-hardy propagules such as root tubers. Thus, the production of storage organs would be the ideal method for restoration or reintroduction programs of this orchid species. The protocol for *in vitro* root tuber formation for *H. bractescens* established by Medina et al. (2009) provides a novel system for the controlled and reliable development of root tubers. In summary, MS medium supplemented with 87.6 mM sucrose plus 4.4 μM BAP was one of the most effective for stimulating root tubers, the ideal explant for successful *ex vitro* transplantation without any acclimation process.

4.6 General Conclusion

Considering that South America is a megadiverse continent in orchid species as well as in other plant families, there is no doubt that orchids are a key group for biodiversity conservation. They are visible and fascinating examples of the natural world and they are seriously threatened by human activities (deforestation, habitat fragmentation, illegal trade, and possibly global warming). However, there is still time to conserve the high orchid diversity that remains and efforts can be successful if we act immediately. Moreover, orchids can play a key role on conservation efforts due to their importance as charismatic species and as a "flag" group whose conservation will help protect many other species and habitats. The task of orchid conservation is multi-faceted and must combine protection of habitats, increase in knowledge about the species and their distribution, coordinated efforts on both *in situ* and *ex situ* actions, disclosure of information, promotion of knowledge and awareness networks, among others.

This chapter reviewed the progresses of different *in vitro* approaches for orchid propagation and germplasm conservation, safeguarding the genetic biodiversity of these species. Several case studies were presented and described to exemplify the protocols developed in the IBONE for propagating and long-term storing the germplasm of Argentine orchids. Moreover, it has been attempted to put together most of

the available literature on *in vitro* propagation and germplasm conservation for South American orchids using different explants and procedures (Tables 4.1, 4.2, and 4.3). There are researches of good scientific quality that even cover critical insights into the physiology and factors affecting growth and development as well as storage of several orchid materials. However, studies are still necessary to increase the number of species evaluated as well as the use of selected material (clonal) for both propagation and conservation approaches.

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Postharvest Technology of Cut Flowers of Orchids

5

Mantana Buanong and Apiradee Uthairatanakij

Abstract

Orchids are one of the world's most fascinating flowers because they exhibit a wide range of diversity in color, form, weight, and size. Commercial production of cut flowers of orchids has expanded enormously, especially *Dendrobium* hybrids. The major postharvest problems of cut orchids are color fading, flower dropping, and short vase life that related with ethylene production, respiration, and microbial growth in the vase solution. Several methods have been tested to maintain the quality of cut flowers of orchids including ethylene inhibitor, germicides, and plant hormone. However, the responses of cut flowers of orchids to postharvest treatments depend on cultivar, variety, and stage of maturity. Therefore, factors affecting the postharvest physiology, bud opening, and vase life of cut orchids are discussed.

Keywords

Orchid flowers · Postharvest · Vase life · Quality

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5.1 Introduction

Orchids belong to the Orchidaceae family which is one of the largest families. The Orchidaceae contain over 800 known genera, over 25,000 known species of monocotyledonous herbaceous perennial plants, and over 100,000 hybrids and cultivars (Sheehan and Sheehan 1994). They are found throughout the world, except in Antarctica. Orchidaceae is commonly divided into five subfamilies: Apostasiodeae, Cypripediodeae, Vanilloideae, Orchidoideae, and Epidendroideae. Subfamilies are further divided into smaller tribes, which are then divided into subtribes and grouped into genera. Orchidaceae are popular for their structural variations in their flowers. Some orchids have single flowers, but most have a racemose inflorescence. Orchid flowers are all bilaterally symmetrical with three petals and three sepals. The different types vary extremely in size, weight, and color. The flowering stem can be basal like in *Cymbidium* or apical like in *Cattleya* and *Dendrobium* or axillary as in *Vanda*. *Dendrobium* is the second largest genus in the family after *Bulbophyllum* (Puchooa 2004). It has unique characteristics among the cut orchids because of varieties of color, larger number of florets in the inflorescence, and regular flowering (Fadelah et al. 2001). Many varieties are easy to grow as commercial cut flowers such as *Cattleya*, *Cymbidium*, *Phaleanopsis*, *Dendrobium*, *Vanda*, *Ascocenda*, *Arachis* and its hybrids, and *Oncidium* (Jha 2005).

The market of cut orchid flower is a globalized market. Orchid cut flowers are transported for several days to long distances. Thus, the quality of cut orchids at the final destination or consumer will be affected by postharvest handling in the supply chain. The major postharvest losses of cut orchids are (i) flower dropping and (ii) petal wilting and stalk yellowing. Temperature is one of the most important factors in controlling quality losses. It affects both physiological (development and senescence of cut flowers, wound responses of cut stems) and physical (water loss, condensation/drying) processes involved in quality loss and microbial growth in vase solution (Nell and Reid 2000; Kader 2002). In addition, postharvest handling such as sleeving, boxing, placing flowers in water during transport or keeping them dry, and recutting of stems or not will interact with some processes affected by temperature.

Orchid flowers are extremely perishable resulting in hastens senescence and wilting of the flowers. The short vase life is related to excessive water loss, decrease in respiration substrates, and sensitivity to exogenous or endogenous ethylene. Thus, maintaining their physiological functions varies actively even after harvest, and the beginning of their senescence very often depends on ethylene. Cut orchids, especially *Dendrobium* hybrids, are susceptible to ethylene damage which can eventually reduce their longevity (Woltering 1987; Hew 1994). The level of the ethylene sensitivity seems to vary depending on the hybrids. Hence, it is essential to identify the degree of ethylene sensitivity in orchid hybrids and the prospective impact of the exposure to ethylene on the length of their vase life. In addition, understanding of the postharvest physiology assists growers in choosing appropriate methods to ensure that the quality of products is satisfactory, with each crop requiring specific conditions.

5.2 Postharvest Physiology of Orchids

Postharvest life means the period of time from harvest until the flower or plant has lost its decorative value, while vase life means the period during which cut flower or cut foliage retains its appearance in a vase. This is a major consideration in identifying plant species suitable for use in the cut flowers industry; therefore, plants with a long vase life are more desirable than those with a short vase life. The major observable signs of senescence in orchid flowers are yellowing, drooping, epinasty, and venation of florets (Lerslerwong and Ketsa 2008). The vase life of cut orchids depends on cultivar and its hybrids. Ketsa and van Doorn (2009) reported that some *Dendrobium* cultivars produce individual flowers with a long vase life, while flowers from other cultivars have a short life. Figure 5.1A showed the different vase life of three cultivars such as *Dendrobium*'Jinda Sweet' (JS), 'Red Sonia' (RS), and 'White Sanan' (WS). The longevity of *Dendrobium* is often terminated by 30% of senescence in an inflorescence (Fig. 5.1B) such as petal abscission, petal wilting, and withering as shown in Fig. 5.2. The vase life of *Dendrobium* 'Jinda Sweet' orchid lasted 8.4 days, and *Dendrobium* 'Red Sonia' had 7.3 days, while the *Dendrobium* 'White Sanan' had 6.8 days of vase life (Buanong, unpublished data). In *Mokara* orchid, Sartpetch et al. (2010) showed that the display life of *Mokara* 'Panee,' *Mokara* Red, *Mokara* 'Nora Yellow,' *Mokara* 'Chark Kuan Pink,' and *Mokara* 'Nora Pink' was 13, 11.6, 12.4, 12.4, and 12.7 days, respectively. However, the vase life of *Dendrobium* 'Burana Jade' did not significantly differ with *Dendrobium* Sonia 'Earsakul' inflorescences (Obsuwan et al. 2015). In addition, the maturity stage of inflorescence also affects the postharvest life. Obsuwan et al. (2015) reported that *Dendrobium*'Burana Jade'and *Dendrobium* Sonia 'Earsakul' inflorescences harvested at 30 of opened flower had the longest display life when compared with 45% (commercial maturity stage) and 60% of flower opening.

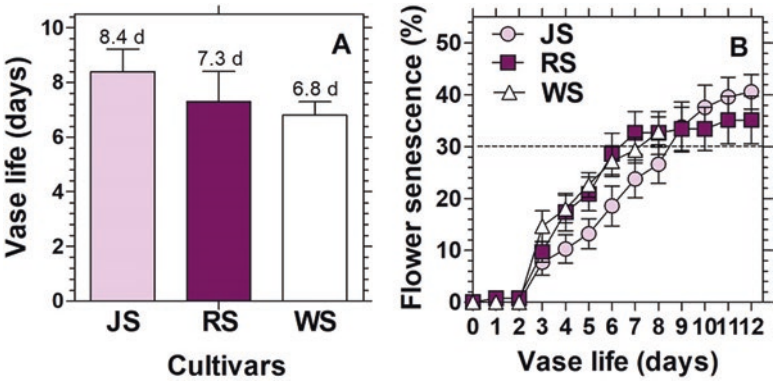

Fig. 5.1 The vase life (**a**) and flower senescence (**b**) of three *Dendrobium* orchid cultivars 'Jinda Sweet' (JS), 'Red Sonia' (RS), and 'White Sanan' (WS)

Fig. 5.2 The senescence symptoms of three cultivars of *Dendrobium* orchid flowers 'Jinda Sweet' (JS), 'Red Sonia' (RS), and 'White Sanan' (WS)

5.2.1 Respiration

Respiration rate in orchid flowers varied significantly among species. Mostly, the young flowers had a higher rate of respiration (Hew 1980). The orchid flowers harvested in the tight bud stage have lower rate of respiration than open flowers. The respiration rates continuously declined during the postharvest period until the flower faded (Sheehan 1954). Theses pattern was found in *Cattleya* (Sheehan 1954) and *Aranda* orchid flowers (Hew 1980) which showed the highest respiratory rate in tight buds and a decline in respiratory rate with age. Also, a marked increase in respiration climacteric accompanied by ethylene climacteric following pollination has been observed in *Dendrobium* 'Pompadour' orchids resulting in premature petal and sepal senescence (Ketsa and Rugkong 1999).

5.2.2 Ethylene Production

Ethylene, the plant hormone, plays an important role in the regulation of flower senescence and, manifested in a range of symptoms including wilting, abscission, coloration, color fading, and yellowing, which also occur in senescing orchid flower species (Goh et al. 1985; Woltering and Van Doorn 1988). Gane (1934) reported that ethylene is produced by many plant tissues, with small amounts of ethylene (0.1–0.2 μl C_2H_4•kg^{-1}•h^{-1}) (Martínez-Romero et al. 2007). Ethylene hastens senescence of petals in Orchidaceae, e.g., *Cymbidium* and *Dendrobium*, that initially stays attached to the flower (Sexton et al. 2000).

Orchids are climacteric flowers which have a climacteric increase in ethylene evolution and respiration during flower senescence, and they respond to exogenous ethylene by enhanced senescence and increased autocatalytic ethylene production

(Mayak and Halevy 1972; Mayak 1987; Rogers 2013). The general pattern of ethylene evolution during development of several orchid species showed a low and stable ethylene production rate in floral buds and young flowers, a sharp increase during flower maturation, opening and senescence, followed by a decrease which remained static (Goh et al. 1985). Endogenous ethylene production has three distinct phases: in the first phase, the production rate is very low, and in the second phase, it increases rapidly and then declines in the third phase (Halevy and Mayak 1981). Endogenous ethylene is produced through the conversion of S-adenosylmethionine (AdoMet) to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS), and ACC is oxidized to ethylene by ACC oxidase (ACO) (Srivastava 2002). Ethylene sensitivity relied on orchid varieties has been reported such as *Dendrobium* hybrids (Almasi et al. 2012), *Phalaenopsis* (Hansen et al. 2013), and *Mokara* (Sartpetch et al. 2010). The responses to ethylene vary widely according to the species (Reid and Wu 1992) although they are often consistent within either families or subfamilies (van Doorn 2001). In *Dendrobium*, the senescence and abscission are mediated by ethylene, particularly after pollination, flowers show the evolution of ethylene production which acts as a signal for the flower to undergo senescence (Chandran et al. 2006). The difference postharvest longevity in *Mokara* cut orchid flowers cv. 'Moo-deang,' 'Jao-pra-ya,' 'Duang-porn,' 'Nora-pink,' and 'Dao-lai' could be categorized by respiration rate and ethylene production pattern as shown in Fig. 5.3A, B: (1) low respiration rate and ethylene production showed in 'Doa-lai,' (2) moderate respiration rate and ethylene production showed in 'Duang-porn' and 'Nora-pink,' and (3) high respiration rate and ethylene production as climacteric flowers showed in 'Jao-pra-ya' and 'Moo-deang.' The increase in ethylene production in *Mokara* flowers closely coincided with a dramatic loss of fresh weight and rapid petal wilting, dropping, venation, and epinasty which terminated by 30% of senescence in an inflorescence (Fig. 5.3C). This indicates that 'Jao-pra-ya' and 'Moo-deang' *Mokara* inflorescences are a climacteric-like flower due to the peak of ethylene production and respiration rate (Wongjunta 2011).

Ethylene sensitivity in cut orchid is high, even exposure to very low level of ethylene (Goh et al. 1985). In *Dendrobium* cultivars, ethylene-sensitive flowers

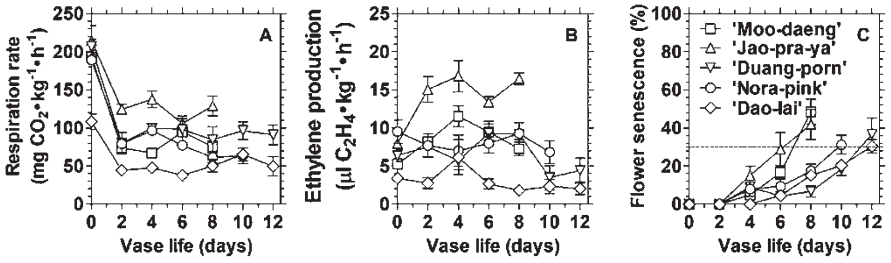

Fig. 5.3 Respiration rate (a), ethylene production (b), and flower senescence (dash line represents the termination of the vase life when 30% of the initial open florets were wilted and drop) (c) of Mokara hybrids cv. 'Moo-deang,' 'Jao-pra-ya,' 'Duang-porn,' 'Nora-pink,' and 'Dao-lai' holding in distilled water in an observation room (21±2 °C, 70–80% RH, under cool-white fluorescent lights 12 h/day) throughout the vase period

showed wilting of flowers and buds during the shipment due to an increase of ethylene inside the packages (Uthaichay et al. 2007). The sensitive to exogenous ethylene in Mokara 'Moo-deang' was tested by pulsing with 10 ppm ethephon, an ethylene releasing compound, for 24 h and compared with flowers pulsed with distilled water. Ethephon treatment accelerated bud opening and induced premature senescence of *Mokara* 'Moo-deang' flowers. Flowers showed visible senescence symptom including venation, wilting, drooping, and bud necrosis (Fig. 5.4A, B) coincident with amount of ACC content (Fig. 5.5A) and accumulated increasing activities of ACC synthase and ACC oxidase (Figs. 5.5B, C) and the burst of ethylene production following ethephon treatment (Fig. 5.5D) in day 4 concomitant with the onset of ethylene production and visible symptom of senescence (Wongjunta 2011). Also, ethylene production has a negative correlation with vase life. The vase life of 'Moo-deang' pulsed with distilled water was 8.1 days twice longer than that of flowers pulsed with ethephon which lasted 4.3 days. In cut *Dendrobium* 'Pompadour' flowers, exogenous ethylene resulted in autocatalytic increase in endogenous ethylene production (Ketsa and Rugkong 1999) and early senescence, especially in sensitive cut orchids (Almasi et al. 2012).

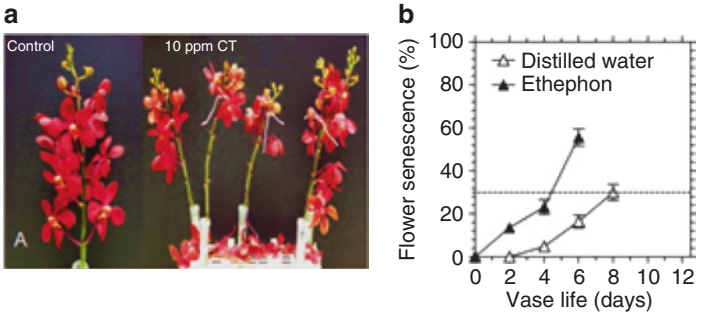

Fig. 5.4 The senescence symptoms of *Mokara* 'Moo-daeng' orchid flowers pulsed with 10 ppm ethephon and distilled water for 24 h at an observation room (21 ± 2 °C, 70–80% RH under cool-white fluorescent lights 12 h/day)

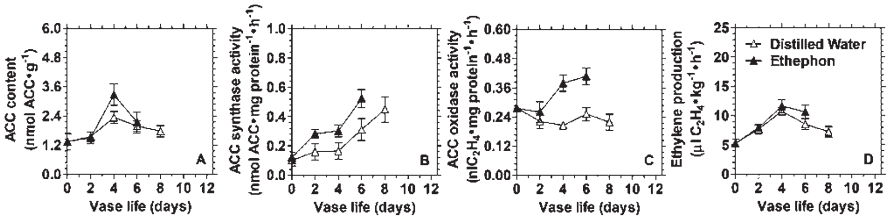

Fig. 5.5 ACC content (a), ACC synthase activity (b), ACC oxidase activity (c), and ethylene production (d) in Mokara 'Moo-Daeng' orchid flowers pulsed with 10 ppm ethephon and distilled water for 24 h, then transferred to distilled water in an observation room (21±2 °C, 70–80% RH under cool-white fluorescent lights for 12 h/day) throughout experimental period

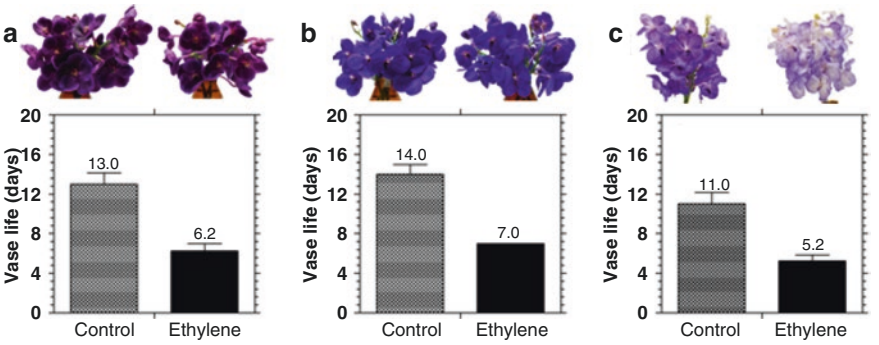

Fig. 5.6 The vase life of cut *Vanda* 'Pure Wax' (a), 'Patchara Delight' (b), and 'Sansai Blue' (c) orchids pretreated with 10 ppm ethylene for 24 h compared to air treatment as a control. After pretreatment, flowers were then placed in the distilled water in an observation room (21±2 °C, 70–80% RH under cool-white fluorescent lights for 12 h/day) throughout experimental period

The ethylene production rates of the *Vanda* 'Sansai Blue' inflorescences were generally low in the range of 2–10 nL•g FW^{-1}•h^{-1} which is common for most orchid flowers including *Vanda*, as measured in individual florets (Burg and Dijkman 1967; Goh et al. 1985; Porat et al. 1994; Kesta and Rugkong 2015; Ketsa et al. 2001; Porat et al. 1995). Exposure of *Vanda* 'Pure Wax' (A), 'Patchara Delight' (B), and 'Sansai Blue' (C) to 10 ppm ethylene treatment significantly reduced by about 50% their vase life. Two visible ethylene-induced symptoms could be observed in the *Vanda* orchid flowers: floret color fading (Fig. 5.6), manifested in reduced anthocyanin content that differed among the three cultivars, and wilting (sleepiness) of petals and sepals due to loss of turgidity, which was used as the visible symptom that determined the end of vase life. However, the ethylene-treated 'Sansai Blue' flowers did not show wilting symptoms during the first 2 days, while the lower florets in the inflorescences of 'Pure Wax' and 'Patchara Delight' cultivars showed sleepiness symptoms 2 days after the ethylene treatment (Fig. 5.6A, B, C). This may indicate that the color fading and wilting symptoms occurring in response to ethylene seem to operate separately in 'Sansai Blue' flowers. The most sensitive *Vanda* cultivar regarding color fading is 'Sansai Blue' indicating that the color fading is a very fast process that occurs within few hours after the increase in ethylene production and the less sensitive cultivar is 'Pure Wax.' Therefore, the responses among the cut orchid flowers to exogenous ethylene were different, based on sensitivity to ethylene (Khunmuang et al. 2018a).

5.2.3 Water Relation

Transient water stress during postharvest handling is common when flowering shoots are held dry; the water transpired is replaced by air, which disrupts water column continuity and impedes water movement in consequence of the loss of turgor pressure (Mayak et al. 2001). The presence of microorganisms such as bacteria,

fungi, or yeast in the vase solutions causes blockage in xylem conduits during vase life of cut flowers, limits water uptake, and reduces the vase life of flowers (Robinson et al. 2009). It might be due to microbial growth, production of tyloses, sediment of materials in the lumen of xylem vessels, and the formation of air emboli in the vascular system (Twumasi et al. 2005). However, the main problem that caused the short vase life of cut orchids was ethylene sensitivity, but not the xylem blockage (Almasi et al. 2015).

5.2.4 Food Supply

Sucrose is the main transport form of sugar to flower bud (Ho and Nichols 1977). Sugars are transported from leaves to flower bud in the phloem. Some of exogenously added sugars in vase solution are transported in the xylem, and the stem may also have phloem loading capacity (Halevy and Mayak 1979; Marissen and La Brijn 1995). Carbohydrates in stem probably also contribute to flower opening. Flower diameter was larger in longer than in shorter rose stems (with leaves removed). A good correlation was observed between the increase in petal fresh weight and the decrease in carbohydrate content in leafless rose stem (Ichimura et al. 1999). Hou et al. (2011) reported that stimulated dark shipping reduced the carbohydrate status of *Phalaenopsis* Sogo Yukidian 'V3' which was positively related to the post-shipping quality. Also, *Oncidium baueri* pretreated with pulsing solution containing sucrose-based for 12 and 24 h has been reported (Favetta et al. 2016).

5.2.5 Hormonal Balance

There are cultivar differences in the level and action of hormones affecting the longevity of the flowers. Auxin plays a role in delaying the abscission, while ethylene clearly promotes the abscission. The interplay between auxin and ethylene in their mutually antagonistic roles in abscission has been reported in several studies. In *Dendrobium* orchid flowers, the abscission of buds and opening flowers is caused by ethylene (Ketsa and van Doorn 2009). Aiamla-Or et al. (2015) revealed that pulsing with 200 mg•L^{-1} HQS + 2% sucrose + 100 ppm BA suppressed and delayed ethylene production in *Mokara* 'Nora Pink' and improved displayed life of *Mokara* inflorescences.

5.3 Postharvest Handling for Cut Orchids

The postharvest life of cut orchid flowers depends on several biotic and abiotic factors that induce the senescence of different plant organs. Thus, control of temperature, relative humidity, light, ethylene, microorganisms, water quality, and others factors are all important to maintaining the quality of cut flowers during their postharvest life (Kader 2007).

5.3.1 Temperature

Temperature is the most important factor since high temperature triggers an increase in the respiration rate, causing rapid senescence of the flower (Kader 2007). Mattiuz et al. (2010) also demonstrated that the temperature effect on extending vase life depends on the length of the storage period and of the floral species. For example, *Oncidium varicosum* 'Samurai' stored at 5 °C showed the better maintenance of flowers quality than at those stored at 10 and 20 °C due to the lower reduction of relative water content and maintained the contents of carotenoids, soluble carbohydrates, and reducing sugars (Mattiuz et al. 2010).

5.3.2 Preservative Solution

The use of preservative solutions and of "cut flower food" containing sugar is generally required to supply an energy source, reduce microbial build up and vascular blockage, increase water uptake, and arrest the negative effect of ethylene (Ketsa and Boonrote 1990; Pun and Ichimura 2003; Rattanawasalanon et al. 2003). There are four different purposes for the chemical solution use followed by (1) conditioning or hardening which was applied to restore the turgidity of cut flowers from water stress during storage and transport. Deionized water with germicides and acidified with citric acid to pH 4.5–5.0 was used in this method. Also, wetting agent such as tween 20 at concentration of 0.01–0.1% can be added to the solution. Flower stems are placed into the solution at room temperature or cold storage for several hours; (2) bud opening, this procedure is to stimulate opening when flowers are harvested at the early stage. The solution contains low concentration of sugar, germicides, and some hormones to increase longevity of flowers and reduce the sensitivity to extreme environment such as low or high temperatures, low humidity, and ethylene contamination.

Sugar Sucrose is the main ingredient to add in the most preservative formulations for orchid flowers, but sometimes other metabolic sugar like glucose is used (Ketsa and van Doorn 2009). In addition, glucose combined with HQS and $AgNO_3$ increased number of bud opening and prolonged vase life of *Dendrobium* 'Youppadeewan' (Ketsa and Boonrote 1990). Mattiuz et al. (2015) noted that inflorescences of *Oncidium varicosum* 'Samurai' treated with the solution containing sucrose 5% plus 8-HQC 100 mg•L^{-1} plus $AgNO_3$ 50 mg•L^{-1} had the highest values for average of relative water content, reducing carbohydrates and soluble sugars, and also had higher number of open flowers resulting in the longest longevity of 22 days. *Dendrobium* 'Lovely Pink' had a longer vase life in the tested vase solution (100 mg•L^{-1} aluminum sulfate, 200 mg•L^{-1} 8-hydroxyquinoline sulfate, and 1.5% sucrose) than in the commercial vase solution, whereas the vase life of *Dendrobium* 'KaoSanan' and *Dendrobium* 'Suree Peach' in tested solution did not differ with commercial solution (Obsuwan et al. 2013).

Germicide The presence of microorganisms in vase solution resulting in xylem blockage, water stress (van Doorn and Perik 1990), releases toxic metabolites and/or enzymes (Accati et al. 1980) or evolves damaging level of ethylene (Fujino et al. 1983). Microorganisms in cut flower stems and in vase solutions are typically composed of yeasts, fungi, and bacteria (Van Doorn 1997). Bacteria in vase solution directly induced wilting by physically blocking the stems and indirectly producing substances that are absorbed by flowers (Marousky 1977). Bacteria suspensions at concentrations of 10^7 CFU•mL^{-1} or higher significantly reduce hydraulic conductance and vase life of cut flowers (Van Doorn 1997). Adding germicides into the vase water maintains clarity in the solution and prevents blockage of xylem elements by microorganisms (Jones and Hill 1993). A preservative solution including a suitable germicide can improve the number of opening flowers and prolong the vase life beyond plain water or a sugar solution. However, the response of many cut flowers to germicides is highly variable among species and variety.

Chlorine Sodium dichloroisocyanurate (DICA), a slow release chlorine, is widely used in flowers handling and vase solutions (He et al. 2006; Faragher et al. 2002; Knee 2000; van Doorn et al. 1989, 1990). Chlorine action involves the oxidation of cellular components in microorganisms, including essential enzymes in cell membranes and protoplasm (Bloomfield and Arthur 1989; Dychdala 1983). Besides, commercial chlorine compounds are influenced by pH; increased acidity increases the available hypochlorite ion, the reactive moiety responsible for sterilization (Hadfield 1954), while high temperature and organic matter cause depletion of available chlorine (Hadfield 1954; Lawrence and Block 1968). Chlorine demand at various concentrations depends on a range of water sources, solution ingredients, cut flower species, and stem numbers. Sattayawong et al. (2010) reported that DICA at 40 mg•L^{-1} significantly reduced total microorganisms in vase solution and increased vase life about 5 days compared to that of control in *Mokara* 'Nora Pink' inflorescence. Adding 50 ppm DICA + citrate phosphate buffer pH 5.4 and 7.4 was the most effective on inhibiting microbial growth in the vase solution of *Mokara* orchid flowers (*Arachins x Ascocentrum x Vanda*) cv. 'Moo-daeng,' while holding flowers in citrate phosphate buffer only had a load of microbial content in the xylem in day 6 of the vase period. Additionally, flowers held in the buffer pH 7.4 produced highest ethylene and shortest vase life of 7.8 days, while the longevity of treatments of DICA + citrate phosphate buffer pH 5.4 and 7.4 were 9.8 and 8.8 days, respectively. Figure 5.7 showed the microbial content in the xylem in day 0 and 6 by scanning electron microscopy (SEM). The microbial growth was undetectable in treatments of DICA + citrate phosphate buffer, while holding flowers in the buffer only had a load of microbial content in the xylem in day 6 of the vase period (Buanong, unpublished data).

Electrolyze Water (EW) Electrolyzed water is generated through the electrolysis of a dilute solution of NaCl and softened tap water passed through on electrolysis

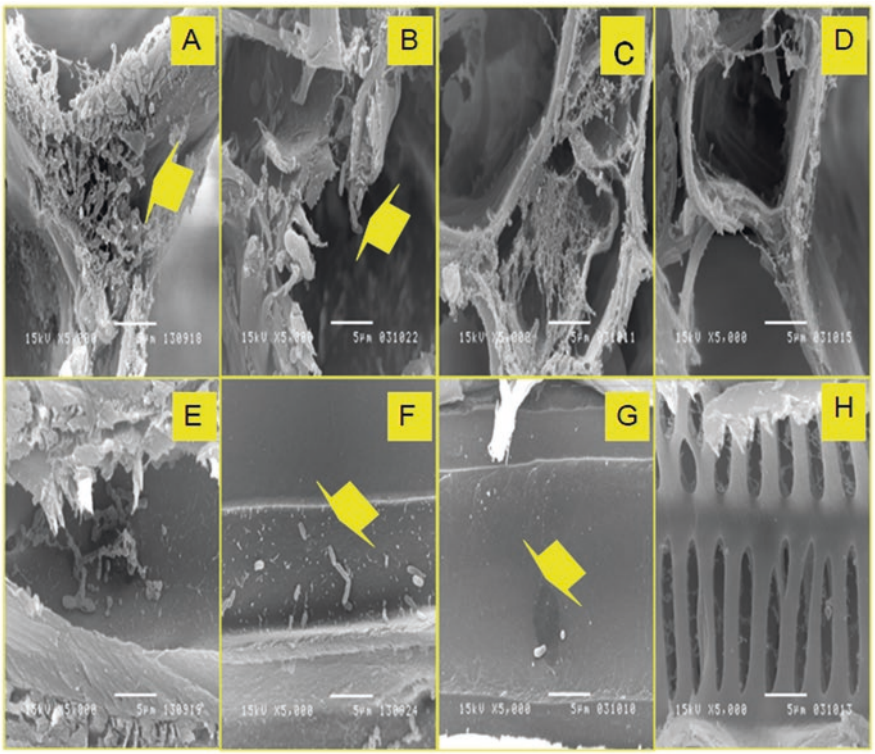

Fig. 5.7 Scanning electron microscope observation of freshly cut rose stems in day 7 showing 5 μm of the cross section (a, c, e, and g) and longitudinal section (b, d, f, and h) on the end cut surface stems held in citrate-phosphate buffer pH 5.4 (a, b), citrate-phosphate buffer pH 7.4 (c, d), 50 ppm sodium dichloroisocyanurate buffered to pH 5.4 (DICA + CPB pH 5.4) (e, f), and 50 ppm sodium dichloroisocyanurate buffered to pH 7.4 with citrate-phosphate (DICA + CPB pH 5.4) (g, h) in an observation room (21±2 °C, 70–80% RH, cool-white fluorescent lights for 12 h/day) throughout experimental period

chamber with bipolar membrane resulting in two solutions: an acidic solution that is characterized by a low pH, high oxidation-reduction potential (ORP), and a free chlorine concentration of approximately 50 ppm and a basic solution which is composed of a high pH and low ORP (Kim et al. 2000). The basic mechanisms have been attributed to kill bacteria. In general, bacteria grow in pH range of 4–9. Aerobic bacteria grow mostly at ORP range + 200 to 800 mV, while anaerobic bacteria grow well at +700 to +200 mV. High ORP level in the electrolyzed water causes the modification of metabolic fluxes and ATP production, probably due to the change in the electron flow in cells (Kim et al. 2000). Neutral electrolyte water (NEW) is efficient antimicrobial by totally inhibiting the growth of bacteria in the vase solution in association with high level of ORP and chlorine content. Maithong et al. (2018) reported that holding flowers in of 15–30% NEW with higher oxidation-reduction potential (ORP) level and chlorine content significantly inhibited the microbial growth, but

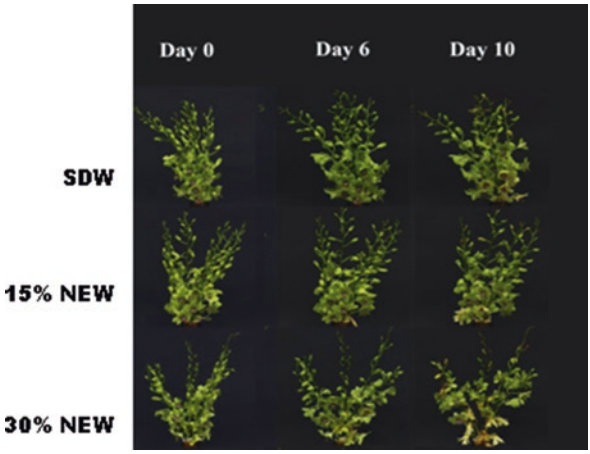

Fig. 5.8 Visual appearance of *Dendrobium*'Burana Jade' inflorescence held in sterile distilled water (SDW), 15 and 30% neutral electrolyzed water (NEW) at 21 ± 2 °C, 70–80% RH with cool white-fluorescent lights for 12 h/day

Table 5.1 Microbial load in vase solution of *Dendrobium* 'Big White' held in sterile distilled water (SDW) and 100 ppm chlorine available electrolyzed acidic water (EAW) in an observation room (21 ± 2 °C, 70–80% RH, cool-white fluorescent lights for 12 h/day) throughout the vase period

Treatments	Microbial load in vase solution (logCFU.mL^{-1})							
	D0	D2	D4	D6	D8	D10	D12	D14
SDW	3.57	6.09	5.52	5.86	5.87	6.30	6.34	6.34
EAW	0	0	0	0	0	0	0	0
T-test	*	**	**	**	**	**	**	**

* = Significantly different at $P < 0.05$, ** = Significantly different at $P \leq 0.01$

application of 15% NEW seemed to have better visual appearance in the quality of *Dendrobium* 'Burana Jade' inflorescence (Fig. 5.8). Electrolyzed acidic water (EAW) is a weakly acidic electrolyzed water with pH ca. 6–8 produced by non-diaphragm-type electrolyzed devices (Harada and Yasui 2003). Holding *Dendrobium* 'Big White'inflorescence in 100 ppm chlorine available electrolyzed acidic water (EAW) pH 5.5 was effective on inhibiting microbial growth in the vase solution for 14 days as shown in Table 5.1 and maintaining the fresh weight and water uptake and increasing the number of opening buds. However, the vase life of *Dendrobium* orchid flowers in SDW and EAW was 12.2 days and 11.8 days, respectively, which was not significantly different (Tonboot et al. 2015). The response of cut flowers to EAW as a germicide is highly variable among species and variety.

5.3.2.1 Plant Growth Regulators

Auxin Abscission results from cell separation in the abscission zone which is related to the activity of several cell wall-degrading enzymes. These enzymes are regulated by ethylene (Ketsa and van Doorn 2009) 0.1-Naphthaleneacetic acid (NAA) is a plant hormone in the auxin family widely used in many commercial postharvest horticultural products (Dimitrios et al. 2008). Flower abscission in

Fig. 5.9 The flower abscission of *Mokara*'Moo-daeng' inflorescences when pulsing in synthetic auxin treatments, 200 μM 2,4-D and NAA, compared with distilled water as a control flowers. All flowers were kept in an observation room (21 ± 2 °C, 70–80% RH, cool-white fluorescent lights for 12 h/day)

Mokara 'Moo-daeng' was significantly prevented by a pulse treatment of 200 μM NAA, while 2,4-dichlorophenoxyacetic acid (2,4-D) was less effective. In contrast, 200 μM 2,4-D accelerated flower abscission up to 50% within 5 days of the vase period, while 200 μM NAA extended the vase life to 11.8 days (Suksamran et al. 2011) (Fig. 5.9). 2,4-D has been extensively used in modern agriculture as a herbicide, at low concentrations (<0.1 mg•L^{-1}), to stimulate growth by cell division and elongation, but in higher concentration (>0.1 mg•L^{-1}), it may cause induced abnormalities culminating with the symptomatic herbicide effect (Chinalia et al. 2007). Lower concentration of 50 μM 2,4-D was more effective on improving the quality of *Mokara* 'Moo-daeng' orchid flowers than other concentrations due to lower ethylene production and flower abscission.

Cytokinin BA has been reported to suppress ethylene production and retard senescence in many cut flowers such as morning glory (Kende and Hanson 1976) and carnation (Eisinger 1977). BA also blocked the conversion of ACC to ethylene in carnation petal (Mor et al. 1983). In *Cattleya* alliance cv. 'York' and 'Sweet Afton,' BA might be substituted for endogenous cytokinin to suppress ethylene production (Yamane et al. 1997). Aiamla-Or et al. (2015) reported that pulsing Mokara 'Nora Pink' inflorescences with 200 mg•L^{-1} HQS + 2% Sucrose + 100 ppm

BA suppressed and delayed ethylene production as shown in Fig. 5.10. *Mokara* 'Nora Pink' responded positively to BA by improving the display quality of flowers after harvest. Treatment of BA prevented open flower abscission, but did not prolonged vase life of In *Dendrobium*'Eiskul' (Rungruchkanont 2011). However, the response of cut flowers to BA as a preservative is highly variable among species and variety.

Others such as Calcium chloride ($CaCl_2$) in vase solution – increased vase life, promoted flower opening, enhanced initial fresh weight, and delayed its reduction rate. Calcium treatment delayed the decrease in petal membrane proteins and phospholipids and slowed down the rate of electrolyte leakage from petals. It also suppressed ethylene production (Torre et al. 1999). Figure 5.11 showed the visual

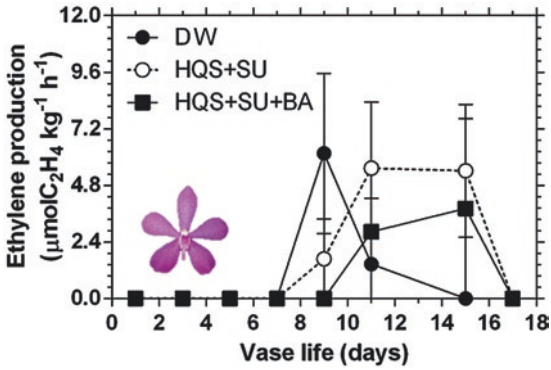

Fig. 5.10 Ethylene production of *Mokara* orchid pulsed with distilled water (DW, control), 200 mg•L^{-1} HQS + 3% sucrose (HQS + SU) and 200 mg•L^{-1} HQS + 3% sucrose + 100 ppm BA (HQS + SU + BA) for 24 h at 21 ± 2 °C, then transferred to distilled water in an observation room (21 ± 2 °C, 70–80% RH, cool-white fluorescent lights for 12 h/day)

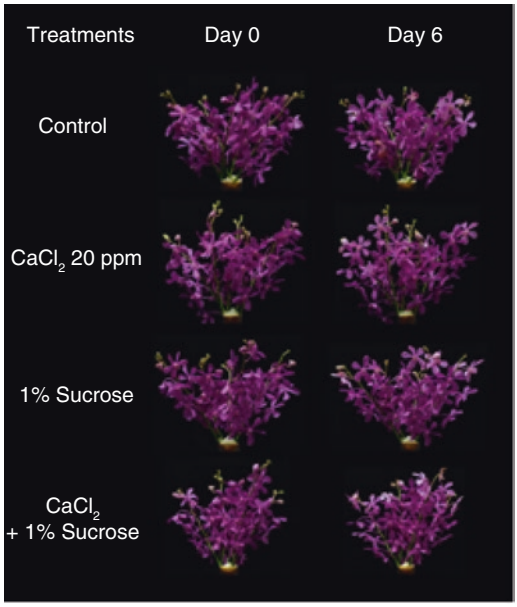

Fig. 5.11 Visual appearance of *Mokara* 'Nora Pink' inflorescences dip in 50 ppm 6-benzylaladine (BA) for 1 min before holding in distilled water (control), 20 ppm $CaCl_2$, 1% sucrose and $CaCl_2$ + sucrose. All flowers were kept in an observation room (21 ± 2 °C, 70–80% RH, cool-white fluorescent lights for 12 h/day)

appearance of dipping *Mokara* 'Nora Pink' inflorescences in 6-benzylaladine (BA) at 50 ppm before holding in 20 ppm $CaCl_2$ + 1% sucrose significantly maintained the fresh weight of flowers and water uptake, reduced the ethylene production throughout the vase period, and then extended the vase life to 11.0 days, while flowers holding in 20 ppm $CaCl_2$ alone had 9.2 days of vase life compared to the control flowers which had 7.8 days of vase life. However, adding 1% sucrose alone and $CaCl_2$ + sucrose in the vase solution did not affect flower opening when compared

to the control flowers (Buanong 2014). Almasi et al. (2015) revealed that aqueous ozone noticeably contributed to inhibit microbial growth in vase solution of cut orchid hybrids, namely, *Dendrobium* 'Darren Glory' and *Mokara* 'Calypso Jumbo' flowers. *Dendrobium* Sonia 'No. 17' inflorescence sprayed six times at weekly intervals with chitosan at the concentrations of 0 (water), 200, 400, or 600 mg•L^{-1}showed no significant differences in the displayed life, percentage of bud opening, and respiration rate. On the other hand, spraying chitosan at 400 mg•L^{-1} increased weight of inflorescence and 600 mg•L^{-1} of chitosan increased petal width (Uthairatanakij et al. 2008). Moreover, *Cattleya* 'Lc. Spring Clima × Christina' treated with 50% carbon dioxide (CO_2) combined with pre-cooling at 5 °C showed the lowest respiration rate and enhanced the vase life (Burana and Yamane 2017).

5.3.3 Inhibitor of Ethylene Biosynthesis and Action

Exposure to exogenous ethylene or endogenously produced ethylene can be controlled by inhibiting either ethylene biosynthesis or ethylene action. Aminooxyacetic acid (AOA), aminoethoxyvinylglycine (AVG), silver thiosulphate (STS), and 1-methylcyclopropene (1-MCP) are effective compounds to delay senescence of climacteric flowers.

Aminooxyacetic acid (AOA) is an inhibitor of pyridoxal phosphate-requiring enzymes including 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, a key enzyme of ethylene biosynthesis (Abeles et al. 1992). Exogenous ethylene may induce autocatalytic ethylene production *Dendrobium* orchid flowers via ACC synthase and ACC oxidase, resulting in premature senescence. Application of AOA may reduce ACC synthase activity and produce lower ethylene, thus reduce the effects of endogenous ethylene. The vase solution containing 4% sucrose + 0.5 mM AOA delayed the discoloration, thinning of petal, and hyponasty in pollinated *Dendrobium* 'Heang Beauty' and extended double of vase life (Chandran et al. 2006). Inclusion of AOA in the vase solution together with sugar gives positive effects on delaying the senescence process of *Dendrobium*'JawYuayTew' flowers (Rattanawasalanon et al. 2003). Also, AOA can act as an antimicrobial agent which is attributed to the maintenance of low pH in the solution resulting in a non-conducive environment for bacterial growth (Rattanawasalanon et al. 2003).

1-Methylcyclopropene, a potent ethylene action inhibitor (Serek et al. 1995a, b; Çelikel and Reid 2002; Blankenship and Dole 2003), significantly inhibited the responses of various orchid species to exogenous ethylene, emasculation, or pollination, thereby extending their flower vase life (Porat et al. 1995; Yamane et al. 2004; Hansen et al. 2013; Sapbua et al. 2013; Yoodee and Obsuwan 2013). 1-MCP acts as an efficient inhibitor against endogenous and exogenous ethylene action: therefore it could be considered as a good alternative for STS because of no harmful heavy metal residue. Application of 1-MCP has been successful in extending vase life several orchid flowers such as cut mini *Phalaenopsis* (Sun et al. 2009), *Cattleya* (Singh and Jaroenkit 2011; Yamane et al. 2004), and *Dendrobium* (Uthaichay et al. 2007). The responses to 1-MCP may depend on cultivar and varieties. For example, in *Vanda* orchid flowers,

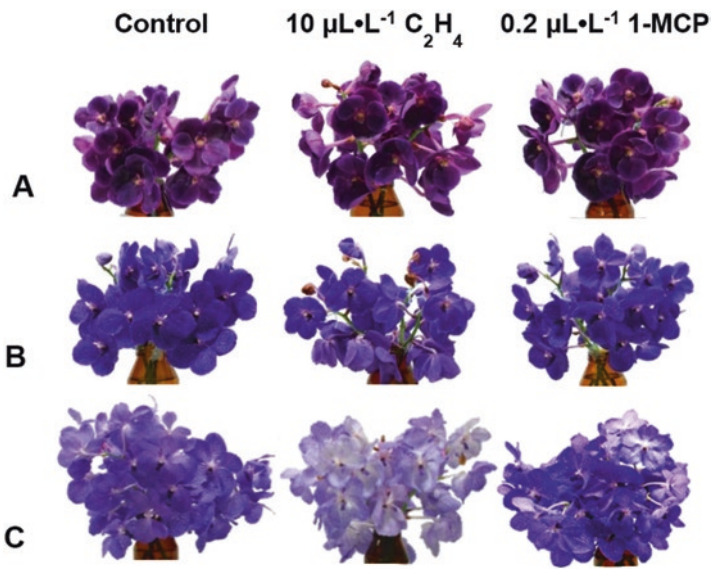

Fig. 5.12 Effect of ethylene or 1-MCP pretreatments on the visual appearance of cut *Vanda* 'Pure Wax' **(a)**, 'Patchara Delight' **(b),** and 'Sansai Blue' **(c)** orchids after 7 days in vase life. The flowers were exposed with air (control), 10 µL•L^{-1} ethylene for 24 h, or 0.2 µL•L^{-1} 1-MCP for 6 h and then placed in vase with distilled water in an observation room throughout the experimental period. Vase life was terminated when 30% of the initial open florets were wilted and showed sleepiness symptoms

the color fading in response to ethylene was very fast and the anthocyanin breakdown occurred during the first 24 h of ethylene treatment. 1-MCP protected *Vanda*'Sansai Blue' flowers from exogenous ethylene treatment when applied before ethylene in the combined treatment (Fig. 5.12) (Khunmuang et al. 2018a).

Phalaenopsis is very sensitive to even low concentration (0.1 µl•L^{-1}) of ethylene, thus applying 0.5 nl•L^{-1} 1-MCP significantly reduced quality losses of mini *Phalaenopsis* 'Allen' and 'Venice' that subjected to 0.1 µl•L^{-1} ethylene (Hansenet al. 2013). Beside, *Phalaenopsis* 'Allen' and 'Venice' were exposed to 200 nL•L^{-1} of 1-MCP after 24–27 h of ethylene exposure showed to be the threshold time span that application of 1-MCP can be delayed the negative effects of exogenous ethylene (Favero et al. 2016). 1-MCP was also effective in prolonging vase life of *Cymbidium* orchids by against accelerated senescence flowers with damaged pollinia (Heyes and Johnston 1998). Raffeiner et al. (2009) demonstrated that pre-treated with 200 nL•L^{-1} 1-MCP for 6 h at 20 °C improved the postharvest quality of *Oncidium* and *Odontoglossum* inflorescences exposed to exogenous ethylene at 1 µL•L^{-1}. In addition, application of 1-MCP at 1000 nL•L^{-1} presented larger values of water content, soluble carbohydrates, and reducing sugars and carotenoids in flowers and also reduced the respiration rates of *Oncidium varicosum* (Mattiuz et al. 2012a, b). In *Dendrobium* 'Darrenn Glory' and *Mokara* 'Calypso Jumbo' orchid hybrids, pretreatment with 300 nL•L^{-1} 1-MCP, followed by using 5.2 mg•L^{-1} aqueous ozone as the vase solution,

could be recommended as postharvest treatments to maintain quality and extend vase life by increasing percentage bud opening (Almasi et al. 2015). Conversely, fumigation of 1-MCP at 0, 250, 500, 1000, 1500, and 2000 nL•L^{-1} for 4 h at 25 °C had no effect on vase life of cut *Dendrobium* 'Burana Jade' inflorescences, but all 1-MCP treatments delayed bud drop and open flower abscission (Yoodee and Obsuwan 2013). Khunmuang et al. (2016) also found that the vase life of *Vanda* 'Sansai Blue' fumigated with 1-MCP at 200 nL•L^{-1} for 6 h was similar to that of control flowers.

Silver thiosulfate (STS) is widespread commercial used to inhibit effects of ethylene and prolongs vase life in many ornamentals including orchid (Beyer Jr. 1976). Silver is more effective when applied as silver thiosulfate (STS) – $[Ag(S_2O_3)_2]$, because the mobility of STS is faster and less phytotoxic to the tissues than silver nitrate ($AgNO_3$) (Van Altvorst and Bovy 1995). The mode of action of silver is to complete with copper for receptor binding sites (Ciardi and Klee 2001; Kumar et al. 2009). However, the limitation of STS treatment is the narrow range of concentrations at which STS is not phytotoxic and is effective as an ethylene inhibitor (Nell 1993). The relatively low concentration and short immersion time is needed for treatments. Ethylene production in emasculated *Cymbidium* flower was partially inhibited by STS (Goh et al. 1985). In *Phalaenopsis*, STS pulsing also prevented damage from ethylene (De et al. 2014). Pulsing 500 mg•L^{-1}silver nitrate for 30 min extended the vase life of *Oncidium* 'Golden Shower' flowers (Ong and Lim 1983). In addition, the vase life of *Aranda* flowers was significantly increased by pulsing with 4 mM STS for 10 min (Hew et al. 1987), and STS (ca. 0.07 mM) pulse treatment for 30 min extended the vase life of *Dendrobium* from 23 to 30 days (Nowak and Vacharotayan 1980). However, treatment with 4 mM STS for 30 min did not extend the longevity of *Cattleya* flowers (Goh et al. 1985).

STS pulsing combined with chlorine as the vase solution was very effective in inhibiting the endogenous ethylene effects, preserving the quality and extending the longevity of cut *Vanda* 'Sansai Blue' orchids to 19 days. Chlorine and long-life treatments improved the flower quality by increasing the number of open flowers and improving the solution uptake (Khunmuang et al. 2018b) (Fig. 5.13).

5.4 Conclusion

In cut orchid flowers, the sensitivity to ethylene is probably responsible for cultivar variation. Pollination accelerates flower senescence, which is accompanied by a climacteric-like rise in ethylene production. Orchid inflorescences have several open flowers and buds; thus, the maturity stage when harvested is pretty much influence the vase life. The number of open flowers, flower size, and petal pigmentation is important and involves on postharvest quality.Sugar application improves bud opening, but also increase microbial growth. Microbial blockage in xylem is limited water uptake, resulting in flower wilting. Application of germicides might help to decrease this risk. In addition, using ethylene inhibitors such as AOA, AVG, 1-MCP, and STS suppressed ethylene production. Single postharvest treatment may not remarkably prolong the vase life. These research findings led us to assume the

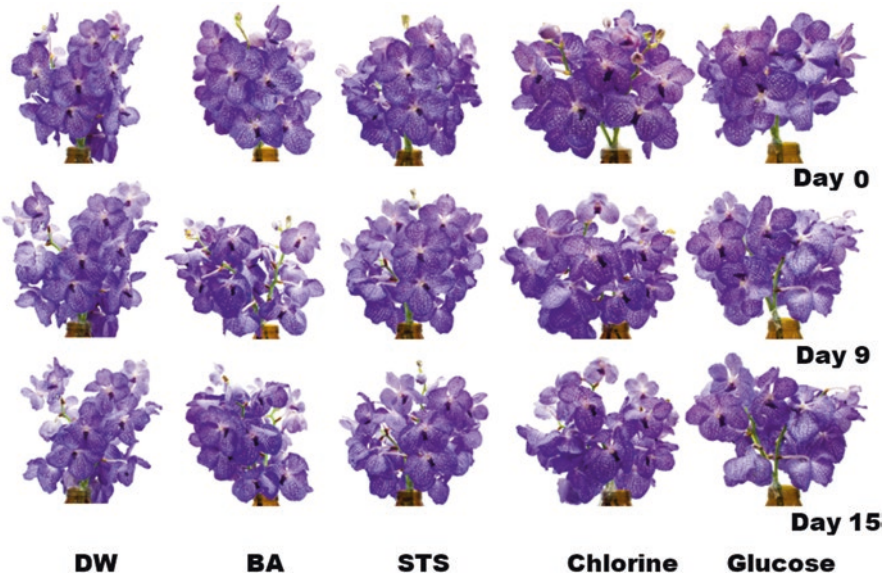

Fig. 5.13 Visual appearances of *Vanda* 'Sansai Blue' pulsed with 110 μMbenzyladenine (BA) (0.25% TOG-L-101®), 0.19 mM STS (0.25% STS-75®), 140 μg•L^{-1} active chlorine (0.025% TOG-6®), and 10 g•L^{-1} glucose (long life, LL, "cut flower food" formulation) for 24 h. After pulsing, all treated flowers were transferred to a vase solution of 0.025% TOG-6. Flowers pulsed with long life remained in the same solution. All flowers were kept in an observation room (21 ± 2 °C, 70–80% RH, cool-white fluorescent lights for 12 h/day)

following situation during postharvest handling system of cut orchids: harvested cut orchid flowers pretreated with postharvest treatment depending on their need before they are packed in a box, stored at low temperature ready for transport.

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Cryobiotechnology of Korean Orchid Biodiversity: A Case Study Using *Cymbidium kanran*

6

Elena Popova and Haeng-Hoon Kim

Abstract

Orchids are exceptionally vulnerable to climatic changes and destruction of natural habitats. Many orchid species become extinct at both national and global levels, and their conservation is a matter of critical importance. In vitro and cryobiotechnology approaches are effectively used to establish medium-term and long-term collections of threatened orchid species. In this study, the feasibility of droplet vitrification and preculture desiccation methods for cryopreservation of the Korean orchid *Cymbidium kanran* was tested using sections, lateral buds and apical tips excised from in vitro grown rhizomes. Preculture with 0.5–1.0 mg/l ABA for 10–21 days followed by treatment in a liquid medium with gradually increasing sucrose concentration enhanced tolerance of rhizome sections and buds to both vitrification solutions and desiccation. Droplet vitrification of rhizome buds and sections using PVS2, PVS3 or alternative vitrification solution A3 was not feasible under conditions tested in the study. High regrowth (86–90%) of rhizome sections after cryopreservation was achieved following the combination of ABA-and-sucrose preculture with desiccation over silica-gel to water content below 34%. This study opens the door for the development and optimization of cryopreservation methods for endangered terrestrial Asian orchids that are usually difficult to propagate and conserve ex situ.

Keywords

Abscisic acid · Cryopreservation · Biodiversity conservation · *Cymbidium kanran* · Droplet vitrification · Rhizome · Preculture desiccation

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6.1 Introduction

Terrestrial orchids native to China, Korea and Japan have long been valued for their elegantly shaped leaves and fragrant flowers of delicate colours (Paek and Yeung 1991). For centuries, these plants have been an integral part of oriental culture. It is not uncommon that modern commercial cultivars of "oriental" orchids such as terrestrial *Cymbidiums* are sold for more than $500 per pot (Paek and Murthy 2002). As a result of their popularity in modern plant trade, some species of terrestrial orchids became nearly extinct in their natural habitats, and urgent conservation measures, both in situ and ex situ, are required to protect their biodiversity (Park et al. 2000; Fang et al. 2011).

Cryopreservation is acknowledged as one of the most effective and feasible options for the long-term conservation of orchid biodiversity (Pritchard and Seaton 1993; Hirano et al. 2006; Popova et al. 2016). Successful cryopreservation of orchids was reported using mature and immature seeds (Pritchard 1984; Koopowitz and Thornhill 1994; Ishikawa et al. 1997; Nikishina et al. 2007; Hirano et al. 2009; Hay et al. 2010; Wu et al. 2013), pollen (Pritchard and Prendergast 1989; Vendrame et al. 2008; Ajeeshkumar and Decruse 2013), vegetative organs such as shoot tips (Thinh and Takagi 2000; Lurswijidjarus and Thammasiri 2004; González-Arnao et al. 2009), protocorms and protocorm-like bodies (Thammasiri 2008; Sopalun et al. 2010; Gogoi et al. 2013; Teixeira da Silva 2013), meristematic clusters (Kondo et al. 2001) and cell suspensions (Tsukazaki et al. 2000). Meanwhile, there are a limited number of studies on cryopreservation of terrestrial Asian orchids, mostly due to significant constraints in their cultivation in vitro. Very low germination of mature seeds under aseptic conditions was reported for terrestrial orchid species (Kokubu et al. 1980; Arditti et al. 1982; Park et al. 2000). The physiology of seed development in terrestrial Asian orchids differs from seed development in epiphytic species. For example, seeds of tropical *Cymbidium spp*. germinate to form protocorms that eventually develop into the whole plants; by contrast protocorms of oriental terrestrial *Cymbidiums* develop into specific organs, rhizomes, and may remain at this stage for several years without progressing to shoot formation. Very slow, compared to tropical epiphytic orchids, growth of rhizomes which may be limited to 2 mm per month was another issue hampering in vitro cultivation and cryopreservation of terrestrial Asian orchid species (Kokubu et al. 1980; Lee and Paek 1996). However, later advances in tissue culture technology and focused work of several researcher groups led to the development of effective micropropagation protocols for ecologically and commercially important oriental orchids of *Cymbidium, Geodorum* and some other genera; most of these methods depend on rhizome multiplication in vitro (Paek and Yeung 1991; Sheelavantmath et al. 2000; Chiang et al. 2010; Fang et al. 2011). Active rhizome growth and branching under favourable culture conditions were reported for some species including *Cymbidium kanran* (Paek et al. 1989, 1992; Lee and Paek 1996; Fang et al. 2011). Even short sections of rhizomes carry multiple vegetative buds each of those having capacity to develop into new shoots. This makes rhizomes a promising material for cryopreservation with potentially higher postcryogenic multiplication ability than the individual

shoot tips or protocorms. Meanwhile, to our best knowledge, there were no successful attempts to cryopreserve orchid rhizomes.

In this work we explored two approaches, droplet vitrification (DV) and preculture desiccation, to cryopreservation of orchid rhizomes using *C. kanran*, an endangered Asian orchid, as a model species. The effects of both "classical" (PVS2 and PVS3) and alternative vitrification solutions (VS) were studied, and preculture with different concentrations of ABA and sucrose was used to improve tolerance of rhizome parts to cryoprotectant and desiccation treatments.

6.1.1 Plant Material

In vitro plants of *C. kanran* Makino with rhizomes were received from the Yeomiji Botanic Gardens and maintained on Hyponex medium (6.5–6-6, Hyponex Co. Ltd., Tokyo, Japan) with 30 g/l sucrose, 1.0 g/l activated charcoal and 2.3 g/l phytagel, pH = 5.6 (Standard medium), in 220 ml Magenta jars. Cultures were kept in a growth room at 23 ± 1 °C under a 16-h photoperiod provided by cool white fluorescent lamps at light intensity of 35–40 μmol m^{-2} s^{-1}. Subcultures were done every 6 weeks by transplanting newly formed plants with rhizomes to a fresh medium. Single lateral buds (5 mm in length) and sections (15–18 mm in length, containing 5–6 lateral buds) were excised from actively growing rhizomes 6 weeks after the last subculture and used in cryopreservation experiments.

6.1.2 Droplet Vitrification

Individual lateral buds and sections of rhizomes selected as described above were cryopreserved using the droplet vitrification method (Panis et al. 2005) following a two-step preculture. Preculture step 1 was performed on Hyponex medium with 2.3 g/l phytagel, 3 or 10% sucrose and 0–1.0 mg/l ABA for 3 or 10 days. At preculture step 2, buds and sections were treated sequentially in liquid MS medium (Murashige and Skoog 1962) with 0.3, 0.5 and 0.7 M sucrose for 24, 6 and 16 h, respectively. Precultured materials were osmoprotected in solution C4–35% (17.5% glycerol +17.5% sucrose, w/v, Kim et al. 2009a) for 40 min and exposed to vitrification solutions A3 (37.5% glycerol +15% dimethylsulfoxide (DMSO) + 15% ethylene glycol +22.5% sucrose, w/v, Kim et al. 2009b), PVS2 (30% glycerol +15% DMSO +15% ethylene glycol +0.4 M sucrose, w/v, Sakai et al. 1990) or PVS3 (50% glycerol +50% sucrose, w/v, Nishizawa et al. 1993) for various durations at 0 or 23 °C. One minute before the end of the VS treatment, half of the materials was transferred onto aluminium foil strips (6 × 30 mm), five buds or sections per foil, and covered with several drops of the same VS. Foils were quickly immersed into liquid nitrogen and kept for a minimum of 60 min before rewarming for 20 s in 20 ml of MS medium with 0.8 M sucrose solution (unloading solution) preheated at 40 °C. After that, an equal volume of unloading solution at 23 ± 2 °C was added; foils were removed by forceps, and materials were left for 30 min at 23 ± 2 °C on a

rotary shaker (90 rpm). Another half of the materials from the vitrification solution was transferred directly to the unloading solution and served as a non-cryopreserved control. After unloading, rhizome buds and sections were blotted dry on sterile filter paper and recovered on standard medium containing 1.0 mg/l GA_3 and 0.5 mg/l BA in darkness for 1 week then transferred to 35 $\mu Em^{-2}\ s^{-1}$ light intensity.

Apical tips, 1.5–2 mm in length, were excised from actively growing rhizomes 6 weeks after the last subculture, precultured in liquid MS medium with 17.5% sucrose for 31 h, then with 25% sucrose for 17 h and osmoprotected in solution C4-35% for 15 min followed by solution C11-60% (30% glycerol +30% sucrose) for 25 min. Apical tips were cryoprotected with PVS3 for 90 min at room temperature and cryopreserved using aluminium foil strips as described above for 1 day. Apical tips were rewarmed in an unloading solution composed of MS medium with 0.1 M sucrose preheated at 40 °C followed by 40 min unloading and postculture under conditions described above.

All cryoprotectant solutions used at different steps of the protocol were prepared based on liquid MS medium and sterilized by vacuum filtration through 0.2 μm filters (Falcon, USA).

6.1.3 Preculture Desiccation of Rhizome Sections

As an alternative to droplet vitrification, the preculture desiccation method was tested for rhizome sections. After preculture on standard medium with 0.5–1.0 mg/l ABA for 3–4 weeks (preculture step 1), the sections were transferred to liquid MS medium with 0.3–0.7 M sucrose for varied durations (preculture step 2) and then placed on sterile filter paper in a desiccator above 250 g activated silica gel (40 sections per desiccator) for 0–21 h. Dried sections were put in cryovials that were quickly immerged into liquid nitrogen. After cryogenic storage for 24 h, vials were rewarmed in a 40 °C water bath for 90 s and rhizome sections were recovered on standard medium with 1.0 mg L^{-1} GA_3 and 0.5 mg L^{-1} BA in darkness for 1 week and then transferred to 35 $\mu Em^{-2}\ s^{-1}$ light intensity.

6.1.4 Water Content Measurement and DSC Thermal Analysis

Water content (WC) in rhizome sections at various stages of preculture, cryoprotectant treatment and desiccation was determined after drying at 104 °C for 24 h and calculated as a percentage of fresh weight basis (%FW). Measurements were performed in three replications for each data point; each replication consisted of minimum ten sections. A differential scanning calorimetry (DSC) system DSC822 (TA8000 Mettler-Toledo, GMbh, Switzerland) was used for recording enthalpies during rewarming. Rhizome sections were placed in 40 μl aluminum pans that were cooled at the rate of 10 °C min^{-1} from 25 °C to −85 °C, followed by isothermal hold at −85 °C for 2 min and heated up to 25 °C at the rate of 10 °C min^{-1}. DSC analyses were performed using an average of 3 or 4 samples and at least three replications for each experimental condition.

6.1.5 Recovery Growth

Survival was measured 24 h after rewarming using the 2,3,5-triphenyltetrazolium chloride reduction (TTC) test. For each treatment, 15 rhizome buds or sections were placed in 5 ml of 1% TTC solution in phosphate buffer, pH = 5.7, in glass tubes (5 explants per tube) and incubated at 30 °C for 16 h. Viability was evaluated in marks from "–" to "++" depending on the area and saturation of the developed red colour. Regrowth was measured 40 days after treatment as number of buds or sections that resumed growth and produced new bright green rhizomes at least 1 cm in length.

All experimental treatments were performed in three replications; each replication consisted of 15 sections, if not stated otherwise. Data were analysed by ANOVA, followed by Duncan's Multiple Range Test (DMRT) at $p = 0.05$ using SAS University edition 3.4 (SAS Institute Inc. Cary, North Carolina, USA). Data in tables and figures are presented as mean values with standard errors. Mean values followed by different letters are significantly different at $p < 0.05$ (DMRT).

6.1.6 Droplet Vitrification of Rhizome Lateral Buds, Sections and Apical Tips

In the first experiment, two types of rhizome explants, i.e., individual lateral buds and rhizome sections with 5–6 lateral buds, were tested for their response to droplet vitrification protocol without cryopreservation. Explants were precultured for 3 or 10 days with various concentrations of sucrose and ABA (Table 6.1). Individual lateral buds were found to be significantly more susceptible to the toxic effect of PVS2 than rhizome sections ($P = 0.029$). Regrowth of 16–40% was recorded for lateral buds precultured with 3 or 10% sucrose irrespective of preculture duration. By contrast, 10-day precultures with ABA improved regrowth of PVS2-treated lateral buds to 81%, which was significantly higher than regrowth in any variant of sucrose preculture ($P = 0.010$, Table 6.1). For rhizome sections, the duration of the preculture was an important factor: 10-day precultures with sucrose or ABA produced significantly higher regrowth than 3-day precultures on the same medium ($P < 0.0001$). With both types of materials, highest regrowth after PVS2 treatment was observed following preculture on ABA-containing medium for 10 days (Table 6.1). Based on these results, rhizome sections precultured on medium with 3% sucrose and 0.5 mg/l ABA for 10 days were used in further experiments.

In the next experiment, alternative cryoprotectant solutions were tested, aiming to improve regrowth of rhizome sections after cryopreservation. Vitrification solutions A3 and PVS3 were applied to rhizome sections selected and precultured as described above; treatments were performed for 0–240 min at room temperature or in an ice bath. The toxic effect of VS A3 applied at room temperature was evident as regrowth of non-cryopreserved rhizome sections decreased from 100% in control (0 min) to 64% and 32% after 60 and 80 min of exposure, respectively (Fig. 6.1). Effect of VS A3 applied on ice was similar to the effect of PVS3 applied at room temperature: with both VS, regrowth was reduced below 50% only after 2 h of

Table 6.1 Effect of different concentrations of sucrose and ABA at preculture step 1 on the regrowth of individual rhizome buds and rhizome sections after exposure to PVS2 without cryopreservation

Material type and size	Preculture step 1 duration (days)	Preculture medium	Regrowth
Rhizome buds, 5 mm	3	3% sucrose	40.0 ± 13.0[b]
	3	10% sucrose	31.1 ± 8.9[b]
	10	3% sucrose	16.3 ± 9.5[b]
	10	10% sucrose	20.0 ± 0.0[b]
	10	3% sucrose +0.5 mg/l ABA	81.3 ± 4.4[a]
Rhizome sections, 15–20 mm	3	3% sucrose	38.4 ± 7.6[b]
	3	10% sucrose	39.3 ± 4.0[b]
	3	3% sucrose +0.5 mg/l ABA	41.1 ± 5.3[b]
	10	3% sucrose	66.1 ± 4.8[a]
	10	10% sucrose	61.9 ± 2.9[a]
	10	3% sucrose +0.5 mg/l ABA	78.7 ± 9.7[a]

Note: Individual buds or sections were excised from 6-week-old rhizomes and precultured on Hyponex medium with different concentrations of sucrose and ABA as showed in the table (preculture step 1). After that, materials were treated in a series of sucrose solutions, 0.3, 0.5 and 0.7 M sucrose for 24, 6 and 16 h, respectively (preculture step 2), osmoprotected in solution C4–35% (17.5% glycerol +17.5% sucrose) for 40 min and exposed to PVS2 (30% glycerol +15% dimethylsulfoxide +15% ethylene glycol +0.4 M sucrose) for 20 min at 23 °C. After unloading in 0.8 M sucrose for 40 min, buds and rhizomes were recovered on Hyponex medium with 1.0 mg/l GA_3 and 0.5 mg/l BA in darkness. Data presented as mean values with standard errors. Values followed by different letters are significantly different at $p = 0.05$ (DMRT)

exposure (Fig. 6.1). Contrarily to our expectations, none of the treatments resulted in regrowth after cryopreservation.

Additional tests demonstrated that increasing the duration of preculture step 1 to 3 weeks and doubling ABA concentration in the preculture medium (1.0 mg/l instead of 0.5 mg/l) enhanced rhizome tolerance to VS treatment (data not shown). Therefore, in the third experiment, rhizome sections were precultured on medium with 1.0 mg/l ABA for 21 days followed by the series of sucrose solutions and osmoprotective treatments and then exposed to VS A3 and PVS3 for as long as 210–360 min (Table 6.2). Short-term survival of rhizomes after VS treatment and cryopreservation was monitored using the TTC test. Water content of rhizome sections before cryopreservation and enthalpy changes during the rewarming process were also measured. As Table 6.2 shows, water content of rhizome sections was relatively high (53–56%) after treatment with PVS3 for 4 h and VS A3 for 6 h. Not surprisingly, ice melting peaks of 56–60 J/g were detected in these materials during rewarming (Table 6.2). During TTC test performed 24 h after cryopreservation, red colour indicating survival was observed in meristematic regions of rhizome sections cryoprotected with VS A3 (Fig. 6.5a); however, no survival or regrowth of cryopreserved materials were recorded in any of the treatments (Table 6.2 and Fig. 6.5b). These results suggested that even after 4–6-h-long treatment with highly

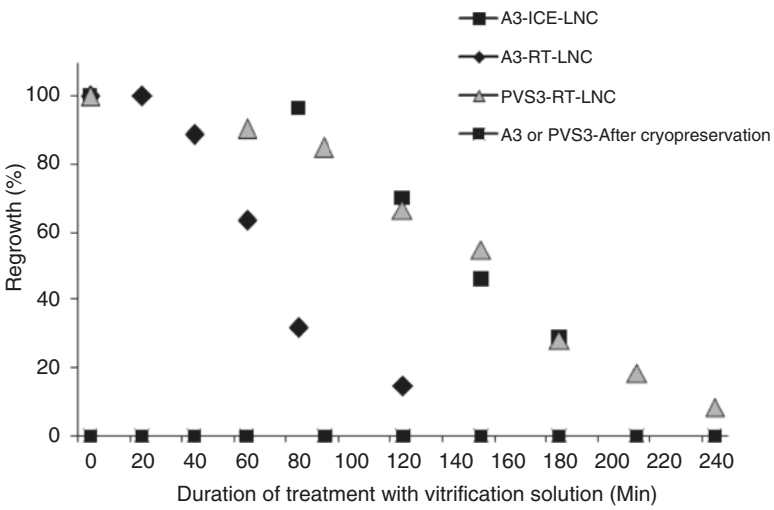

Fig. 6.1 Regrowth of rhizome sections after exposure to vitrification solutions PVS3 and A3 at 0 °C (ICE) or 23 °C (RT) for various durations before (LNC) and after (LN) cryopreservation Before being exposed to vitrification solutions, rhizome sections excised from 6-week-old rhizomes were precultured with 0.5 mg/l ABA for 10 days and then treated in the series of sucrose solutions (0.3, 0.5 and 0.7 M sucrose for 24, 6 and 16 h, respectively) and osmoprotected in solution C4–35% (17.5% glycerol +17.5% sucrose) for 40 min. After vitrification solution treatment, rhizome sections were placed into 0.8 M sucrose solution for 40 min for unloading and then recovered on regrowth medium containing 1.0 mg/l GA_3 and 0.5 mg/l BA in darkness. PVS3 was composed of 50% sucrose +50% glycerol, w/v; A3 was composed of 37.5% glycerol +15% DMSO +15% ethylene glycol +22.5% sucrose, w/v. No regrowth after cryopreservation was observed in any of the treatments and for simplicity, these treatments are shown as a single line. Data presented as mean values of two replications; each replication consisted of 15 rhizome sections (n = 30 for each data point)

concentrated vitrification solutions, rhizome sections were not sufficiently cryoprotected and dehydrated to withstand cryopreservation. Meanwhile, due to the toxic effects of VS during the long exposure, regrowth of rhizome sections was reduced to 66% without cryopreservation (Table 6.2). Additional experiments demonstrated that increasing the duration of osmoprotection with solution C4–35% from 40 to 80 or 100 min had no positive effect on the regrowth (data not shown).

After several years, experiments on droplet vitrification were revisited using apical tips of rhizomes and a modified procedure, now including two-step osmoprotection. All rhizome apical tips survived after preculture, osmoprotection and cryoprotection, and 90% of them looked healthy 10 days after cryopreservation (data not shown). Cryopreserved apical tips remained green for a month or longer but most of them failed to proliferate and eventually died.

Based on these observations, it was decided that cryopreservation of rhizome parts via droplet vitrification method may not be feasible under the conditions tested in the study, and alternative cryopreservation methods should be investigated.

Table 6.2 Water content, TTC-test based viability, regrowth and enthalpy at rewarming of rhizome sections after exposure to vitrification solutionsA3 and PVS3 (-LN) followed by cryopreservation (+LN)

Vitrification solution (VS)	Duration of VS treatment (min)	Water content (%FW)	Viability based on TTC test		Regrowth ± SE (%)		Enthalpy at rewarming (Jg^{-1})
			−LN	+LN	−LN	+LN	
A3	210	61.6 ± 0.4	+	+	82.6 ± 5.8	0.0	85.0
	270	n/d	+	+	71.6 ± 16.0	0.0	66.7
	360	55.5 ± 3.6	n/d	n/d	66.7 ± 0.0	0.0	60.3
PVS3	180	54.8 ± 0.4	++	–	89.7 ± 5.2	0.0	95.1
	240	53.1 ± 2.8	++	–	66.2 ± 8.3	0.0	56.1

Note: Rhizome sections were excised from 6-week-old rhizomes and precultured on Hyponex medium with 1.0 mg/l ABA for 3 weeks (preculture step 1) then treated in the series of sucrose solutions, 0.3, 0.5 and 0.7 M sucrose for 24, 6 and 16 h, respectively (preculture step 2), osmoprotected in solution C4–35% (17.5% glycerol +17.5% sucrose) for 40 min and exposed to VS A3 on ice or PVS3 at 23 °C for the indicated durations. After unloading in 0.8 M sucrose for 40 min, rhizome sections were recovered on Hyponex medium containing 1.0 mg/l GA_3 and 0.5 mg/l BA in darkness. TTC colour gradation: –, no colour; +, pale red colour in meristematic regions; ++, dark red colour in meristematic regions, n/d, not determined. Data presented as mean values with standard errors

6.2 Preculture Desiccation of Rhizome Sections

In the first experiment, rhizome sections were precultured on medium with 1.0 mg/l ABA under light, dark or combination of light/dark conditions then desiccated over silica-gel (Fig. 6.2). After, 13 h of desiccation, rhizome sections that were kept in darkness during the whole preculture period had significantly higher WC (53%) than sections precultured under light or light/dark conditions (27–29% WC). Not surprisingly, regrowth of rhizome sections that were kept in the dark was also higher than regrowth of the materials precultured under light or light/dark combination. Based on these results, in further experiments both preculture and desiccation steps were performed in darkness.

The water content of rhizome sections after ABA and sucrose preculture was 75% and decreased to 21% after 21 h desiccation over silica gel (Fig. 6.3). 100% regrowth of control (noncryopreserved) rhizome sections was recorded at WC above 40%. After 13 h of desiccation (WC = 34%), regrowth gradually declined and reached 67% after 21 h of desiccation. After cryopreservation, regrowth of 20–25% was observed only for rhizome sections desiccated to WC below 34% (Fig. 6.3). Exothermal peaks of 2–12 J/g were recorded during rewarming of sections

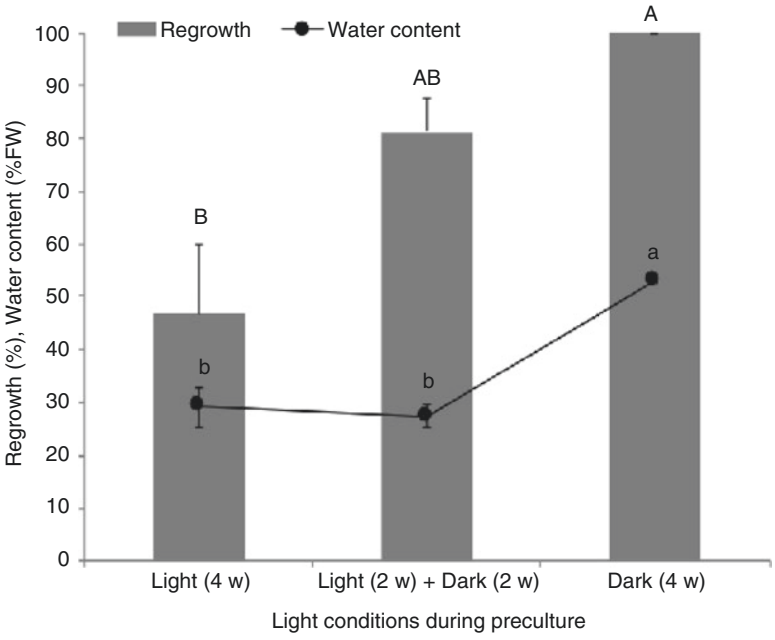

Fig. 6.2 Effect of light and darkness during preculture of rhizome sections on medium with 1.0 mg/l ABA for 4 weeks (preculture step 1) on their water content and regrowth after desiccation for 13 h without cryopreservation; *w* weeks

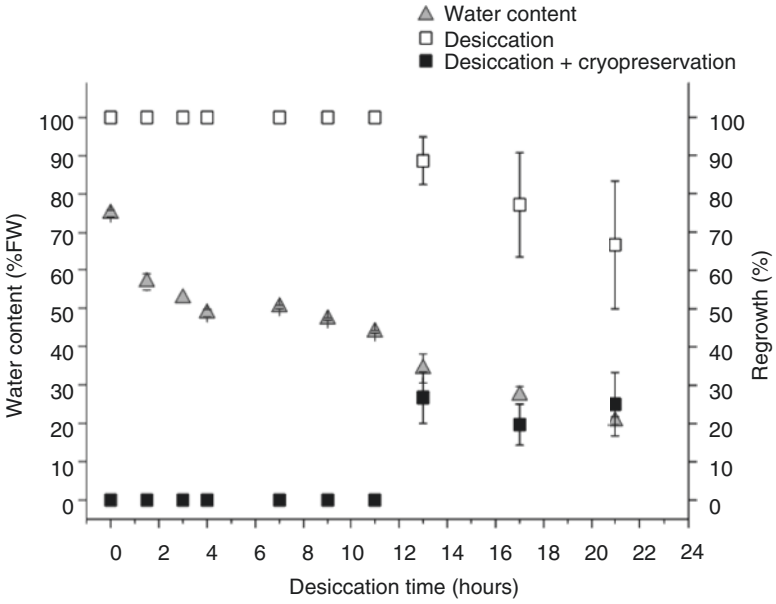

Fig. 6.3 Water content and regrowth of control and cryopreserved rhizome sections as affected by duration of desiccation over silica gel. Prior to desiccation, rhizome sections were precultured on medium with 0.5 mg/l ABA for 3 weeks in darkness then in liquid MS medium with 0.3 M sucrose for 24 h followed by 0.7 M sucrose for 24 h

desiccated for 13 h while no peaks were detected for rhizomes desiccated for longer durations (data not shown). However, no significant difference in the postcryopreservation regrowth was observed between rhizome sections desiccated for 13, 17 and 21 h.

Significant improvement of regrowth after cryopreservation was achieved when preculture with sucrose was prolonged to several days (Fig. 6.4). In the best treatment, rhizome sections precultured on ABA-containing medium for 3 weeks were placed into 0.3 M sucrose for 5 days followed by 0.5 M sucrose for 2 days, and 0.7 M sucrose for 24 h (preculture 2 in Fig. 6.4). Additional preculture step with 1.0 M sucrose for 7 h also improved regrowth. Following these treatments, regrowth after cryopreservation reached 86–90% which was comparable to regrowth of desiccated non-cryopreserved rhizome sections (89–100%). As shown in Table 6.3, each rhizome section taken to the experiments contained 5–6 lateral buds from which 2–4 buds were able to resume growth after cryopreservation and produce new rhizomes. Therefore, each section after cryopreservation produced several new rhizomes (Fig. 6.5c) that could be further multiplied and developed into normal plants.

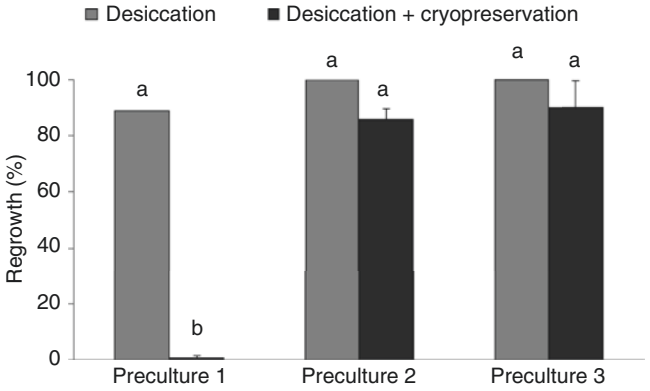

Fig. 6.4 Regrowth of rhizome sections after desiccation and cryopreservation following three variants of sucrose preculture. Rhizome sections excised from 6-week-old rhizomes were precultured on medium with 1.0 mg/l ABA in darkness for 3 weeks and then in the series of sucrose solutions with gradually increased concentrations (precultures 1–3). Precultured materials were desiccated over silica gel for 17 h and cryopreserved in 2-ml cryovials. After 24 h of cryogenic storage, cryovials were rewarmed in a water bath at 40 °C for 90 s, and rhizome sections were recovered on medium with 1.0 mg/l GA_3 and 0.5 mg/l BA in darkness. Data presented as mean values with standard errors. Values followed by different letters are significantly different at $p = 0.05$ (DMRT). Preculture 1: 0.3 M sucrose (120 h) ➔0.5 M sucrose (79 h); preculture 2: 0.3 M sucrose (120 h) ➔ 0.5 M sucrose (48 h) ➔ 0.7 M sucrose (31 h); preculture 3: 0.3 M sucrose (120 h) ➔0.5 M sucrose (48 h) ➔ 0.7 M sucrose (24 h) ➔ 1.0 M sucrose (7 h)

Table 6.3 Average number of survived buds on rhizome sections after desiccation and cryopreservation following two variants of preculture

Preculture*	Treatment	Average number of buds per rhizome section taken to experiment	Average number of buds per rhizome section that showed regrowth
Preculture 2	Desiccation	5.62 ± 0.25^{a}	3.17 ± 0.50^{ab}
	Desiccation + cryopreservation	6.05 ± 0.49^{a}	3.14 ± 0.35^{ab}
Preculture 3	Desiccation	6.22 ± 0.32^{a}	3.78 ± 0.46^{a}
	Desiccation + cryopreservation	5.60 ± 0.56^{a}	2.10 ± 0.38^{b}

Data presented as mean values with standard errors. Values followed by different letters are significantly different at $P = 0.05$ (DMRT).
*Preculture treatments as in Fig. 6.4. Preculture 2: 0.3 M sucrose (120 h) ➔ 0.5 M sucrose (48 h) ➔ 0.7 M sucrose (31 h); preculture 3: 0.3 M sucrose (120 h) ➔0.5 M sucrose (48 h) ➔ 0.7 M sucrose (24 h) ➔ 1.0 M sucrose (7 h)

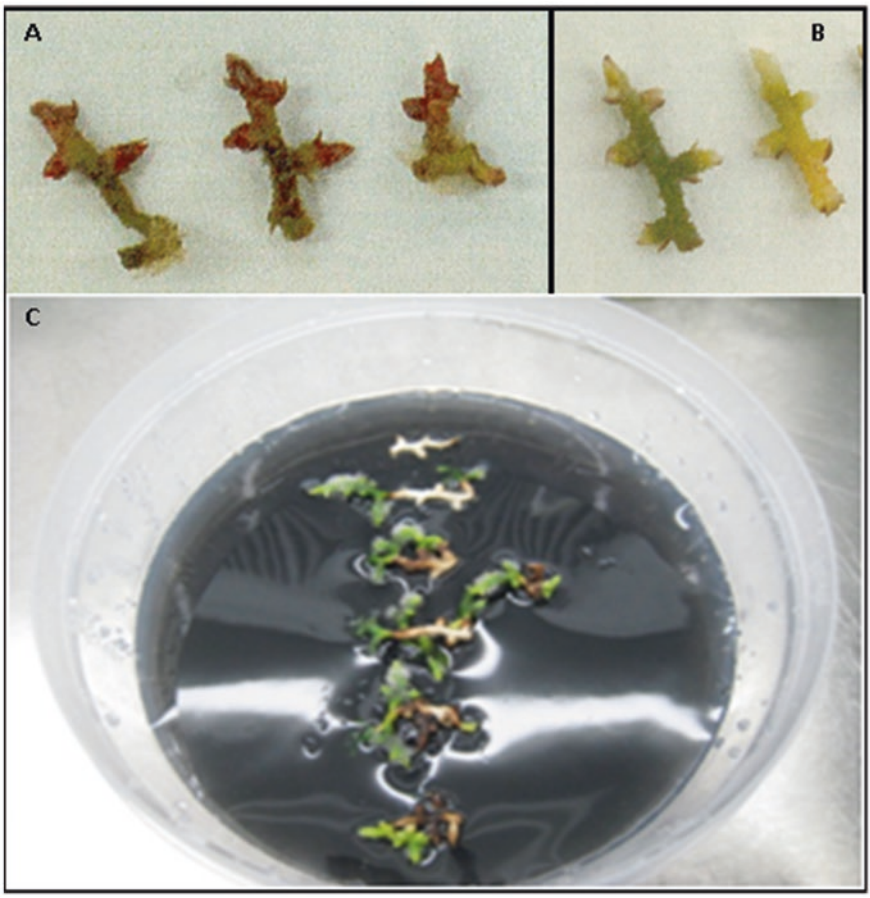

Fig. 6.5 (**a**, **b**) TTC test of rhizome sections cryoprotected with VS A3 for 270 min before (**a**) and 24 h after (**b**) cryopreservation. Treatments as described in Table 6.2. (**c**) Regrowth of rhizome sections cryopreserved using the preculture desiccation method (preculture 2 in Fig. 6.4)

6.3 Cryobiotechnological Methods for Orchids

Cryopreservation of in vitro cultured rhizomes and tubers attract increasing attention as an alternative method for the long-term conservation of both crops and endangered wild plants. Recent examples include cryopreservation of rhizomes of *Asparagus officinalis* (Carmona-Martín et al. 2018) and microtubers of potato (Uchendu et al. 2016). In this study, we explored the feasibility of cryopreservation for the conservation of rhizome-producing terrestrial orchids native to Asia using *C. kanran* Makino as a model species. Among the two cryopreservation methods tested, preculture desiccation was more effective than droplet vitrification. Our results showed that even after long (4–6 h) cryoprotectant treatment with highly concentrated vitrification solutions, rhizome sections were not sufficiently

dehydrated and cryoprotected and were lethally damaged by cooling in liquid nitrogen and subsequent rewarming. Ice melting peaks were detected in rhizome sections at the rewarming stage confirming that freezable water remained in them after all cryoprotectant treatments. This may be a result of the specific structure of rhizome tissues which makes them less permeable to cryoprotectants compared to other orchid materials commonly used in cryopreservation such as shoot tips or protocorms. At the same time, 4–6-h exposure to VS reduced regrowth of rhizome sections from 100% in control to ca. 66% without cryopreservation. None of the combinations of preculture, osmoprotection and various VS tested in the study resulted in regrowth after cryopreservation. By contrast, desiccation of rhizome sections over silica gel reduced their WC to 21% without dramatically decreasing their viability. In addition, the preculture desiccation method is relatively simple compared to the multi-step process of droplet vitrification. It is known that some methods may be more effective than others for cryopreservation of specific orchid tissues. For example, vitrification was more effective than programmed freezing for cryopreservation of *Dactylorhiza fuchsii* protocorms (Nikishina et al. 2007). Sopalun et al. (2010) found that cryopreservation of *Grammatophyllum speciosum* protocorms was the most successful by using droplet vitrification method (38% regrowth) followed by encapsulation dehydration (24% regrowth) and encapsulation vitrification (14% regrowth) methods. At the same time, there are many examples demonstrating that the same orchid species can be effectively cryopreserved utilizing different methods and materials (see Hirano et al. 2006 and Popova et al. 2016 for review). We believe that the process of developing and adapting cryopreservation methods for the new material should be focused on the simplicity of the protocol and time and efforts required for its optimization. Green colour of rhizome apical tips observed 1 month after their cryopreservation using droplet vitrification suggests that this method still has some potential, and further modifications of the protocol steps may improve regrowth. Meanwhile, we suggest the preculture desiccation method to be used for cryopreservation of *C. kanran* rhizomes and, possibly, of other Asian terrestrial orchids due to its simplicity and high regrowth achieved after cryopreservation.

In both methods tested in this study, precultures with ABA and high concentrations of sucrose were critically important for regrowth of rhizome parts after cryoprotection or desiccation treatments and cryopreservation. The beneficial effect of ABA preculture to increase desiccation and cryopreservation tolerance of in vitro cultured orchid materials has been frequently reported. For example, preculture of *Dendrobium gratiosissimum* protocorms with 1 mg/l ABA for 7 days resulted in higher survival (67%) after cryopreservation than preculture with high sucrose concentrations (9%) (Bunnag et al. 2009). Preconditioning on medium containing 0.1–1.0 mg/l ABA before cryopreservation was reported to be important for survival of shoot primordia of *Cattleya loddigesii* var. hassisonian, *Cattleya walkeriana* and *Dendrobium* cv. "Yukidaruma" (Kondo et al. 2001) as well as for cell suspension of *Doritaenopsis* cv. New Toyohashi (Tsukazaki et al. 2000). Preculture of *D. candidum* protocorms on medium with 0.5 mg/l ABA for 3 weeks followed by cryopreservation using the vitrification protocol resulted in 88% regrowth (Wang et al.

1998). Bian et al. (2002) recorded extensive accumulation of soluble sugars, heat-stable proteins and dehydrins in PLBs of *Dendrobium candidum* following pretreatment with 0.1 mg/l ABA for 3 days. In our study, preculture with ABA for 21 days significantly improved tolerance of rhizome individual buds and sections to both vitrification solution treatments and desiccation. Positive effects of preculture were more prominent when it was performed in darkness.

Preculture with sucrose is an essential step in the majority of cryopreservation protocols developed for orchids. For example, positive effect of the step-wise preculture in sucrose-enriched medium on regrowth after cryopreservation was reported for protocorms of *Oncidium bifolium* (Flachsland et al. 2006) and *Dendrobium virgineum* (Maneerattanarungroj 2009). In our work, the terminal concentration of sucrose in the preculture medium was critical for regrowth of rhizome sections after desiccation and cryopreservation (Fig. 6.4). Terminal sucrose concentrations of 0.7 and 1.0 M resulted in significantly higher regrowth than 0.5 M. Similarly, Gogoi et al. (2013) reported that preculture of *Cymbidium* protocorms with 0.7 M sucrose for 20 h led to 70% post-cryopreservation regrowth compared to 3–4% regrowth after preculture with 0.3–0.5 M sucrose. By contrast to protocorms and meristematic tissues of other orchid species where 1–3-day preculture with 0.3–0.5 M sucrose is usually sufficient for induction of stress tolerance, *C. kanran* rhizomes required over 8 days (199 h) of 0.3, 0.7 and 1.0 M sucrose preculture for successful cryopreservation. This is, again, may be a result of the specific structure of rhizome tissues that are less permeable to osmotically active compounds in preculture and cryoprotectant solutions than other orchid organs. Histological studies and evaluation of cryoprotectant concentration in rhizome tissues at different stages of preculture and cryoprotectant treatments would be helpful for better understanding of rhizome responses to different cryopreservation protocols.

6.4 Conclusion

In this study, successful cryopreservation was developed for *C. kanran* Makino, an endangered Asian terrestrial orchid, using sections of in vitro cultured rhizomes. The preculture desiccation method composed of preculture with 1.0 mg/l ABA for 3 weeks followed by 8-day treatment in 0.3, 0.5 and 0.7 M sucrose solutions and 17 h of desiccation over silica gel resulted in 90% regrowth after cryopreservation. By contrast, zero regrowth of cryopreserved rhizome sections and lateral buds was observed following various combinations of preculture and cryoprotectant treatments in the droplet vitrification protocol. We suggest preculture desiccation as an effective and simple method for cryopreservation of orchid rhizomes that is worth to be tested with other Asian terrestrial orchid species of both commercial and ecological value.

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Part II

Orchid Biodiversity and Conservation

7 Species Diversity and Distribution of Orchids in Rudraprayag District, Uttarakhand, India

V. P. Bhatt

Abstract

This present investigation deals with the orchid flora of Rudraprayag district, Uttarakhand, Himalaya during 2014–17. A total of 35 genera with 77 species were recorded from the district, of which 40 were terrestrial, 25 were epiphytic, and 10 were recorded as epiphytic and lithophytic. The diversity represents 30% of the orchids reported from the state of Uttarakhand. A maximum number of orchid species were found in subtemperate zone between 1500 and 2000 m a.s.l. The result also shows that the species diversity decreases with increase in altitude. Temperate open forest had the maximum number of orchid species, while rocky/boulders habitat had the least orchid species richness.

Keywords

Orchids diversity · Rudraprayag · Distribution pattern · Conservation

7.1 Introduction

Orchids are among the most significant ornamental plants in the world and known for the attractive color and shape of their flowers. Orchidaceae includes about 788 genera (Mabberley 1997) and 24,500 species (Dressler 2006) and is the second largest family of flowering plants in the world. They are concentrated in three areas, notably Tropical America, Indo-Malayan, and the Eastern Himalaya. The majority of the species are epiphytic (72%), most abundant in tropical forests. Besides a wide range of the environmental factors in which they are distributed all over the world, barring a few isolated islands and frozen continent of the Antarctica, in India,

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northeastern region and Western Ghats have a maximum concentration of orchids. While epiphytic orchids occur in the Eastern Himalaya and Western Ghats, the terrestrial species abound in the middle elevation (1500–2500 m) zone of the Western Himalaya and are represented by 1129 species and 184 genera (Karthikeyan 2000). The state of Uttarakhand ranks fifth among the Indian states in terms of orchid richness and has about 237 species (Jalal et al. 2008). This is high compared to the rest of the North Indian states. In the state of Uttarakhand, the distribution of the orchids is extremely patchy. They are mainly concentrated along the riverine areas and pockets of moist forests (Jalal et al. 2007). Several valleys in the state are rich in terms of orchid species richness. Rudraprayag district is one among those which support a good number of orchid species. The present study was thus planned with a view of assessing the species diversity of orchids of Rudraprayag district of Uttarakhand, analyzing its distribution pattern and biological status, and suggesting appropriate strategies for their conservation.

The Rudraprayag district is located between 30°21′ and 31°16′ latitude and 78°59′ and 79°56′ longitude in the northern part of Western Himalaya of Uttarakhand (Fig. 7.1). The district covers the total geographical area of *2439 km*^{2}. The district covers a wide altitudinal range from 600 m a.s.l. to 6000 m a.s.l. and mean annual temperature range from −40C to 34°. The forests are mainly dominated by different oak species, viz., *Quercus glauca* (Harinj), *Quercus leucotrichophora* (Banj), *Quercus floribunda* (Moru), and *Quercus semecarpifolia* (Kharsu), which form the climax vegetation at different climatic zones. Oak forests are not only fulfilling the

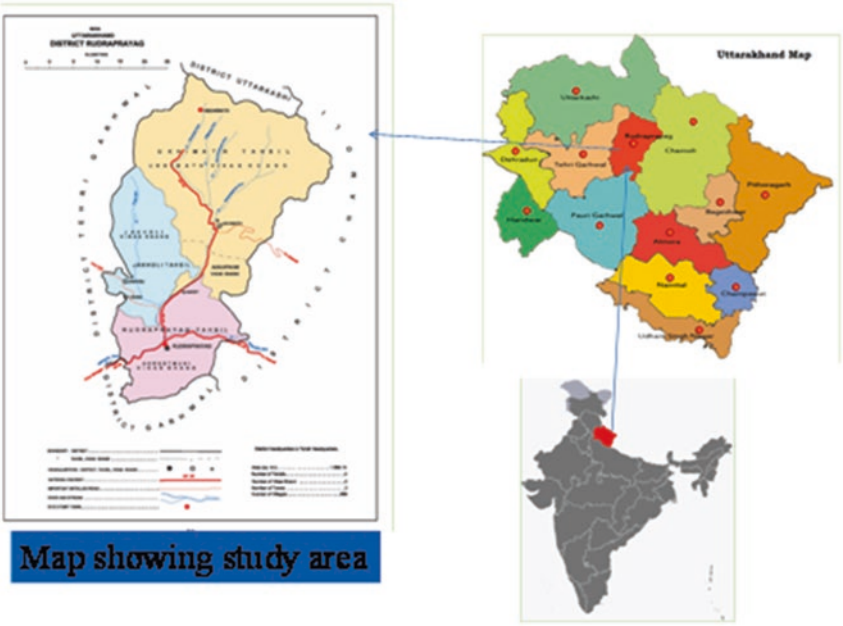

Fig. 7.1 Map showing the study area (Rudraprayag district)

day-to-day requirements of local inhabitants but also associated with the ecological and hydrological balance and support other species (orchids, ferns) to grow luxuriantly. The available information on the region (Semwal and Gaur 1981; Kala and Gaur 1982; Naithani 1984; Semwal 1984; Gaur 1999; Joshi et al. 2004) indicates the Rudraprayag district has not been investigated thoroughly for its floristic diversity including orchids.

Extensive surveys of orchids were conducted in various ecoclimatic zones of the Rudraprayag district during 2015–2017 covering summer and monsoon seasons. For each species encountered, information on habit, habitat, and distribution pattern was collected following a standard technique (Jain and Rao 1977). The collections were identified using standard regional floras (Naithani 1984; Deva and Naithani 1986; Polunin and Stainton 1984; Pangtey et al. 1991), and unidentified specimens were consulted to herbarium of the Forest Research Institute (FRI), Dehradun, and Botanical Survey of India (BSI), Dehradun, for identification. For each species, the distribution pattern was analyzed along the altitudinal gradient and within different habitats. The voucher specimens in the form of herbarium sheets and photographs have been deposited at the Govt. PG College Gopeshwar, Chamoli, Uttarakhand.

7.2 Orchid Diversity

Orchid species of 77 were recorded from Rudraprayag district, Uttarakhand, of these 40 were terrestrial, 25 were epiphytic, and 10 epiphytic and lithophytic (Table 7.1). *Dendrobium* (11 species, Fig. 7.2), *Bulbophyllum* (7 species), *Calanthe* (6 species), *Habenaria* (4 species), *Malaxis* (4 species), and *Goodyera* (3 species, Fig. 7.2) are the dominant genera of the district. Twenty genera, namely, *Aerides*, *Cephalanthera*, *Dactylorhiza* (Fig. 7.2), *Diplomeris*, *Eria* (Fig. 7.3a), *Epipactis*, *Galearis*, *Gastrochilus* (Fig. 7.3d), *Gymnadenia*, *Herminium*, *Kingidium*, *Nervilia*, *Pinalia*, *Pleione*, *Ponerorchis*, *Rhynchostylis*, *Satyrium*, *Spiranthes*, *Thunia*, and *Zeuxine*, were represented by a single species each.

7.3 Distribution Pattern

The distribution pattern of species along an altitudinal gradient suggests that the highest number of species (38) belonging to 19 genera was recorded from subtemperate zone and the lowest number of orchid species recorded in subtropical zone, whereas after subtemperate again the number decreases with increase in altitude. The high diversity of species in subtemperate zone may be due to the availability of multiple habitats and suitable climatic conditions (high humidity and temperature) for orchid growth (Fig. 7.4).

Table 7.1 Species diversity and distribution of orchid species in Rudraprayag district of Uttarakhand

S. no.	Species	Habit	Habitat	Flowering	Distribution range (m)	Conservation status
1	*Aerides multiflora* Roxb.	E, L	A, B	Apr–Sept	1000–2000	
2	*Bulbophyllum affine* Wall. ex Lindl.	E	B, C	May–July	800–1700	
3	*B. cariniflorum* Rchb. f.	L	B, C	July–Aug	800–2500	
4	*B. leopardinum* (Wall.) Lindl ex wall	E, L	B, C	May–Nov	1200–3000	LC
5	*B. muscicola* Rchb. f.	T, E, L	B, C	Aug–Sept	1600–1700	
6	*B. reptans* (Lindl.) Lindl ex wall.	E/L	A, B	July–Oct	1700–2500	
7	*B. umbellatum* Lindl.	E	B	May–July	1600–2000	
8	*B. wallichii* Rchb. f.	E	B	Apr–June	1600–1800	
9	*Calanthe alpina* Hook. f. ex Lindl.	T	A, B	July–Aug	1500–3500	Rare
10	*C. davidii* Franch.	T	A, B	July–Aug	1500–2000	Endangered
11	*C. mannii* Hook. f	T	A, B	May–June	1400–1800	
12	*C. plantaginea* Lindl.	T	A, C	Apr–May	1500–2000	
13	*C. puberula* Lindl.	T	A	July–Sept	1500–2000	
14	*C. tricarinata* Lindl.	T	B	Apr–July	2000–3500	
15	*Cephalanthera longifolia* (L.) Fritsch.	T	A	May–July	1500–3500	
16	*Coelogyne cristata* Lindl.	E	B, C	Apr–Aug	1600–1800	
17	*C. stricta* (D. Don) Schltr.	E	B, C	Sept–Oct	1500–1700	
18	*Cymbidium erythraeum* Lindl.	E	A, B	Sept–Nov	1500–1800	
19	*C. iridioides* D. Don	E	A, B	May–Oct	1600–2200	
20	*C. macrorhizon* Lindl.	E	A, B	June–July	1600–2000	
21	*Cypripedium cordigerum* D. Don	T	E	May–July	2000–3500	Rare
22	*C. elegans* Rchb. f.	T	E	July–Sept	3300–3500	
23	*C. himalaicum* Rolf	T	E	July–Sept	3300–3800	
24	*Dactylorhiza hatagirea* (D. Don) Soo	T	E	May–Aug	3200–3550	
25	*Dendrobium amoenum* Wall. ex Lindl.	E	B	April–June	600–2500	
26	*D. bicameratum* Lindl.	E/L	B, D	July–Aug	600–1600	
27	*D. densiflorum* Lindl.	E	B, D	June–Aug	600–1800	
28	*D. moniliforme* (L) Sw.	E	B, D	Apr–May	1500–2500	
29	*D. chrysanthum* Wall. ex Lindl.	E	B, D	May–June	800–1800	
30	*D. denudans* D. Don	E/L	B, D	Sept–Oct	800–1800	

(continued)

Table 7.1 (continued)

S. no.	Species	Habit	Habitat	Flowering	Distribution range (m)	Conservation status
31	*D. hesperis* (Seidenf.) Schuit. & Peter B. Adams	E	B, D	June–July	800–1500	Endangered
32	*D. heterocarpum* Wall. ex Lindl.	E	B	May–June	600–1600	
33	*D. macrostachyum* Lindl.	E	B	June–Aug	600–800	
34	*D. monticola* Hunt & Summerh.	E	B	Aug–Sept	1800–2000	
35	*D. fimbriatum* Hook.	E	B	May–June	800–2500	
36	*Diplomeris hirsuta* (Lindl.) Lindl	T/L	B	July–Aug	800–1000	Vulnerable
37	*Epipactis helleborine* (L.) Crantz.	T	A, B	June–Oct	1800–2500	
38	*Eria occidentalis* Seid	E	B	July–Aug	800–1000	Rare
39	*Galearis spathulata* (Lindl.) P.F. Hunt	T	B, D	July–Aug	3000–3500	
40	*Gastrochilus calceolaris* (Sm.) D. Don.	T	B, D	Sept–Oct	1500–1800	
41	*Goodyera foliosa* (Lindl.) Benth. ex Hook. f.	T	A, B	Aug–Sept	2500–2900	Endangered
42	*G. repens* (L.) R. Br.	T	B, D	Aug–Oct	1500–3000	
43	*G. fusca* Hook. f.	T	E	Sept–Oct	3200–3500	
44	*Gymnadenia orchidis* Lindl.	T	E	May–Oct	3300–3550	
45	*Habenaria edgeworthii* Hook. f.	T	A, B	July–Aug	2500–3000	
46	*H. intermedia* D. Don	T	A, B	July–Aug	2500–3000	
47	*H. latilabris* Hook. f.	T	A, B, D	Aug–Sept	1500–3300	
48	*H. pectinata* D. Don	T	A, B	July–Sept	1500–2500	
49	*Herminium lanceum* (Thunb. ex Sw.) Vuijk	T	B	July–Oct	1500–2500	
50	*H. mackinnonii* Duthie	T	B	July–Aug	1600–2000	
51	*Kingidium taenialis* (Lindl.) Hunt.	E	A, B	May–June	1600–2000	
52	*Malaxis acuminata* D. Don	T	C	July–Oct	1600–2200	
53	*M. cylindrostachya* (Lindl.) Kuntz.	T	B	July–Aug	2000–3000	
54	*M. muscifera* (Lindl.) Ktze.	T	B, D, E	June–Oct	2900–3500	

(continued)

Table 7.1 (continued)

S. no.	Species	Habit	Habitat	Flowering	Distribution range (m)	Conservation status
55	*M. purpurea* (Lindl.) Kurtz.	T	C	July–Aug	1600–1900	
56	*Neottia acuminata* Schltr.	T	B, D	July–Aug	1600–3500	Rare
57	*N. microglottis* (Duthie) Schlr.	T	A, B	Aug–Sept	200–2500	Rare
58	*Nervilia mackinnoni* (Duthie) Schultz.	T	A, B	Aug–Oct	1600–1900	
59	*Oberonia pachyrachis* Rchb. f. ex Hook. f.	E	B	Aug–Oct	1000–1700	
60	*O. pyrulifera* Lindl.	E	A, B	June–Sept	2000–2500	
61	*Oreorchis indica* (Lindl.) Hook.	T	A, B	Sept–Oct	2800–3000	
62	*Or. micrantha* Lindl.	T	A, B	June–July	2500–3300	
63	*Peristylus elisabethae* (Duthie) Hunt	T	B	July–Aug	1800–2200	
64	*P. fallax* Lindl.	T	B	July–Aug	2200–2500	
65	*Pholidota articulata* Lindl.	E/L	A, B	June–July	1000–2000	
66	*P. imbricata* Lindl.	E/L	A, B	July–Aug	700–1600	
67	*Pinalia spicata* (D. Don) S.C. Chen & J.J. Wood	E	B	Aug–Oct	2300–2600	
68	*Pleione hookeriana* (Lindl.) More	E/L	D	May–June	2700–3000	
69	*Ponerorchis chusua* (D. Don) Soo	T	D	July–Aug	3200–3450	
70	*Rhynchostylis retusa* (L.) Blume	E/L	B	June–Aug	500–1000	
71	*Satyrium nepalense* D. Don	T	D, E	July–Sept	2600–3400	
72	*Spiranthes sinensis* (Pers.) Ames	T	B, D	June–Oct	2600–3000	
73	*Thunia alba* (Lindl.) Rchb. f.	E/L	B, C	June–July	1500–1700	
74	*Vanda alpina* Lindl.	E	E	June–July	800–3500	
75	*V. cristata* Lindl.	E	B	Aug–Sept	1500–1800	
76	*V. testacea* (Lindl.) Rchb. f.	E	B	June–July	600–800	
77	*Zeuxine flava* (Lindl.) Trimen	T	A, B	Feb–Mar	1500–1600	

T terrestrial, *E* epiphyte, *L* lithophyte, *A* Banj oak dense forests, *B* Banj oak open forests, *C* riverine, *D* Kharsu oak forests, *E* Alpine pastures

Fig. 7.2 **(a)** *Dendrobium amoenum*, **(b)** *D. bicameratum*, **(c)** D. heterocarpum, **(d)** Goodyera biflora, **(e)** Goodyera viridiflora **(f)** Dacttylorhiza hatagirea

7.4 Habitat-Wise Distribution

Five different orchid habitats (Banj oak open forests, Banj oak dense forests, riverine, Kharsu oak forests, and Alpine pastures) were identified in the study area. The maximum species richness was in the Banj oak open forests (62) followed by Banj oak dense forests (26) and Kharsu oak forest (17) (Fig. 7.5).

7.5 Conservation Status of the Taxa of the Region

Nayar and Shastry (1987, 1988, 1990) reveals that *Calanthe davidii* Franch and *Dendrobium hesperis* (Seidenf.) Schuit. & Peter B. Adams were recorded as Endangered, *Diplomeris hirsuta* (Lindl.) Lindl as Vulnerable, while *Cypripedium*

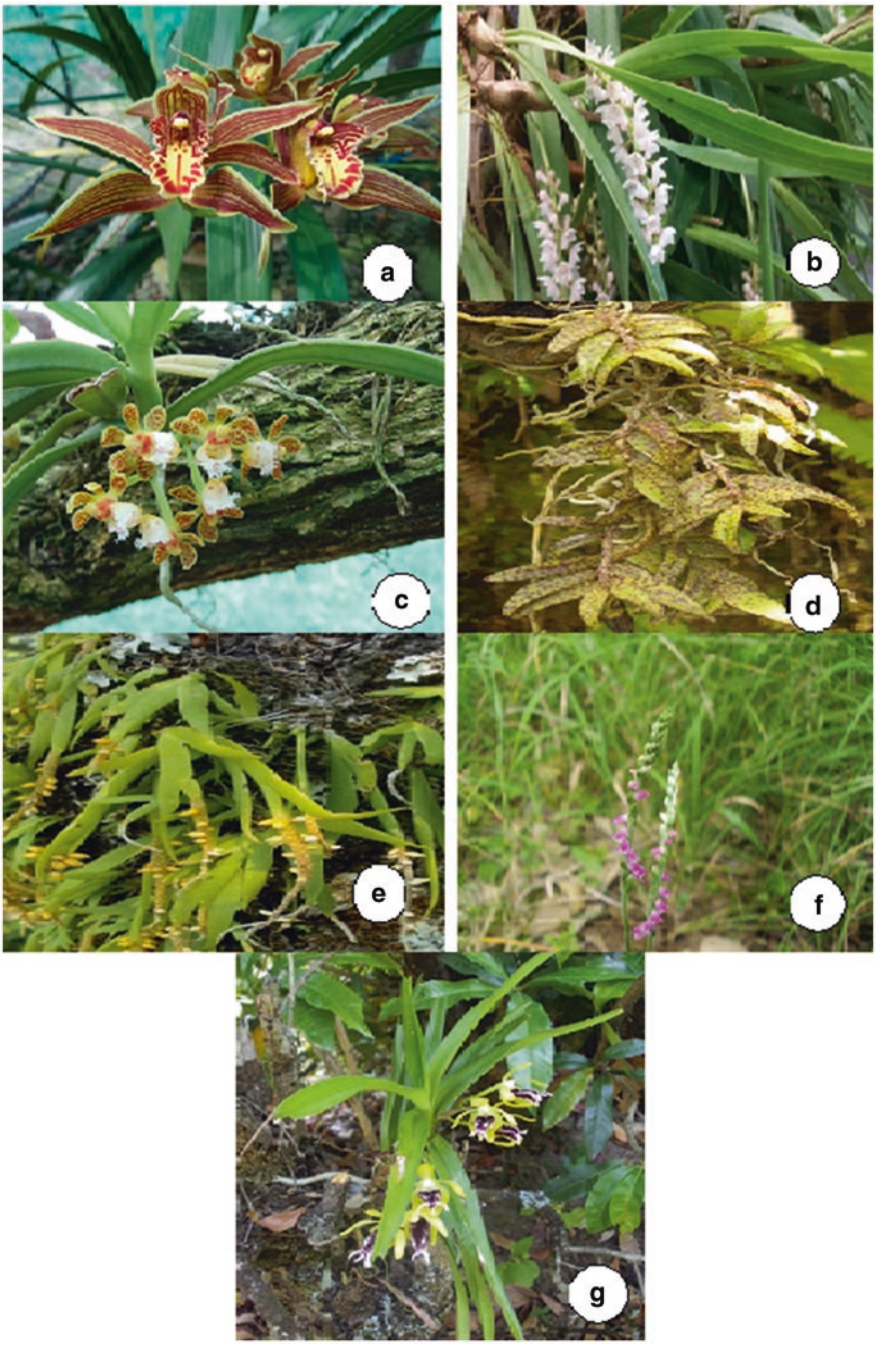

Fig. 7.3 (a) Cymbidium iridioides (b) Eria spicata (c) Gastrochilus acutifolius (d) Gastrochilus distichus (e) Oberonia pachyrachis (f) Spiranthes sinensis (g) Vanda cristata

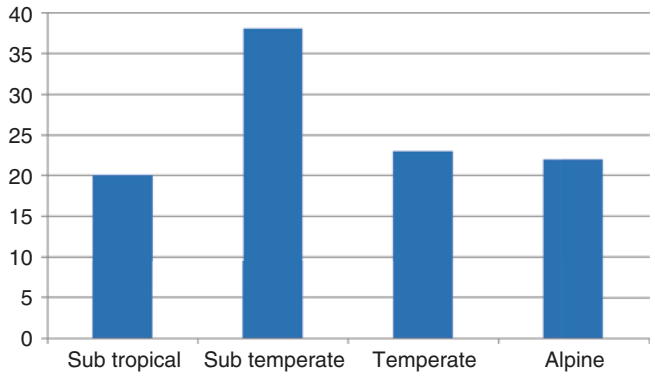

Fig. 7.4 Distribution of orchids in different climatic zones

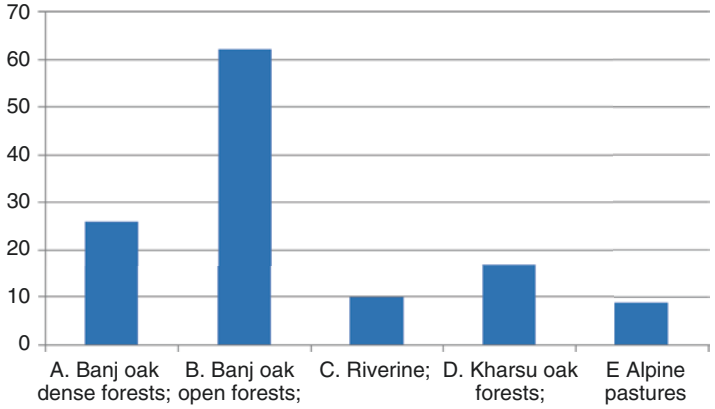

Fig. 7.5 Distribution of orchids in different habitats

elegans Rchb. f., *Cypripedium himalaicum* Rolfe, *Eria occidentalis* Seid, *Calanthe alpina* Hook. f. ex. Lindl., *Cypripedium cordigerum* D. Don, *Neottia acuminata* Schltr., and *Neottia microglottis* (Duthie) Schlr. as Rare. In addition, loss of habitat and low regeneration was also recorded during the study which further adds the serious threat to orchid population.

7.6 Conclusion

The present study highlights the diversity and distribution and conservation status of orchids in Rudraprayag district of Uttarakhand. The high diversity of terrestrial and epiphytic orchids in the temperate open forests shows the ideal habitat for the orchid growth with high humidity and temperature. The high richness of the orchids shows good condition of the forests (Jalal 2005). The conservation status of the

orchids, low regeneration and loss of habitat in the region call the attention for the conservation strategies and therefore, it needs proper conservation measures and sustainable utilization of these forests for the long-term management of these orchids.

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8 Status of Genetic Diversity and Its Characterization in Genus *Bulbophyllum* (Orchidaceae) from North-Eastern India

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Abstract

Orchids, which are cosmopolitan in distribution, belong to family Orchidaceae, having 1000 genera and up to 35,000 species, and are genetically diverse and of great economic importance. Bulbophylum, a medicinally important orchid, has quite intriguing aspects related to chromosome counts and structure. Several orchid enthusiasts have worked on cytogenetic diversity of the genus, chromosome banding pattern, besides developing some useful interspecific hybrids. However, molecular aspects of chromosomal DNA, an important genetic study, are by and large lacking. The review is an attempt to collate all the relevant cytogenetic information available and assess the extent of genetic diversity in genus *Bulbophyllum.*

Keywords

Bulbophyllum · Genetic diversity · Cytogenetics · Heterochromatin

8.1 Introduction

Orchids e cosmopolitan in distribution, occurring in every habitat, except in Antarctica and desert areas of the world. The great majority are found in the tropics, mostly in Asia, South America and Central America. Orchidaceae is considered as the largest and most diverse family among the monocotyledons comprising epiphytes/lithophytes/shrubs/herbs, and the most highly advanced in terms of floral specialization (Yonzone et al. 2011, 2012). About 1000 genera having 25,000–35,000 species (Yonzone and Kamran 2008) of orchids are reported throughout the world. Of these, 1331 species are

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grown/cultivated in India (Yonzone and Kamran 2008; Yonzone et al. 2011, 2012). North-eastern region of India is enriched with 151 genera of orchids comprising from 800 (Tondon and Kumaria 2010) to 876 species (Rao and Singh 2015). The taxa are mainly found in the eastern, north-eastern and north-western Himalayas, Peninsular India and Andaman and Nicobar Islands (De et al. 2015).

The immense variety of the climatic, edaphic and altitudinal variations in India has resulted in a great range of ecological habitats which in turn is reflected in the diversity of orchids as can be seen in genera, viz., *Dendrobiums*, *Cymbidiums*, *Bulbophyllums, Paphiopedilum delophyllum*, etc. Among these *Bulbophyllum* Thouars is the largest genus of the family Orchidaceae (Sieder et al. 2007; Chen and Vermeulen 2009) comprising about 1900 species, largely distributed in tropical regions of both new and old worlds (Chen and Vermeulen 2009), and these species are widely distributed in East Africa, India, Malaysia, East Asia, Australia and tropical America (Suzuki 1986). There are about 100 species reported from India, in which 63 of them are available in the north-eastern region of India (Augustine et al. 2001; Misra et al. 2007). Thirty-three to fifty-seven species of *Bulbophyllum* are reported from Meghalaya (Kataki 1986; Pandey et al. 2015; Rao and Singh 2015). Verma et al. (2015) recently reported a new species of *Bulbophyllum* from Meghalaya. About 30% of Indian species such as *B. hirtum*, *B. leopardinum*, *B. roseopictum* and *B. odoratissimum* (Fig. 8.1a–d) are reported to be endemic in nature (Augustine et al. 2001), while the remaining are known to be grown worldwide. Some of these species of *Bulbophyllum* found in the north-eastern region are, viz., *B. obrienianum*, *B. hastatum*, *B. scabratum*, *B. eublepharum*, *B. bisetum*, *B. cornu-cervi*, *B. elassonotum*, *B. leptanthum*, *B. cauliflorum*, *B. iners*, *B. clarkeanum*, *B. caudatum*, *B. rothschildianum*, *B. piluliferm*, *B. conchiferum*, *B. rigioum*, *B. striatum*, *B. ebulbum* and *B. parryae*. However, it is noteworthy that one of the species, *B. rothschildianum*, which was reported to be extinct from nature, has been recently rediscovered by Kumar (1992). Many of the Bulbophyllum species are epiphytic with pseudobulbs (enlarged stems) which are generated on the rhizome. These structures, function as water and nutrient storage organs (Saito 2006). Nishida et al. (2004) and Teixeira et al. (2004) have observed that species of Bulbophyllum inhabit different geographical regions ranging from subtropical dry forests to wet mountain cloud forests.

8.2 *Bulbophyllum*: Genetic Diversity and Its Characterization

The genus *Bulbophyllum* has high medicinal and horticultural value (Pant 2013). Quite a good number of *Bulbophyllum* species available in Meghalaya are economically important from an ornamental point of view, and they are prized for their incredible diversity in flower size, shape, form, colour and attractiveness which make them great ornamental plants (De et al. 2015). A few species like *B. keangtungense*, *B. leopardinum*, *B. maximum* and *B. odoratissimum* are reported to be of significant medicinal value and are used to treat fever, tuberculosis, inflammation,

Fig. 8.1 *Bulbophyllum.* (**a**) *B. hirtum*, (**b**) B. leopardinum, (**c**) B. roseopictum, (**d**) B. odoratissimum

etc. (Kumari et al. 2012; Pant 2013) and counter sorcery effects. Similarly, *B. neilgherrense*, *B. odoratissimum* and *B. sterile* are reported to be effective in curing leucoderma and rheumatism (Rajendran et al. 1997; Shanavaskhan et al. 2012).

Chromosome variation in the genus *Bulbophyllum* is quite intriguing because of inconsistency in the chromosome counts reported for the same species. The somatic numbers reported a range from $2n = 38$ to $2n = 42$ (Lim and Jones 1981). Although quite a good number of reports have been published on world species of *Bulbophyllum*, the same cannot be said for the species of north-eastern India. The basic number for the genus is still uncertain, making it difficult to estimate the ploidy level of various species and also to understand the karyological evolution of the genus. Triploid (3x) and pentaploid (5x) chromosome numbers of $2n = 3x = 57$ and $2n = 5x = 95$ are reported for the *B. patans* and *B. vagans*, respectively (Lim and Jones 1981; Augustine et al. 2001). Quite a good number of species of *Bulbophyllum* (about 18) were karyologically studied in detail, and karyotype formula was worked out by the late Prof. Viz and his students at the Department of Botany, Panjab

University, Chandigarh. However, these species are mostly from Western Himalayan region. Augustine et al. (2001) studied the karyomorphology and deduced the karyotypic formula of some species of *Bulbophyllum* from Meghalaya. However, their studies did not include many important taxa like *B. reptans*, *B. penicillium*, *B. cauliflorum*, *B. carniflorum*, *B. repens*, etc. A major noteworthy feature is that the above reports are confined only to karyological studies, while information on genome homology, recombination frequencies and disjunctional pattern at anaphase I and II stages of meiosis are completely lacking.

Despite the occurrence of huge inter- and intraspecific variation, there are a few reports detailing the molecular basis of genetic diversity in the genus *Bulbophyllum*. Most of the studies are restricted to morphological (Hawakawa et al. 2014) and allozyme (Ribeiro et al. 2007) variation. However, none of the species from Meghalaya were included in such analyses. Similarly, a couple of serious efforts were undertaken by Fischer et al. (2007) and Hosseini et al. (2016) which are remarkable, but their studies included only three species from northeast India. Thus, the accurate systematic position of many of the species grown in India and Meghalaya are ambiguous and need urgent clarification.

Staining with base-specific fluorochromes has been recognized as a reliable method of distinguishing some types of heterochromatin in plants (Vosa 1970, 1976; Schweizer 1976). The fluorochromes quinacrine (Q), Hoechst 33258 (H) and 4′-6-diamidino-2- phenylindole (DAPI) preferentially stain AT-rich heterochromatin, while mithramycin (MM) and chromomycin A3 (CMA) preferentially stain GC-rich heterochromatin (Schweizer 1976). Heterochromatin detection together with physical mapping of DNA sequences by fluorescence in situ hybridization (FISH) has been proven to be a useful technique to analyse the patterns of karyotype evolution (Maluszynska and Heslop-Harrison 1993; Wolny and Hasterok 2009), to develop chromosome markers to delimit species (Moscone et al. 1993; Raina et al. 2001; Robledo et al. 2009) and to investigate the species relationships (Thomas et al. 1997; Robledo and Seijo 2010) in many plant groups with poor differentiation in chromosome morphology.

It is quite important to note that till date very scant reports are available about the heterochromatin distribution in orchids. Steady diminution of haploid chromatin matter as a characteristic feature has been reported (Lim and Jones 1981) in genera such as *Cymbidium*, *Dendrobium*, *Bulbophyllum*, *Eulophia*, *Vanda* and *Phalaenopsis*. In situ hybridization of radioactive satellite DNA and complementary RNA to nuclei, as well as staining of nuclei with AT-specific fluorochromes, confirmed the various types of heterochromatin-rich regions in chromocenters.

Later on, Nagl (1977) also described the heterochromatin elimination in *Dendrobium*. Chromocenters are also reported in *Dendrobium*, *Bulbophyllum* and *Cymbidium* species and found to be heterochromatic by heterochromatin staining methods. On the other hand, euchromatic domains were earmarked with the presence of histone H3K4me2 and H3K27me3 immuno-signals in orchids. Heterochromatin blocks ranging from few to many were also reported in *Maxillaria* species using double staining and CMA/DAPI banding pattern. Such information will provide a clearer view towards the possible role of epigenetic

factors in influencing complex cellular phenomenon. The spatial and temporal chromosomal distribution heterochromatin landmarks at both mitosis and meiosis of *Bulbophyllum* species may reveal a unique insight into the global chromatin environment and their plausible cellular function in cell division and development and will throw a light on the evolutionary pattern and phylogenetic relationship of the genus *Bulbophyllum*. Presently such studies are underway which will be focusing on resolving the ambiguities pertaining to phylogenetic relationship between and within the species of *Bulbophyllum* vis-a-vis quantum of existing genetic variation among them with the help of some powerful molecular tools for understanding the phylogeny and variation.

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Orchid Diversity in Darjeeling Himalaya, India: Present Status and Conservation

9

Mohammed Rahamtulla, Ashis Kumar Roy, and S. M. Khasim

Abstract

Darjeeling Himalaya, one of the biodiversity hotspots of the world (Eastern Himalaya) is a virtual goldmine of orchids. A comprehensive study on the orchid diversity in Darjeeling Himalaya (West Bengal) was conducted from 2016 to 2018 with the objective to assess the presence of orchids. A total of 25 species belonging to 18 genera were identified in the different forested regions during the study, of which 80% (20 species) were epiphytic and 20% (5 species) were terrestrial. This study noticed that both epiphytic and terrestrial orchids showed altitudinal variation ranging from 701 ft to 6009 ft *Bulbophyllum leopardinum* was observed at lowest altitude (701 ft), whereas the *Phalaenopsis mannii* was found at 6009 ft (highest altitude). In the present study, the collected orchid species were arranged alphabetically; their author citation, synonyms, common names, distribution in the world as well as in the study area along with its altitude, habitats, host plants (in case of epiphytic orchids), date of collection, voucher specimen numbers, and botanical description were given with a special focus on present IUCN conservation status (2018) and population trend. Out of 25 orchid species recorded in the present study, 4 orchid species are reported in the IUCN Red List of Threatened Species Version 2018–1. These are *Paphiopedilum insigne, Bulbophyllum leopardinum, Dendrobium aphyllum, and Vanda tessellata.* The vegetation of this region is under significant threat from a combination of logging, construction of river valley projects, agriculture, and human population increases. Orchids are collected on a "massive scale" by the local population for sale. In addition to this, forests in the region have suffered from a very high level of habitat loss, fragmentation, and illegal collection for the

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horticultural trade which threatens many orchids in this region. The present study also aimed at to document the most endangered species that were not reported in IUCN Red List of Threatened Species.

Keywords
Orchid diversity · Darjeeling Himalaya · Northeast India · Conservation

9.1 Introduction

Orchids are the most beautiful and wondrous flowers among all the flowering plants in the world. The exquisite beauty of orchid flowers, variety of fragrance, brilliance in colors, remarkable range of sizes, manifold shapes, variation in the form, attractive habitats, and wide distribution in the earth have aroused highest appreciation throughout the world. Theophrastus, popularly known as Father of Botany, gave the name *orchids* to the bizarre group of plants, on the basis of the resemblance of paired underground tubers of these plants to masculine anatomy (the testes). These gorgeous plants belong to the family Orchidaceae, in the plant group the monocotyledons. The family Orchidaceae has about 28,000 currently accepted species, distributed in about 763 genera (Christenhusz and Byng 2016). Most of the orchids are perennial herbs with simple leaves. Although their specialized flower structure conforms to a standard plan, the vegetative parts show great variations, a large number of them being epiphytes or terrestrials and a few being saprophytes and leafless in nature. These plants are habitat specific (Linder 1995) and have specialized pollinators (Darwin 1862; Cozzolino and Widmer 2005) as well as specific mycorrhizal associations (Taylor and Bruns 1997; McCormick et al. 2004; Shefferson et al. 2007). The seeds of orchids lack endosperm, and involvement of fungi in seed germination is a well-known fact (Bernard 1909), but the specificity in orchid mycorrhizae has been controversial (Harley and Smith 1983). Mycorrhizal association may be species specific (Clements 1987; Taylor and Bruns 1997) or generalist (Hadley 1970; Smreciu and Currah 1989; Masuhara and Katsuya 1989, 1991; Masuhara et al. 1993; Rasmussen 1995) or variable among species (Muir 1989). This observable fact of specialization makes orchids diverse and rare (Gill 1989; Shefferson et al. 2005; Otero and Flanagan 2006). This specialty may increase the chance of extinction for those orchids most specific to endangered or rare habitats (Shefferson et al. 2008). However there are some advances in understanding orchid family, but threats to survival of orchids continue to increase day by day. Global IUCN Red Lists of Threatened Species have so far conducted 948 (3.3%) of the estimated 28,484 orchid species worldwide (Govaerts et al. 2017), but more than half (56.5%) of these were found to be threatened with extinction. Major threatening process includes habitat destruction and degradation, burning, weed encroachment, disease, and pests.

9.2 Darjeeling Himalaya

Darjeeling Hill area is unique from environmental point of view. There are different climatic zones with distinctive attributes and endangered animals like red panda, etc. along with attractive orchids and medicinal plants. About 1300 orchid species belonging to 140 genera are found in India with temperate Himalayas as their natural home (Yonzone and Kamran 2008). Darjeeling is a part of the Eastern Himalayan zoogeographic zone (Negi 1992). The region is well known for its diverse range of vegetation and is one of the richest in India (Bhujel 1996).

Darjeeling Hill district of West Bengal is situated between 27°13′–28°31′ N latitude and 87°59′–88°53′ E longitude in the Eastern Himalayan region of India. The word "Dorji" in Tibetan means "thunderbolt" and "Ling" means "place". Therefore Darjeeling was so named to mean "the place of thunderbolt." Geographically Darjeeling is bounded on its north by the state of Sikkim, on its east by Bhutan, on its west by Nepal, and Terai and Dooars in the south. The district includes the town of Darjeeling, Kurseong subdivision, Mirik, and the Terai region (lower plains), among other areas. Kalimpong, which was earlier a subdivision of Darjeeling district, has become a separate district of West Bengal effective 14 February 2017 with an area of 1056 km^2 of its own. The average altitude of Darjeeling Town is 6710 ft (i.e., about 2045 m). However, the highest point in the whole of Darjeeling district is Sandakphu (close to 12,000 ft) which also happens to be the highest altitude point in the whole of West Bengal. The two best seasons in Darjeeling are one between March and May (i.e., spring and summer) and the other between October and November (i.e., autumn). The summer is short from May up to June. The winter is from December to February.

Field observations and botanical collections were carried out in the forested regions of the Darjeeling Himalaya including farms and floral nurseries, which were traversed on foot from 2016 to 2018 in different seasons. Regular visits to the wild habitat of these rare plants, studying the morphological features of the plants, measuring the flowers, and drawing sketches of the live plants in situ were done. Once a species of interest (orchid) was observed in the forested region, several consecutive visits were made to that particular region to study the morphological features, environmental adaptability, and behavioral aspects of the species as well as its location (latitude, longitude, and altitude); it was done by using Google Maps and My Elevation app (version 1.49) offered by RDH Software. Later Field Book application (version 4.0.3) offered by PhenoApps was used for collecting phenotypic notes. Efforts were made to study plants in their flowering condition so as to identify them properly. The specimens were collected as living collections. The live specimens which were in nonflowering state were identified once they flowered. Charming photographs of live specimens (orchids) were taken in their natural habitats as well as in the greenhouse with the help of Kodak PIXPRO Astro Zoom AZ401-BK 16MP Digital Camera with 40X Optical Zoom. Whole plants along with their flowers were preserved in FAA (Formalin-Acetic acid-Alcohol), and they were mounted on herbarium sheets also to study the morphological details. Herbarium specimens were preserved by following the process of Jain and Rao (1977). Voucher specimens are

deposited at Department of Botany and Microbiology, Acharya Nagarjuna University. Collected specimens were critically examined and identified with the help of standard orchid flora (King and Pantling 1898; Pradhan 1976, 1979; Lucksom 2007; Som Deva and Naithani 1986; Abraham and Vatsala 1981; Bose and Bhattacharjee 1980; Bentham 1881). After the identification of plants, they were also checked in The IUCN Red List of Threatened Species Version 2018–1 (https://www.iucnredlist.org) which gives a good snapshot of the current status of species.

9.3 Phytogeography of Darjeeling Himalaya

Flora around Darjeeling comprises sal, oak, semievergreen, temperate, and alpine forests. The natural forests of Darjeeling district may be grouped into the following broad categories: (a) tropical semievergreen forest, (b) tropical moist deciduous forest, (c) subtropical hill forest, (d) Eastern Himalayan wet temperate forest, and (e) alpine forest.

Depending upon the various forest types of Darjeeling, the orchid habitats can be broadly categorized into 5 zones (Table 9.1). They are:

1. **Tropical Zone** (between 400 and 1200 m)
2. **Subtropical Zone** (between 1200 and 2200 m)
3. **Temperate Zone** (between 2200 and 3200 m)
4. **Upper Temperate Zone** (3200 and 3600 m)
5. **Alpine Zone** (between 3600 and 6000 m)

1 *Tropical Zone*: This type of forest is characterized by dense tree covered with rich floral and faunal diversity with annual rainfall lying in between 2700 and 3000 mm. The humidity is usually between 85 and 100%, and the temperature ranges from 29 to 35 °C. During winter the temperature ranges from 20 to 25 °C. In this zone forests are multistoried. The uppermost layer trees that provide the topmost canopy to the forest in this area are *Albizia, Gmelina, Shorea*, and *Terminalia*. The lower layer consists of *Callicarpa, Dendrocalamus*, and *Schima*. These combinations with their close canopy form a dense, dark humid environment, which forms an ideal habitat to support the luxuriant growth of many epiphytes. Some of the common epiphytic orchids are *Acampe rigida* (Buch.-Ham. ex J.E.Smith) P.F. Hunt, *Aerides multiflorum* Roxb., *Bulbophyllum andersonii* (Hook. f.) J.J.Smith, *Cymbidium aloiflolium* (L.) Sw., *Dendrobium aphyllum* (Roxb.) C.E.C Fischer, *D. farmerii* Paxt, *D. formosum* Roxb., *D. moschatum* (Buchanan-Hamilton) Sw., *Papilionanthe teres* (Roxb.) Schltr., *Vanda testacea* (Lindl.) Rchb. f., and many other species of the various genera. The lower vegetation supports the orchid species of *Bulbophyllum roxburghii* (Lindl.) Rchb.f., *B. leptanthum* Hook. f., *B. tortuosum* (Bl.) Lindl., *Phalaenopsis lobbii* (Rchb. f.) H.R. Sweet., and *P. mannii* Rchb. f. The shady thick humus forest floor of this zone supports the growth of orchids like *Acanthephippium sylhetense*

Table 9.1 Distribution of some orchids in the forested regions of Darjeeling Himalayas in different phyto-climatic zones

S. no	Phyto-climatic zone	Name of orchid species present in the zone
1.	Tropical Zone (400–1200 m)	*Acampe rigida*, *Acanthephippium sylhetense*, *Aerides multiflorum*, *Anoectochilus roxburghii*, *Arundina graminifolia*, *Bulbophyllum andersonii*, *B. leptanthum*, *B. roxburghii*, *B. tortuosum*, *Cymbidium aloiflolium*, *Dendrobium aphyllum*, *D. farmerii*, *D. formosum*, *D. moschatum*, *Galeola cathcartii*, *Goodyera hispida*, *G. procera*, *Nervillia macroglossa*, *N. plicata*, *Paphiopedilum venustum*, *Papilionanthe teres*, *Phalaenopsis* lobbii, *P. mannii*, *Tropidia angulosa*, *Vanda testacea*, *Zeuxine affinis*, etc.
2.	Subtropical Zone (1200–2200 m)	*Anthgonium gracile*, *Bulbophyllum guttulatum*, *B. hirtum*, *B. reptans*, *Calanthe mannii*, *C. plantaginea*, *C. puberula*, *Coelogyne cristata*, *C. elata*, C. *flaccida*, *Cymbidium macrorhizon*, *Dendrobium chrysanthum*, *D. densiflorum*, *D. moschatum*, *Diplomeris hirsuta*, *Eria confusa*, *E. graminifolia*, *Gastrochilus affinis*, *G. calceolaris*, *Goodyera clavata*, *Goodyera procera*, *Malaxis acuminate*, *Nervilia gammieana*, *N. hookeriana*, *Oberonia auriculata*, *O. micrantha*, *O. pachyrachis*, *Ornithochilus difformis*, *Paphiopedilum fairrieanum, P. venustum*, *Podochilus khasianum*, etc.
3.	Temperate Zone (2200–3200 m)	*Aphyllorchis montana*, *A. alpina*, *Bulbophyllum eublepharum*, *B. hymenanthum*, *B. reptans*, *Cephalantheropsis longipes*, *Galeola lindleyana*, *Gastrochilus distichus*, *Pleione humilis*, *Spiranthes sinensis*, *Stigmatodactylus paradoxus*, etc.
4.	Upper Temperate Zone (3200–3600 m)	*Bulbophyllum dyeranum*, *B. griffithii*, *B. hymenanthum*, *Calanthe alpina*, *C. tricarinata*, *Eria pusilla*, *Habenaria stenopetala*, *Liparis perpusilla*, *Neottia listeroides*, *Satyrium nepalense*, etc.
5.	Alpine Zone (3600–6000 m)	*Aorchis spathulata*, *Androcorys puioniformis*, *Chusua nana*, *Diphylax urceolata*, *Goodyera fusca, Gymnadenia orchidis*, *Habenaria diphylla, Herminium orbiculare*, *H. josephi*, and *H. macrophyllum*

Lindl., *Galeola cathcartii* Hook. f., *Nervillia macroglossa* (Hook. f.) Schltr., *N. plicata* (Andrew) Schltr., *Tropidia angulosa* (Lindl.) Blume. The shady river banks are rich with orchids like *Anoectochilus roxburghii* Lindl., *Goodyera hispida* Lindl., and *Zeuxine affinis* (Lindl.) Benth. ex Hook. f. The landslide and the sandy sloppy areas are colonized by *Goodyera procera* (Ker Gawler) Hook. The sandstone rocks support the growth of *Arundina graminifolia* (D.Don) Hochr. and *Paphiopedilum venustum* (Wall.) Pfitzer.

2 *Subtropical Zone*: This zone is somewhat cooler zone. In this zone the summer precipitation is heavy, and the day temperature lies between 25 and 28 °C. The night temperature drops below 18 °C. The forest types in this zone are either evergreen or semievergreen. Here the trees are shorter and bushy in appearance and stratification is not clear. The undergrowth is heavy. The common trees are *Bauhinia, Betula, Bombax, Camellia, Castanopsis, Cedrela, Cinnamomum, Daphniphyllum, Engelhardtia, Eurya, Evodia, Ficus, Fraxinus, Machilus, Magnolia, Mahonia, Prunus, Rhus, Saurauia, Terminalia*, etc. Most common

epiphytic orchids are *Bulbophyllum guttulatum* (Hook. f.) Balakrishnan., *B. hirtum* (J.E. Smith) Lindl., *B. reptans* (Lindl.) Lindl., *Coelogyne cristata* Lindl., *C. elata* Lindl., *C. flaccida* Lindl., *Dendrobium chrysanthum* Wall.ex Lindl., *D. densiflorum* Lindl., *D. moschatum* (Buch.-Ham.) Sw., *Eria confusa* Hook. f., *E. graminifolia* Lindl., *Gastrochilus affinis* (King & Pantling) Schltr., *G. calceolaris* (Buchanan-Hamilton ex J.E. Smith) D.Don., *Oberonia auriculata* King & Pantling, *O. micrantha* King & Pantling, *O. pachyrachis* Rchb. f., *Ornithochilus difformis* (Wall. ex Lindl.) Schltr., *Podochilus khasianum* Hook. f., etc. Here the forest floor is rich in humus and supports the growth of *Calanthe mannii* Hook. f., *C. plantaginea* Lindl., *C. puberula* Lindl., *Cymbidium macrorhizon* Lindl., *Goodyera clavata* N. Pearce & P.J. Cribb, *Nervilia gammieana* (Hook. f.) Schltr., *N. hookeriana* (King & Pantl.) Schltr., etc. Stiff rocky crevices of dolomite and limestone origin support the growth of orchids like *Diplomeris hirsuta* (Lindl.) Lindl. *Paphiopedilum fairrieanum* (Lindl.) Stein, *P. venustum* (Wall.ex Lindl.) Pfitzer, etc. Landslide areas, river banks, and sandy loam hills are rich with orchids like *Goodyera procera* (Ker Gawler) Hook., *Anthgonium gracile* Wall. ex Lindl., *Malaxis acuminata* D. Don., etc.

3 *Temperate Zone*: This zone has heavy summer rainfall, presence of heavy fog and mist almost through the year, and a severe winter with irregular winter snow along with scanty rainfall during January and February are the main uniqueness of this zone. Its summer temperature hardly exceeds 18 °C. Depending on the altitudinal variation, the floral composition differs. The broad leaved forest occupies the lower temperate zone. The natural occurring species are *Acer Species, Betula, Castanopsis, Echinocarpus, Engelhardtia, Eurya, Juglans, Lithocarpus, Machilus, Michelia, Prunus, Quercus*, etc. The common epiphytic orchids are *Bulbophyllum eublepharum* Rchb. f., *B. hymenanthum* Hook. f., *B. reptans* (Lindl.) Lindl., *Gastrochilus distichus* (Lindl.) Kuntze, and *Pleione humilis* (J.E. Smith) D.Don. The humid shady forest floor supports the growth of *Aphyllorchis montana* Rchb. f., *A. alpina* King & Pantling, *Cephalantheropsis longipes* (Hook. f.) Ormerod, *Galeola lindleyana* (Hook. f. & Thomson) Rchb. f., *Spiranthes sinensis* (Persoon) Ames, and *Stigmatodactylus paradoxus* (Prain) Schltr.

4 *Upper Temperate Zone*: This zone is characterized by dominance of conifer trees in the top canopy and the *Rhododendron* sharing the middle canopy with other broad leaved trees. The common tree species are *Acer, Betula, Carpinus, Larix, Magnolia, Pyrus, Taxus, Tsuga*, etc. In this zone the distribution of epiphytic orchids is very less. As the terrain gains higher altitude, the epiphytic orchids are slowly replaced by ground orchids. The common epiphytic orchids present in this zone are *Bulbophyllum dyeranum* (King & Pant.) Seidenf., *B. griffithii* (Lindl.) Rchb. *B. hymenanthum* Hook. f., *Eria pusilla* (Griff.) Lindl., *Liparis perpusilla* Hook. f., etc. The most common ground orchids found are *Calanthe alpina* Hook. f. ex.Lindl., *C. tricarinata* Lindl., *Neottia listeroides* Lindl., *Habenaria stenopetala* Lindl., *Satyrium nepalense* D. Don, etc.

5 *Alpine Zone*: In this zone the precipitation is in the form of snow, and the region remains under snow cover almost for nearly 5–8 months. The rainfall is less and

the light intensity is high. Soil type is sandy to sandy loam. The soil depth is shallow with exposed boulders. These factors together with freezing temperature support the dwarf growth of vegetation. The Subalpine zone lies just above the upper temperate zone and is characterized by the growth of dwarf *Abies, Rhododendrons* along with *Hydrangea, Pyrus, Sorbus, Hypericum, Ribes, Sambucus*, etc. Here the soil type is sandy to sandy loam. These species do not support the growth of epiphytic orchids. Only ground orchids are found above this level. Some of the orchids found in this zone are *Corybas himalaicus* (King & Pantling) Schltr., *Didiciea cunninghamii* King & Prain ex King & Pantling, *Diphylax urceolata* (C.B. Clarke) Hook. f., *Malaxis muscifera* (Lindl.) Kuntze, *Oreorchis foliosa* (Lindl.) Lindl., *O. indica* (lindl.) Hook. f., and *Tipularia josephi* Rchb. f. ex Lindl. on top of moss covered rocks or in crevices of rocks. The alpine zone can be divided in two fragments; they are Hilly well-drained slopes and wet flat valleys. Hilly slopes support the growth of the orchid species such as *Diphylax urceolata* (C. B. Clarke) J. D. Hooker., *Goodyera fusca* (Lindl.) Hook. f., *Gymnadenia orchidis* Lindl., *Habenaria diphylla* Dalzell, *Herminium orbiculare* King ex Rolfe., etc. The alpine flat valleys support the growth of orchids like *Aorchis spathulata* (Lindl.) Vermeulen, *Androcorys puioniformis* (Lindl. ex Hook. f.) K.Y. Lang, *Chusua nana* (King & Pantling) Pradhan, *Herminium josephi* Rchb. f., and *H. macrophyllum* (D.Don) Dandy.

Darjeeling hills are endowed with countless orchid species. The present study was conducted in an attempt to document the diversity of Orchid species in Darjeeling Himalaya of West Bengal, India, and to identify those species needing targeted recovery efforts but also for focusing the conservation agenda by identifying the key sites and habitats that need to be protected.

9.4 Enumeration of Orchid Species in Darjeeling Himalaya

The collected orchid species are arranged alphabetically; their author citation, synonyms, common names, distribution in the world as well as in the study area along with its altitude, habitats, host plants (in case of epiphytic orchids), date of collection, voucher specimen numbers, and botanical description were given with a special focus on present IUCN conservation status (2018) and population trend.

1. ***Acampe papillosa*** (Lindl.) Lindl. Fol. Orch. Acampe 21,853; *Saccolabium papillosum* Lindl. in Edw. Bot. Reg. 18:t. 1552.1833; Hook. f., 6:63. 1890; King & Pantl. 219, t.290. 1898. Duthie, 147. 1906.
   Common name: Small Warty Acampe.
   Distribution in the world: Himalaya (Garhwal to Arunachal Pradesh), Assam, Bengal, Meghalaya, Tripura, Burma, Bangladesh, Bhutan, Laos, Myanmar, Nepal, Thailand, and Vietnam.

Species examined: Sukna wild life, Sukna forest, West Bengal. **Altitude**: 735 ft **Habitat**: epipyte; **Host Plant**: bark of *Juniperus recurva*. **Date of collection**: 23-05-2016.
Voucher specimen number: ANUBH1201. Flowering**:** June to August.
IUCN conservation status (2018): Unknown. **Population trend**, decreasing.

Stem is erect, clustered, rigid, and stout. Leaves are coriaceous, 7.5–10 × 1.5–2 cm, fleshy, deeply channeled, apex truncate, or deeply notched. Inflorescence leaf-opposed, stout, 1–1.5 cm long, umbellate or subcorymbose, 4–8 flowered. Flowers are 9 mm across. Bracts are triangular. Sepals subequal, 4 mm long, yellow with brown blotches, oblong, subacute, spreading. Petals narrower, linear-spathulate, yellow with brown blotches. Lip is longer than the sepals, adnate to the base of the column; lateral lobes none; mid-lobe decurved, oblong-ovate, obtuse, crenate, upper surface transversely rugulose, white with transverse pale-purple bars. Spur half as long as the ovary, cylindric, straight, pale yellow, hairy within. Capsules are fusiform, rigid, less than 2.5 cm in length (Fig. 9.5a).

2. ***A. rigida*** (Buch.-Ham.ex J.E. Smith) P.F. Hunt in Kew Bull. 24(1): 98. 1970; Hara et al. 1:31. 1978; Jayaweera 2: 229. 1981; Hajra & Kothari 160, Pl.2. 1983; Singh & Dawre, 1021. 1983; *Aerides rigida* Buch.-Ham. Ex J. E. Smith in Rees Cyclop. 39: *Aerides* n. 12. 1819; *Vanda longifolia* Lindl. Gen. & Sp. Orch. 215. 1833; *Acampe longifolia* (Lindl.) Lindl. Fol. Orch. *Acampe* 1. 1853; *Saccolabium longifolium* (Lindl.) Hook. f., Fl. Brit. Ind. 6:62. 1890 King & Pantl. 220, t.292. 1898; *Vanda multiflora* Lindl. Collect. Bot. t. 38. 1825. *Acampe multiflora* (Lindl.) Lindl. Fol. Orch. Acampe 1. 1853.
Common name: Stiff Acampe.
Distribution in the world: Himalaya (Kumaun to Arunachal Pradesh), Assam, Meghalaya, Nagaland, Maharashtra, Karnataka, Kerala, Goa. Burma, China, the Philippines, Thailand, Indo-China, Malaya, Sri Lanka.
Species examined: near sacred water body, Lamahatta, Tukdah Forest, West Bengal. **Altitude**: 6255 ft. **Habitat**: epiphyte. **Host plant**: bark of *Rhododendron arboretum*. **Date of collection**: 13-06-2016.
Voucher specimen number: ANUBH1202. **Flowering**: May to June.
IUCN conservation status (2018): unknown. **Population trend**: decreasing.

Stem stout, 60–90 cm long. Leaves thick, many, 25–35 × 3.5–5 cm, coriaceous, elongated, oblong, unequal at the apex. Inflorescence up to 18 cm long, erect, leaf-opposed, stout, with one or two short lateral branches. Flowers crowded, fleshy, 12–18 mm across. Bracts small, rounded. Sepals subequal, broadly oblong, obtuse, 13–14 × 7–8 mm, yellow with transverse stripes of crimson. Petals ovate-oblong, falcate, 12 × 4 mm, yellow with transverse stripes of crimson. Lip saccate, white with purple spots. Three-lobed; lateral lobes erect, narrow, and hairy within; mid-lobe ovate, obtuse, slightly recurved, and concave. Capsules are greenish-yellow, obconical, and 4–6 cm long (Fig. 9.5b).

3. ***Aerides multiflora*** Roxb. Pl. Coromand. 3:68 t.271.1820; Hook. f., 6:44. 1890; King & Pantl. 212, t. 283. 1898; Duthie, 142, 1906; *Aerides affine* Lindl. Gen. & Sp. Orch. 239. 1833.
   Common name: Many Flowered Fox Brush Orchid.
   Distribution in the world: Himalaya, Himachal Pradesh, Arunachal Pradesh, Assam, Nagaland, Meghalaya, Manipur, Mizoram, Tripura, Orissa, Madhya Pradesh. Andaman Island, Bangladesh, Burma, Java, Indo-China, Malaya, Thailand.
   Species examined: near Latpanchar Forests, Kurseong, West Bengal. **Altitude**: 3579 ft.
   Habitat: epiphyte. **Host plant**: bark of *Ficus religiosa*. **Date of collection**: 28-05-2016.
   Voucher specimen number: ANUBH1203. **Flowering**: April to May.
   IUCN conservation status (2018): Unknown. **Population trend**: decreasing.

Stem stout, 10–25 cm long. Leaves are linear-oblong, 15–25 × 1.5–3 cm, conduplicate, apex obliquely bifid and often tinged with reddish-purple. Racemes longer than the leaves, simple, axillary, densely many flowered. Flowers 2 cm across, white, flushed with pink or purple. Sepals and petals are subequal, oblong, blunt, 8–9 mm long. Lip twice as long the sepals, adnate to the base of the column, indistinctly three-lobed, pointing forward, margin denticulate; mid-lobe broad, hastate, ovate, apex rounded to acute, mouth covered by a callus from the base of the lip. Spur is small, almost straight (Fig. 9.5c).

4. ***Arundina graminifolia*** (D. Don) Hochr. In Bull. New York Bot. Gard. 6:270. 1910; *Bletia graminifolia* D. Don, Prodr. Fl. Nepal 291,825; *Arundina bambusifolia* Lindl. Gen. & Sp. Orch. 125. 1831; Hook. f., 5:857. 1890; King & Pantl. 113, t. 156. 1898.
   Common name: Bamboo orchid.
   Distribution in the world: Himalaya, Arunachal Pradesh, Assam, Meghalaya, Manipur, Nagaland, Mizoram, South India, Bangladesh, Burma, China, Thailand, Malaysia, Sri Lanka.
   Species examined: NHPC, NH31A, road side, Kalijhora, West Bengal. **Altitude**: 745 ft.
   Habitat: terrestrial. **Date of collection**: 23-08-2016.
   Voucher specimen number: ANUBH1204. **Flowering**: January to November.
   IUCN conservation status (2018): Unknown. **Population trend**: decreasing.

Stem tall, woody, reed-like, erect, 1–2 m long and about 2.5 cm in diameter at the base. Leaves are many, membranous, narrowly lanceolate, acuminate, sheathing at base, 15–30 cm × 2–2.5 cm. Inflorescence racemose, branched, the large showy flowers located at the terminal part and opening one or two at a time. Flowers are 6–7 cm across, rosy-purple. Bracts broad, acute 12 × 8 mm sepals free, spreading, subequal, the dorsal erect, oblong, acute, the laterals lying parallel behind the lip. Petals are rhomboid-elliptic, acute, spreading, shorter, and broader than the sepals.

Lip cucullate, its base convolute round the column, the mouth large, expanded, three-lobed, mid-lobe divided into rounded lobules, apex irregularly toothed, keel 2–3, crested(Fig. 9.5d).

5. ***Bulbophyllum guttulatum*** (Hook.f.) N.P. Balakr., J. Bombay Nat. Hist. Soc. 67:66. 1970; *Cirrhopetalum guttulatum* Hook.f., Fl. Brit. India 5: 776.1896.
   Common name: Small-Spotted Bulb-Leaf Orchid.
   Distribution in the world: Arunachal Pradesh, Meghalaya, Nagaland, Manipur, Mizoram, Burma, and Thailand.
   Species examined: Sittong Forest, West Bengal. **Altitude**: 2736 ft.
   Habitat: epiphyte. **Host plant**: bark of *Rhus typhina*. **Date of collection**: 21-08-2017.
   Voucher specimen number: ANUBH1205. **Flowering**: June to August.
   IUCN conservation status (2018): Unknown. **Population trend**: decreasing.

   However, this orchid is in endangered state from our observation.

   Rhizome creeping, pseudobulbs are 1 cm apart from each other, narrowly ovoid to ovoid-conic, ribbed, with a terminal leaf and 2–2.5 cm, 7–10 mm in diameter. The leaf blade is oblong-elliptic, leathery with an obtuse-rounded apex, and with base contracted into petiole. The scape from the base of pseudobulb is slender, erect, and 10–15 cm; the umbel is often with two or three flowers. The floral bracts are 5 mm, with an acute tip and ovate-lanceolate in shape. The yellow flowers are spotted with red. The dorsal sepal is concave, broadly ovate, the apex is subtruncate to mucronulate and 8–10 × 6–7 mm in size. The lateral sepals are narrow, twisted near the base, obliquely ovate-lanceolate, with an obtuse apex, with their lower margin connate toward each other at the base. Petals have an entire margin with a mucronate apex; they are broadly ovate-triangular in shape, 4.5 × 4mm in size and the base ~1/3 width closely attached to column foot. Lip is recurved, subovate in outside, with a dilated base, adaxially with three longitudinal keels, with an emarginate apex and immobile. The column is triangular, ~3 mm and with acute wings on the lower margins. The foot is ~5 mm, with a free part which is ~2 mm and upcurved (Fig. 9.5e)

6. ***B. leopardinum*** (Wall.) Lindl. Gen. & Sp. Orch. 48. 1830; Hook.f., 5:756. 1890; King & pantl. 67, t.92; *Dendrobium leopardinum* Wall. Tent. Nepal 1:39.t.28. 1824; *Sarcopodium leopardinum* (Wall.) Lindl. Paxt. Fl. Gard. 1:155. 1850; *Phyllorchis leopardina* (Wall.) Kze. Rev. Gen. Pl. 2:677. 1891.
   Common name: Leopard Spotted Bulbophyllum.
   Distribution in the world: Himalaya (Kumaun to Arunachal Pradesh), Meghalaya, Nagaland, Manipur, Mizoram, Burma, and Thailand.
   Species examined: near road sides of Tista Bazar, West Bengal. **Altitude**: 701 ft.
   Habitat: epiphyte. **Host plant**: bark of *Shorea robusta*. **Date of collection**: 21-07-2016.
   Voucher specimen number: ANUBH1206; Flowering: June to August.
   IUCN conservation status (2018): Least Concern ver 3.1. **Population trend**: decreasing.

Rhizome stout, covered with numerous fibrous sheaths. Pseudobulbs obpyriform, compressed, crowded, smooth, 2.5–3.5 cm, placed obliquely. Leaves are elliptic-oblong, 8–15 × 2.5–5 cm, apex blunt but shortly mucronate, base narrowed to the short petiole. Flowers one to three, 2.5 cm across, from the base of the pseudobulb, pale ochraceous, spotted with purple. Bracts are broad, sub-spathaceous. Sepals are ovate-lanceolate, subacute, nine-nerved. Petals are much smaller than sepals, seven-nerved. Lip shorter than petals, thick, secreting nectar profusely, decurved from below the middle, lanceolate, the base having small toothed auricles (Fig. 9.5f).

7. ***Coelogyne cristata*** Lindl. Coll. Bot.sub t.33. (Text only). 1821; Hook.f., 5:829. 1890; King & pantl. 133, t.184. 1898; Duthie, 113. 1906; Seindenf., 32. 1975;Das & Jain 9, fig. 14 a–c. 1980; *Cymbidium speciosissimum* D. Don., Prodr. Fl. Nepal.: 35. 1825; *Pleione speciosissima* (D. Don) Kuntze., Revis. Gen. Pl. 2: 681.1891.
   Common name: Crested Coelogyne.
   Distribution in the world: Himalaya (Garhwal to Arunachal Pradesh), Meghalaya, Nagaland, Tibet, Indo-China.
   Species examined: Pankhabari road side, Kurseong, West Bengal. **Altitude**: 4852 ft.
   Habitat: epiphyte. **Host plant**: bark of *Pinus roxburghii*. **Date of collection**: 23-02-2016.
   Voucher specimen number: ANUBH1207. **Flowering**: January to April.
   IUCN conservation status (2018): unknown. **Population trend**: decreasing.

Plants are pendulous. Pseudobulbs are present at a distance of about 5–6 cm, ovoid-oblong. Two leaves are present, subsessile, 12–30 × 1.5–3 cm, linear-oblong, acute. Inflorescence is raceme, 12–30 cm long, drooping, (−4) 5–8 flowered. Flowers are 6–9 cm across, white, fragrant. Bracts are persistent, oblong, and acute. Sepals subequal, obtuse, 3.5–5.3 × 0.6 × 1.7 cm, undulate. Petals are 3.5–5 × 0.7–1.7, undulate. Lip oblong, 3.5–4.5 × 2.4–4.2 cm, white, tinged with yellow, three-lobed; mid-lobe distinct, rhomboid, entire; lateral lobes broadly oblong, rounded; keels 4–5, lacinate or deeply fimbriate, segments hairlike, yellow, two lateral extending to mid-lobe, forming raised plates (Fig. 9.6a).

8. ***Cymbidium aloifolium*** (Linn.) Sw. in Nov. Act. Sci. Ups. 6:73. 1799; Hook.f., 6:8. 1890; King & Pantl. 189, t.252. 1898; Duthie, 136. 1906. Seth, 399. Pl. 26, fig. A.C. 1982; Seidenf., 72:77 fig. 43.1983; *Epidendrum aloifolium* Linn. Sp. Pl. ed. 1:953. 1753; *Epidendrum pendulum* Roxb. Pl. Coromandel, 1:35. 1759; *Epidendrum aloides* Curtis, Bot. Mag. 11:t. 387. 1797, sphalm. for *E. aloifolium*; *Cymbidium pendulum* (Roxb.) Sw., Nov. Act. Soc. Sci. Ups. 6:73. 1799; Abrahm & Vatsala, 325. Fig. 70. 1981.
   Common name: Aloe Leaf Cymbidium.
   Distribution in the world: Himalaya (Kumaun to Arunachal Pradesh), Assam, Meghalaya, Mizoram, Nagaland, Tripura, Manipur, Madhya Pradesh, South

India, Andaman Islands. S. China, Hong Kong, Burma, Thailand, Malaya, Sumatra, Java, Borneo, Vietnam, Taiwan, and Sri Lanka.

Species examined: 11th Tinchuley Lopchu Road, Tukdah Forest, West Bengal. **Altitude**: 5006 ft. **Habitat**: epiphyte. **Host plant**: bark of *Engelhardtia spicata.* **Date of collection**: 04-05-2016.

Voucher specimen number: ANUBH1208. **Flowering**: April to June.

IUCN conservation status (2018): unknown. **Population trend**: decreasing.

Pseudobulbs laterally compressed, 6–7 mm long, leaves 30–35 cm long and more than 3 cm broad, linear – oblong, curved, obtuse, fleshy, obliquely notched at the apex. Inflorescences from the base of pseudobulbs, somewhat shorter than the leaves, peduncle 7–10 cm long, bearing several tubular acute scarious sheaths near the base, raceme many flowered, decurved, 30–40 cm long. Flowers are 3.5 cm across. Bracts are minute, ovate, and much shorter than the ovary. Sepals subequal, oblong slightly lanceolate, blunt 2 cm long or less. Petals are as long as the sepals, ovate-lanceolate, blunt. Lip as long as the sepals, oblong, three-lobed, lateral, lobes acute, longer than column; mid-lobe ovate-oblong, decurved, striped with pink or purple (Fig. 9.6b).

9. ***Dendrobium aphyllum*** (Roxb.) Fischer in Gamble, Fl. Pres. Madras 3:1416. 1928; *Limodorum aphyllum* Roxb. Pl. Coromand. 1:34.t.41. 1795; *D. pierardii* Roxb. Hort. Beng. 63. 1814. nom. Nud. ex Hook., Exot. Fl. 1:t. 9.1822; Hook. f., 5:738. 1890; King & Pantl. 51, t. 72. 1898; Duthie, 97. 1906.

Common name: Leafless Dendrobium.

Distribution in the world: Himalaya (Garhwal, Nepal to Arunachal Pradesh), Madhya Pradesh, Orissa, Assam, Meghalaya, Nagaland, Manipur, Mizoram, Tripura, Bengal, Peninsular India, Andaman Island. Burma, Thailand, Indo-China, W. China, and Malaya.

Species examined: NH 10, Teesta river sides, Rangpo Forest, West Bengal. **Altitude**: 1320 ft.

Habitat: epiphyte. **Host plant**: bark of *Terminalia myriocarpa*. **Date of collection**: 20-03-2016.

Voucher specimen number: ANUBH1209. **Flowering**: March to May.

IUCN conservation status (2018): Least Concern ver 3.1. **Population trend**: decreasing.

Pseudobulbs are 60–120 × 0.5–0.8 cm, pendulous, leafy throughout, compressed. Leaves 5–10 × 2.5–3.5 cm, decreasing in length upward, lanceolate, ovate-lanceolate, acuminate. Flowers in fascicles of 1–3, from the swollen nodes, 3–4 cm across, pale-rose, lip yellow. Sepals subequal, oblong-lanceolate, subacute. Petals much broader than sepals, elliptic, rounded, sepals and petals rose colored. Lip suborbicular, base shortly convolute, pubescent, ciliate on anterior margin, apical portion pale-yellow with radiating streaks of purple at base (Fig. 9.6c).

10. ***D. fimbriatum*** Hook., Exot. Fl. 1: t. 71. 1823; *Callista fimbriata* (Hook.) Kuntze, Revis. Gen. Pl. 2: 653.1891; *Dendrobium normale* Falc., Ann. Nat. Hist. 3: 196.1839; *Dendrobium paxtonii* Paxton, Paxton's Mag. Bot. 6: 169.1839; *Callista normalis* (Falc.) Kuntze, Revis. Gen. Pl. 2: 655. 1891; *Callista oculata* (Hook.) Kuntze, Revis. Gen. Pl. 2: 653.1891.
    Common name: Fringe-Lipped Dendrobium.
    Distribution in the world: Himalaya (Kumaun to Arunachal Pradesh), Assam, Meghalaya, Nagaland, Manipur, Mizoram, Tripura. Bangladesh, Burma, Thailand.
    Species examined: road sides of river Rangeet, West Bengal. **Altitude**: 1890 ft. **Habitat**: epiphyte.
    Host plant: bark of *Shorea robusta*. **Date of collection**: 23-08-2017.
    Voucher specimen number: ANUBH1210. **Flowering**: March to May.
    IUCN conservation status (2018): Unknown. **Population trend**: decreasing.

Stem is long and grows up to 4 ft, tapering toward the apex, suberect. Leaves are oblong-lanceolate, pointed at its apex and narrowed toward its base, 4–7 cm long and 1–2 cm in width at its mid portion. Racemes appear laterally from the nodes of the leafy stems, pendulous, with 5–9 flowers, the peduncle with many sheathing bracts at its base. Flowers are 3–4 cm across, sepals and petals bright yellow, lip of the same color with a large orbicular reddish-brown patch around its middle portion. Sepals are oblong, blunt, spreading. Petals are wider than the sepals, oblong. Lip is orbicular, with undulate and fringed margins (Fig. 9.6d).

11. ***D. moschatum*** (Buch.-Ham.) Sw., Schrad. N.Journ. 1:94. 1806; King & Pantl. 60, t.84. 1898; Duthie, 103. 1906; *Epidendrum moschatum* Buch.-Ham. in SymesAcc. Emb. Kingd. Ava: 478 t.1800; *D. calceolaria* Carey ex Hook. Exot. Fl. 3:t. 184. 1826; Hook.f., 5:744. 1890.
    Common name: Musk Dendrobium.
    Distribution in the world: Himalaya (Kumaun to Arunachal Pradesh), Assam, Meghalaya, Nagaland, Manipur, Mizoram, Tripura. Bangladesh, Burma, Thailand.
    Species examined: Rangli Forest, West Bengal. **Altitude**: 1906 ft. **Habitat**: epiphyte.
    Host plant: bark of *Castanopsis indica*. **Date of collection**: 23-06-2017.
    Voucher specimen number: AMUBH1211. **Flowering**: March to June.
    IUCN conservation status (2018): Unknown. **Population trend**: decreasing.

Pseudobulbs are stout, erect, tufted, 90–180 × 1–2 cm, tapering moderately from the middle on the both sides. Leaves are several, alternate, 10–15 × 3–5 cm, ovate to lanceolate-ovate, acute or faintly notched. Inflorescence is near the apex of leafy or leafless pseudobulb, 10–30 cm long and 8–16-flowered. Flowers are 5–10 cm across, fragrant, pale pinkish-yellow. Sepals 3 cm long, broadly ovate, obtuse. Petals

are larger than sepals, broadly ovate, obtuse. Lip is shorter than petals, forming a globular or pear-shaped pouch with the upturned margins (calceolar); anterior part very hairy, base with two dark maroon blotches (Fig. 9.6e).

12. ***Eria lasiopetala*** (Willd.) Ormerod, Opera Bot. 124: 22. 1995; *Aerides lasiopetala* Willd., Sp. Pl. 4(1): 130. 1805; *Epidendrum lasiopetalum* (Willd.) Poir. J.B.A.M.de Lamarck, Encycl., Suppl. 1: 384.1810; *Dendrolirium lasiopetalum* (Willd.) S.C.Chen & J.J.Wood, in Fl. China 25: 351.2009.
    Common name: The Shaggy Petaled Eria.
    Distribution in the world: Bangladesh, Himalaya to Hong Kong, and West Malaysia.
    Species examined: Sukna Forest, West Bengal. **Altitude**: 790 ft. **Habitat**: epiphyte.
    Host plant: bark of *Shorea robusta*. **Date of collection**: 23-06-2017.
    Voucher specimen number: ANUBH1212. **Flowering**: June to July.
    IUCN conservation status (2018): Unknown. **Population trend**: decreasing.

Pseudobulbs are much compressed with sheathed bases, vertically ribbed, 4–7 cm long and 2–3 cm in width, arranged at a distance of 3–4 cm apart on a stout and clothed rhizome. Leaves arise from the apex of the bulbs, three to five in numbers, almost oblong, tapering to the base, 6–9 cm long and 2–3 cm in width. Raceme from the base of the bulbs, erect, stout, fully covered with soft hairs. Flowers are 4–10, about 2 cm across. Sepals are hairy on the outer surface; dorsal small, lanceolate, arching; lateral large, triangular, and spreading. Petals are parallel over the column, narrowed at the base. Lip oblong, three-lobed; lateral lobes very narrow, terminal lobe oblong, acute, slightly deflexed, the disc with two ridges (Fig. 9.6f).

13. ***Eria stricta*** Lindl., Coll. Bot. t. 41 B; in Journ. Linn. Soc. III, 52; *Eria secundiflora* Griff. Notul. III, 302; Ic. Pl. Asiat., t 301, 1851; *Mycarathes stricta* Lindl. in Wall. Cat, 1970; Gen. and Spec. Orchid., 63; Wight Ic. 1733.
    Common name: Erect Eria.
    Distribution in the world: Assam, Bangladesh, China South-Central, East Himalaya, Myanmar; Nepal, Thailand, Tibet; Vietnam.
    Species examined: Near opposite Road side of Mahananda wild Sanctuary, West Bengal.
    Altitude: 1568 ft. **Habitat**: epiphyte. **Host plant**: bark of *Juniperus recurva*.
    Date of Collection: 23-02-2017. **Voucher specimen number**: ANUBH1213.
    Flowering: February to May. **IUCN conservation status (2018)**: Unknown.
    Population trend: decreasing.

Pseudobulbs are crowded, without a rhizome, often cohering, cylindrical, 3.5–5 in. long and about 3 in. in diam., with loose fibrous sheaths at the base, and at the apex two oblong elliptic sub-coriaceous sessile leaves tapering to each end, 2.5–4 in. long and 5–9 in. broad. Spike solitary, erect, rising from between the leaves and exceeding them in length, the peduncle is naked, the raceme many

flowered. Flowers 1–15 in. long, crowded; the floral bract ovate, acute, glabrous, much shorter than the woolly ovary. Sepals are densely woolly outside, subequal, ovate. Petals are ovate-rotund, blunt, as long as the sepals. Lip suborbicular, concave, three-lobed; the apical lobe very short, broad, truncate; the side lobes are shallow. Column is short and broad; the foot is very short (Fig. 9.7a).

14. ***Panisea uniflora*** (Lindl.) Lindl., Fol. Orchid. 5: 2.1854; *Coelogyne uniflora* Lindl., Gen. Sp. Orchid. Pl.: 42. 1830; *Pleione uniflora* (Lindl.) Kuntze, Revis. Gen. Pl. 2: 680. 1891; *Coelogyne thuniana* Rchb.f., Allg. Gartenzeitung 23: 145. 1855; *Coelogyne biflora* E.C.Parish ex Rchb.f., Gard. Chron. 1865: 1035. 1865; *Pleione thuniana* (Rchb.f.) Kuntze, Revis. Gen. Pl. 2: 680. 1891;*Chelonistele biflora* (E.C.Parish ex Rchb.f.) Pfitzer in H.G.A. Engler (ed.), Pflanzenr., IV, 50 II B 7: 139. 1907.
    Common name: One-Flowered Panisea.
    Distribution in the world: China, India, Nepal, Cambodia, Laos, Myanmar, Thailand, Vietnam.
    Species examined: NH 10, Birik Forest,West Bengal. **Altitude**: 862 ft. **Habitat**: epiphyte.
    Host plant: Bark of *Terminalia myriocarpa*. **Date of collection**: 23-06-2017.
    Voucher specimen number: ANUBH1214. **Flowering**: March to June.
    IUCN conservation status (2018): Unknown. **Population trend**: decreasing.

Pseudobulbs as long as 2–4 cm, caespitose, ovoid, mammillate, wrinkled throughout, attached with a few fibrous sheaths at the base. Leaves in pairs, narrowly oblong, acuminate, slightly narrowed at the base, sessile, 4–6 cm long and 1–1.5 cm in width. Flowers are solitary, on a very short peduncle from the base of the pseudobulb. Flowers are 2 cm across, sepals and petals greenish-yellow, lip of the same shade on its apex and gradually progressing to bright yellow on the base side, also with few orange spots. Sepals oblong-lanceolate, sub-acute, spreading, five veined. Petals shorter than the sepals, subacute, broadly lanceolate, mid-veined. Lip oblong, with a very short claw at the base, the side lobes erect and narrow with their acute apices pointing forward (Fig. 9.7b).

15. ***Paphiopedilum insigne*** (Wall. ex Lindl.) Pfitzer, Jahrb. Wiss. Bot. 19: 159. 1888; *Cypripedium insigne* Wall. ex Lindl., Coll. Bot.: t. 32. 1824; *Cordula insignis* (Wall. ex Lindl.) Raf., Fl. Tellur. 4: 46. 1838; *Paphiopedilum macfarlanei* F.G. Mey., Gartenflora 83: 93.1934.
    Common name: Splendid Slipper Orchid.
    Distribution in the world: Assam (Meghalaya) to China (NW. Yunnan), Bangladesh, Myanmar, Thailand.
    Species examined: Tukdah Forest, West Bengal. **Altitude**: 6003 ft. **Habitat**: terrestrial.
    Date of collection: 23-11-2017. **Voucher specimen number**: ANUBH1215.
    Flowering: November to December.

IUCN conservation status (2018): Endangered B1ab(ii, iii, v) + 2ab(ii, iii, v) ver 3.1.
Population trend: decreasing. However, this orchid is in critically endangered condition from our observation.

Paphiopedilum insigne is a terrestrial orchid, grows on dolomite limestone, near waterfalls, and in light and shady areas. Leaves are up to 2.5 cm long, broadly linear, strap-shaped, forming tuffs, pointed, pale green in color. Flowering stem is about 30 cm long. Flowers are yellow-green with purple spots, exterior of hood is white, lip is pitcher-like; dorsal sepal with white tip portion spotted purple, base is light green. Petals are linear-oblong, margin wavy, hairless, yellowish-green. Lip is helmet shaped, light or yellowish-green with a brown shade (Fig. 9.7c).

16. ***Papilionanthe teres*** (Roxb.) Schltr, Orchis. 9: 78. 1915; *Dendrobium teres* Roxb., Fl. Ind. ed. 1832 3: 485. 1832. *Vanda teres* (Roxb.) Lindl., Gen. Sp. Orchid. Pl. 217.1833; Hook. f., Fl. Brit. India 6: 49. 1890; C.E.C. Fisch., Fl. Madras 1444. 1928.
Common name: Cylindrical Vanda.
Distribution in the world: Bangladesh, Bhutan, Borneo, Cambodia, China, Java, Laos, Myanmar, Nepal, Thailand, Vietnam, Andaman & Nicobar Islands, India, Andhra Pradesh, Arunachal Pradesh, Assam, Meghalaya, Mizoram, Nagaland, Odisha, Sikkim, Tamil Nadu, Tripura and West Bengal.
Species examined: Lish Forest, West Bengal. **Altitude**: 1365 ft. **Habitat**: epiphyte.
Host plant: bark of *Pinus sabiniana*. **Date of collection**: 18-05-2017.
Voucher specimen number: ANUBH1216. **Flowering**: May to June.
IUCN conservation status (2018): Unknown. **Population trend**: decreasing.

Stem is slender, terete, branched, leaves fleshy, articulate, obtuse, ca 15 × 0.4 cm. Inflorescence longer than leaves, extra-axillary, 30 cm long; raceme laxly 2–6 flowered. Floral bracts brown with rose dots, broadly triangular, acute, one-veined, 3 × 1.5 mm pedicel with ovary whitish-rose, 4 cm long. Flowers are whitish-rose, 6–9 cm across. Sepals and petals are obovate or oblong-obovate, undulate, rounded, nine-veined; dorsal ca 3 × 2.3 cm; laterals ca 3.6 × 2.4 cm; petals ca 3.6 × 3.2 cm. Lip ca 4 × 3.8 cm, three-lobed; laterals incurved, quadrate or quadrate-ovate; midlobe clawed, obovate-orbicular, deeply bilobed; spur conical, mouth funnel-shaped, rounded, ca 2 cm long. Column is whitish-rose, cylindrical, with two triangular arms at apex, 1 × 0.5 cm; foot ca 2 mm long. Stigma is orbicular-rounded. Pollinia 2, subglobose, 3 × 2.8 mm. Capsules are narrowly clavate and 10 cm long (Fig. 9.7d).

17. ***Phaius tankervilleae*** (L. Herit.) Blume, Bot. Lugd. Bat. 2:177. 1856; Seidenf. & Arora, 24. 1982; *Limodorum tankervalleae* Banks ex L. Herit., Sert. Angl.:28. 1789; *Phaius wallichii* Lindl. in Wall.Pl. Asiat.Rarior. 2:46.t. 158. 1831;

Hook.f., 5:816. 1819; King & pantl. 108,t.150. 1898; *Phaius grandifolius* Lour. Fl. Cochinch. 2:529. 1790; *Phaius blunei* Lindl. Gen. & Sp.Orch. 127. 1830.
Common name: Nun's Orchid, Nun's Cap Orchid.
Distribution in the world: Himalaya (Nepal to Arunachal Pradesh), Assam, Meghalaya, Mizoram, Manipur, Tripura, Bangladesh, Sri Lanka, Burma, Java, Sumatra, Borneo, Thailand, Indo-China, China, New Guinea, Formosa, Samoan, and Fiji Islands.
Species examined: Mazeok Forest, West Bengal. **Altitude**: 2132 ft. **Habitat**: terrestrial.
Date of collection: 12-04-2017. **Voucher specimen number**: ANUBH1217. **Flowering**: April to May.
IUCN conservation status (2018): Unknown. **Population trend**: decreasing.

Phaius tankervilleae is a large terrestrial herb with pseudobulbs, leafy stem, and vermiform roots. Pseudobulbs are about 8 cm long, 2.5–3 cm in diameter, annulate, internodes greenish-white in color. Leaves 2–4 on each pseudobulb, 30–120 cm long from the base of petiole to apex, 4.5–13.5 cm broad, lanceolate or oblong – lanceolate, acuminate, strongly ribbed. Inflorescence many flowered raceme. Flowers 6–11 cm across, varying in color from purplish-orange to pale orange-yellow with an orange-yellow to purplish-white lip. Bracts 4.5 cm long, orbicular-ovate, acuminate or cuspidately acute, 11-veined. Sepals the dorsal 4–5 cm long, lanceolate, acute, seven-veined. Petals 3.7–5.2 cm long, lanceolate or oblong – lanceolate, acute or subacute, seven-veined. Lip 3.5–5 cm long, erect, three-lobed, adnate to the base of the column and embed, adnate to the base of the column and embracing it by the convolute, and recurved crenate lateral lobes; mid-lobe orbicular, crenate, produced into a horn – like spur, sometimes bifid (Fig. 9.7e).

18. ***Phalaenopsis mannii*** Rchb.f. in Gard. Chron., 902. 1871; Rolfe in Gard. Chron. 2: 276. 1886; Hook. F., Fl. Brit. Ind. 5: 30. 1890; King et Pantl. in Ann. Bot. Gard. Calcutta 8: 197, pl. 264. 1898; *Phalaenopsis boxalii* Rchb.f. Gard. Chron. ns 19:274. 1883; *Polychilos mannii* (Rchb.f.) Shim, Malayan Nat. Journ. 36:24. 1982
Common name: Mann's Phalaenopsis.
Distribution in the world: Indian Himalayas, Andaman and Nicobar Island, Assam, Nepal, Bhutan, Sikkim, Myanmar, Southern China, and Vietnam.
Species examined: Tukdah Forest, West Bengal. **Altitude**: 6009 ft. **Habitat**: epiphyte.
Host plant: bark of *Pinus wallichiana*. **Date of Collection**: 06-05-2017.
Voucher specimen number: ANUBH1218. **Flowering**: April to June.
IUCN conservation status (2018): Unknown. **Population trend**: decreasing.

Stem is very short, completely enclosed by imbricating leaf sheaths. Roots many, rather long, flexuous, glabrous. Leaves more or less numerous (4 or 5), drawn up or spread out, a little flask, obovate-oblong or oblanceolate-oblong, abruptly acute, a little sickle-shaped, of a pale green, more or less brown spotted, (especially toward the base),veined, long from 20 to 35 cm, wide from 4 to 7 cm. Flower stalk as long or a little longer than the leaves, erect or a little pendant, robust, hardly compressed, green very finely punctuated of mauve, sometimes simple, more often provided with 2 or 3 short branches, multiflores. Bracts rigid, a little fleshy, very concave, ovate-lanceolate, acute, green, long of 1 cm. Flower spread out, a little fleshy and rigid from 4 to 5 cm. Sepals very spread out, convex, margin a little undulated and strongly revolutate, carrying outside, close to the apex, a strong apiculate bulge. Dorsal sepal is linear-ligulate, sub-obtuse. Lateral sepals larger, lanceolate-sub-spatulate, acute, strongly asymmetrical. Petals almost similar to the sepals, but narrower and a little shorter, little thickened at apex, lanceolate-ligulate, acute (Fig. 9.7f).

19. ***Pholidota articulata*** Lindl. Gen. & Sp. Orch. 38. 1830; Hook. f., 5:844. 1890: King & Pantl. 146, t. 205. 1898; Duthie,116. 1906; Seidenf. & Smitinand 139, 1959; *P. griffithii* Hook. f., Fl. Brit. Ind. 5:845. 1890 et Ic. Pl.t. 1881. 1890; Arora 438, fig. 1–4. 1969; Raizada et al. 25. 1981. *P. articulata* var. *griffithii* (Hook.f.) King & Pantl. Ann. Roy. Bot. Gard. Cal. 8:147, t. 204, 1898. Duthie, 116. 1906.

    Common name: Jointed Pholidota.

    Distribution in the world: Himalaya (Garhwal to Arunachal Pradesh), Assam, Meghalaya, Manipur, Nagaland, Mizoram, Tripura. Burma, China, Indo-China, Malaya.

    Species examined: Mangpong Forest, West Bengal. **Altitude**: 940 ft. **Habitat**: epiphyte.

    Host plant: Bark of *Pinus gerardiana*. **Date of Collection**: 04-02-2017.

    Voucher specimen number: ANUBH1219. **Flowering**: February to June.

    IUCN conservation status (2018): Unknown. **Population trend**: decreasing.

Pseudobulbs are 6–10 × 1.5–2.5 cm, new one arising near the apex of the old, jointed, branching, and furrowed. Leaves are two, 7.5–12 × 2.5–4.5 cm narrowly oblong, sessile or shortly petiolate, tapering to each end, many – nerved, membranous. Inflorescence is a raceme, 20–55 cm long pendulous, densely many flowered. Flowers are 8 mm across, white, suffused with brownish-pink. Bracts are large, distichous, broadly – ovate, convolute, deciduous when flowers open, acute or obtuse, longer than ovary. Sepals are the dorsal suborbicular; laterals ovate, keeled. Petals are ovate-orbicular, blunt. Lip is as long as the sepals, three-lobed, hypochile with rounded lobs, cymbiform, with 5-lamillate nerves; epichile broad bilobulate or not, apex usually apiculate (Fig. 9.8a).

20. ***Rhynchostylis retusa*** (Linn.) Bl. Bijdr. 286. t. 49. 1825; Hook. f., 6:32. 1890; King & Pantl. 213, t. 284. 1898; Duthie, 148. 1906; *Epidendrum retusum* Linn. Sp. Pl. 953. 1753; *Aerides spicatum* D. Don Prodr. Fl. Nepal 31. 1825; *Saccolabium guttatum* Lindl. Gen. & Sp. Orch. 220. 1833.
Common name: Foxtail Orchid.
Distribution in the world: Himalaya (Himachal Pradesh to Arunachal Pradesh), Assam, Meghalaya, Manipur, Nagaland, Mizoram, Tripura, Bengal, Bihar, Orissa, Madhya Pradesh, Western Ghats, Andaman Island. Burma, Sri Lanka, Malaysia, Java, Thailand, and the Philippines.
Species examined: Mazeok Forest, West Bengal. **Altitude**: 2132 ft. **Habitat**: epiphyte.
Host plant: bark of *Quercus alba*. **Date of collection**: 23-05-2016.
Voucher specimen number: ANUBH1220. **Flowering**: March to July.
IUCN conservation status (2018): Unknown. **Population trend**: decreasing.

Stem is 10–20 cm long, woody, covered with persistent sheathing base of the fallen leaves. Leaves are distichous, 15–45 × 2–3.5 cm, dense, linear, praemorse or bluntly bilobed at apex. Raceme is longer than the leaves, cylindrical, drooping, and axillary. Flowers are 2 cm across, many, dens, white or pink with purple, or pink markings. Bracts are ovate – lanceolate, shorter than ovary. Sepals the dorsal 8 mm long, suborbicular, obtuse; the laterals 9 mm long, broadly ovate, obtuse. Petals are ovate-elliptic, obtuse, and narrower than sepals. Lip is 7 mm long, continuous with foot, spurred, bend inward; hypochile narrow; epichile obovate, acute, and concave with erect margin, deep magenta in color (Fig. 9.8b).

21. ***Spathoglottis plicata Blume*** in Bijdr. 401. 1825; *Bletia angustifolia* Gaudich In: Voy. Uranie: 421. 1829; *Calanthe poilanei* Gagnep In: Bull. Mus. Natl. Hist. Nat., sér. 2, 22: 625. 1951; *Spathoglottis angustifolia* (Gaudich.) Benth. & Hook. f. In: Gen. Pl. 3: 512. 1883.
Common name: Philippine ground orchid, large purple orchid.
Distribution in the world: Southern India, Southern China, Southern Japan, Indo-China, Myanmar, Thailand, Peninsular Malaysia, Singapore, Sumatra, Java, Borneo, the Philippines, throughout Indonesia to Australia and the Pacific Islands.
Species examined: NH 10 Birik Forest, West Bengal. **Altitude**: 843 ft.; **Habitat**: terrestrial.
Date of Collection: 01-08-2016. **Voucher specimen number**: ANUBH1221. **Flowering**: March to April.
IUCN conservation status (2018): Unknown. **Population trend**: decreasing.

Pseudobulbs are ovoid-conic, 3 × 1–1.7 cm, enclosed in sheaths, with 3–5 leaves. Leaf blade is linear-lanceolate, 30–80 × 5–7 cm, petiole-like stalk 10–20 cm. Inflorescence is up to 1 m long, with many tubular sheaths; rachis 8–15 cm, 9–16-flowered; floral bracts reflexed, purple, ovate, ca. 1.5 cm. Flowers opening successively, purple; pedicel and ovary purplish, 25–35 mm. Sepals are ovate, concave, sparsely pubescent, apex acute; lateral sepals oblique. Petals are subelliptic, apex acute; lip 3-lobed, 16–23 mm, with two small, triangular, pubescent auricles at base of claw; disk with two short keels arising from base of lip; lateral lobes falcate, apex dilated and truncate; mid-lobe dilated toward apex and flabellate, ca. 10 mm, apex subtruncate, and often shallowly two-lobed, with a distinct claw at base; claw and lower part of lip sometimes yellow; keels expanded toward apex into two large, fleshy, erect, rounded extensions terminating near base of claw (Fig. 9.8c).

22. ***Thunia alba*** (Lindl.) Rchb.f. in Bot. Zeit 10:764. 1852; *Limodorum bracteatum* Roxb. Fl. Ind. ed. 2, 3:466. 1832; *Phaius albus* Lindl. in Wall. Pl. Asist. Rarior 2:85 t.198. 1831; Hook.f., 5:818. 1890; King & Pantl. 110,t.153. 1898; Duthie, 109. 1906; *Thunia venosa* Rolfe, Orch. Rev. 206. 1905; Duthie, 193. 1920; *Thunia bracteata* (Roxb.) Schlechter in Fedde, Repert. Beih. 4:205. 1919.

    Common name: White Thunia, Marshall's Thunia.

    Distribution in the world: Himalaya (Garhwal to Arunachal Pradesh), Assam, Nagaland, Meghalaya, Mizoram, Tripura, Manipur, Madhya Pradesh, Western Ghats, Andaman Island. Burma, China, Indo-China, Malaya, and Thailand.

    Species examined: Latpanchar Forest, West Bengal**. Altitude**: 1809 ft. **Habitat**: terrestrial.

    Date of collection: 01-08-2016. **Voucher specimen number**: ANUBH1222. **Flowering**: May to August.

    IUCN conservation status (2018): Unknown. **Population trend**: decreasing.

Thunia alba is terrestrial, rarely epiphytic; pseudobulbs absent. Stem is stout, erect, tufted, 30–90 cm in height. Leaves are 10–15 cm long, sessile, elliptic-lanceolate, acuminate, 3–7 nerved. Inflorescence drooping, 10–30 cm long, 4–6 flowered. Flowers white, 5–6.5 cm long. Bracts are ovate – oblong, equaling, or exceeding the ovary. Sepals are white, 6–7 cm long, free, subequal, narrowly oblong, acute. Petals are similar to sepals. Lip is yellow or orange with purple lines, broadly oblong-panduriform when spread out, lower half convolute into a tube, upper portion cucullate, forming a rounded shell like mouth, slightly acuminate, margins undulate or unequally dentate; spur short, horizontal, slightly bifid (Fig. 9.8d).

23. ***Vanda coerulea*** Griff. ex Lindl., Edwards's Bot. Reg. 33: t. 30 (1847); *Vanda coerulea* var. *rogersii* Rolfe, Orchid Rev. 22: 31 (1914); *Vanda coerulea delicata* Rolfe, Orchid Rev. 33: 318 (1925); *Vanda coerulea* f. *luwangalba* Kishor, Orchid Rev. 116: 224 (2008); *Vanda coerulea* f. *delicata* (Rolfe) Christenson, Orchid Rev. 117: 222 (2009); *Vanda coerulea* f. *rogersii* (Rolfe) Christenson, Orchid Rev. 117: 222 (2009).
Common name: Blue Vanda.
Distribution in the world: India (Assam, Arunachal Pradesh, Meghalaya, Nagaland), Nepal, Burma, Northern Thailand, Southern China (Yunnan), Bhutan, Laos, and Vietnam.
Species examined: Tukdah Forest, West Bengal.; **Altitude**: 4990 ft.; **Habitat**: epiphyte.
Host Plant: bark of *Pinus roxburghii*. **Date of collection**: 23-12-2016.
Voucher specimen number: ANUBH1223. **Flowering**: July to December.
IUCN conservation status (2018): Unknown. **Population trend**: decreasing, but this orchid is endangered condition according to our observations.

Stems are 6–25 cm long. Leaf blade is very thick and leathery, apex unequally bilobed. About 1–3 inflorescences, with 6–14 flowers are present; peduncle is 16–22 cm; rachis is zigzag, 9–28 cm; floral bracts are broadly ovate, 9–11 × 6–9 mm, apex obtuse-acute. Flowers are thin in texture; wide opening; ovary is white in color with tinged blue, 35–40 mm; sepals and petals are sky-blue in color, tessellated. Sepals are similar, broadly obovate, 36–45× 18–35 mm, base contracted into short claw, apex rounded. Petals are elliptic-obovate, 36–45 × 20–25 mm, base contracted into a short claw, apex rounded; lip is sky-blue in color, shorter than sepals, fleshy, spurred; lateral lobes erect, narrowly falcate, adaxially white with yellow spots; apex is subacuminate; mid-lobe deep blue, ligulate, base with a pair of calli, apex subtruncate and emarginate; disk has 2–3 longitudinal ridges; spur is tubular, slightly conical; apex is obtuse. Column is 4–6 mm in length (Fig. 9.8e).

24. ***V. cristata*** Wall. ex Lindl., Gen. & Sp. Orchid. Pl.: 216.1833; Hook.f., 6:53. 1890; King & Pantl. 216, t.287. 1898; Duthie, 146, 1906; *Aerides cristata* (Wall. ex Lindl.) Wall. ex Hook.f., Fl. Brit. India 6: 53 (1890); *Trudelia cristata* (Wall. ex Lindl.) Senghas ex Roeth, Orchidee (Hamburg) 58: 707 (2007 publ. 2008); *Vanda striata* Rchb.f., Xenia Orchid. 2: 137 (1868); *Luisia striata* (Rchb.f.) Kraenzl. in H. G. Reichenbach, Xenia Orchid. 3:120 (1893).
Common name: The Comb Vanda.
Distribution in the world: Himalaya (Garhwal to Arunachal Pradesh), Meghalaya, Madhya Pradesh. Nepal, Bhutan Bangladesh, China, and Indo-China.
Species examined: Tukdah Forest, West Bengal. **Altitude**: 5999 ft. **Habitat**: epiphyte.

Host Plant: bark of *Terminalia elliptica*. **Date of collection**: 02-05-2016.
Voucher specimen number: ANUBH1224. **Flowering**: May **to** June.
IUCN conservation status (2018): Unknown. **Population trend**: decreasing.

Stem is tall up to 15 cm; erect stout, covered with remains of old sheaths. Leaves bifarious, crowded, 7.5–10 × 1.5–2.8 cm, narrowly oblong, bilobed at the apex. Inflorescence is raceme, equal, or slightly shorter than the leaves, 2–5-flowered. Flowers are 4–5 cm across, yellowish or olive green with purplish blotches. Sepals and petals subequal, oblong, obtuse, the petals narrower than sepals. Lip adnate to the base of the column, longer than the sepals, green blotched with dull purple – brown, 3-lobed; lateral lobes erect, triangular-mid lobe oblong, 2–3 lobulate and with a horn-like fleshy beak pointing downward (Fig. 9.8f).

25. ***V. tessellata*** (Roxb.) Hook. ex G.Don in J.C. London, Hort. Brit.: 372.1830; *Cymbidium tessellatum* (Roxb.) Sw., Nova Acta Regiae Soc. Sci. Upsal. 6: 75 (1799); *Aerides tessellata* (Roxb.) Wight ex Lindl., Gen. Sp. Orchid. Pl.: 240 (1833); *Vanda roxburghii* R.Br., Bot. Reg. 6: t. 506 (1820); *Cymbidium tesselloides* Roxb., Fl. Ind. ed. 1832, 3: 463 (1832);*Vanda tesselloides* (Roxb.) Rchb.f. in W.G.Walpers, Ann. Bot. Syst. 6: 864 (1864); *Vanda roxburghii var. wrightiana* Rchb.f., Gard. Chron., n.s., 20: 262 (1883).
Common name: Checkered Vanda, Grey Orchid.
Distribution in the world: Himalaya (Garhwal to Bhutan), Tripura, Bengal, Bihar, Orissa, Upper Gangetic plain, Madhya Pradesh, Gujarat, Konkan, Southern Peninsular India, Bangladesh, Myanmar, Nepal, Sri Lanka, Indo-China.
Species examined: Near Latpanchar Forests, kurseong, West Bengal. **Altitude**: 3005 ft.
Habitat: epiphyte. **Host Plant**: Bark of *Alnus glutinosa*. **Date of collection**: 13-07-2017.
Voucher specimen number: ANUBH1225. **Flowering**: May to July.
IUCN conservation status (2018): Least Concern ver 3.1. **Population trend**: decreasing, however this orchid is abundant in Eastern Ghats.

Stem is erect, 30 cm long. Leaves are several, distichous, 10–20 × 1–1.2 cm, linear, keeled, articulate at the base, unequally bilobed at the apex. Inflorescence is raceme, axillary; 3–10 flowered, longer than leaves, zigzag. Flowers are 3.5–5 cm across, yellowish-green, with blue tinge, scented. Bracts are suborbicular, obtuse. Sepals and petals are subequal, spreading, clawed, and wavy, more than 15 m long. Lip is shorter than the sepals, yellow, three-lobed; lateral lobes obliquely elliptic, subfalcate, acute or acuminate; mid-lobe pink or purple dotted, thick, panduriform, apex 2–3-lobed. Spur conical, hairy inside (Fig. 9.9).

9.5 Analysis of Orchid Flora

In the present orchid diversity study, 25 orchid species belonging to 18 genera were observed in the different forested regions of the Darjeeling Himalaya (Table 9.2) (Fig. 9.1). The total number of species recorded from different genera were *Acampe* 2, *Aerides* 1, *Arundina* 1, *Bulbophyllum* 2, *Coelogyne* 1, *Cymbidium* 1, *Dendrobium* 3, *Eria* 2, *Panisea* 1, *Paphiopedilum* 1, *Papilionanthe* 1, *Phaius* 1, *Phalaenopsis* 1, *Pholidota* 1, *Rhynchostylis* 1, *Spathoglottis* 1, *Thunia* 1, and *Vanda* 3. Out of 25 orchid species, 5 species belonging to 5 genera were terrestrial, and the rest of the 20 species with 13 genera were epiphytic. Out of 25 orchid species, 80% were epiphytic (Fig. 9.2), and 20% were terrestrial (Fig. 9.2). The number of epiphytic orchids is greater than the terrestrial orchids. The orchids such as *Acampe papillosa, A. rigida, Aerides multiflora, Bulbophyllum guttulatum, B. leopardinum, Coelogyne cristata, Cymbidium aloifolium, Dendrobium aphyllum, D. fimbriatum, D.moschatum, Eria lasiopetala, E. stricta, Panisea uniflora, Papilionanthe teres, Phalaenopsis mannii, Pholidota articulata, Rhynchostylis retusa, Vanda coerulea, V. cristata, and V. tessellata* were observed on different host trees (Epiphytic). *Arundina graminifolia*, *Paphiopedilum insigne, Phaius tankervilleae, Spathoglottis plicata,* and *Thunia alba* were found on the moist hilly rocks (terrestrial). Both epiphytic and terrestrial orchids showed altitudinal variation (Fig. 9.3) ranging from 701 ft. to 6009 ft. *Bulbophyllum leopardinum* was observed at lowest altitude (701 ft.), whereas the *Phalaenopsis mannii* was found at 6009 ft. (highest altitude). In the present study, most of the orchids were observed on shrubby or small trees as well as very large trees. But few trees gave shelter to large number of orchids on them. It may be due to host preference of different orchid species. In this study, orchids were found on trees like *Alnus glutinosa, Castanopsis indica, Engelhardtia spicata, Ficus religiosa, Juniperus recurva, Pinus gerardiana, P. roxburghii, P. sabiniana, P. wallichiana, Quercus alba, Rhododendron arboretum, Rhus typhina, Shorea robusta, Terminalia elliptica, and T. myriocarpa*.

Out of 25 orchid species recorded in the present study, 4 orchids (endangered 1, least concern 3) (Table 9.2 and Fig. 9.4) are reported in the IUCN Red List of Threatened Species Version 2018–1 (https://www.iucnredlist.org); however, the IUCN status of most of the orchids in the present study is unknown (Fig. 9.4), but some orchids such as *Bulbophyllum guttulatum* and *Vanda Coerulea* were found to be threatened. *Paphiopedilum insigne* is an endangered (B1ab(ii, iii, v) + 2ab(ii, iii, v) ver 3.1) orchid found in Tukdah Forest, West Bengal, at an altitude of 6003 ft. on moist hilly and shady rocks. *Bulbophyllum leopardinum* is in IUCN Red List; its present status is Least Concern (ver 3.1) which was observed near the road sides of Tista Bazar, West Bengal, on the moist bark of *Shorea robusta* at an altitude of 701 ft. The present population trend of *B. leopardinum* is decreasing at alarming rate. *Dendrobium aphyllum* is an another orchid observed on the bark of *Terminalia myriocarpa* in Rangpo Forest at an altitude of 1320 ft. whose present status is Least Concern (ver 3.1). *Vanda tessellata* was observed in Latpanchar Forest at an altitude of 3005 ft. on *Alnus glutinosa.* The present status of *V. tessellata* is Least Concern (ver 3.1) in IUCN Red list; however, this orchid is abundant in Eastern Ghats (Figs. 9.5, 9.6, 9.7, 9.8, and 9.9).

Table 9.2 Distribution of Orchids in Darjeeling Himalaya with their habitat, location, altitudinal ranges, flowering month, and IUCN status

S. no	Name of the orchid species with voucher specimen number	Habitat	Location in Darjeeling Himalaya		Flowering month	IUCN status (Red list 2018)
			Location	Altitude (in feet)		
1.	*Acampe papillosa* (Lindl.) Lindl. ANUBH1201	Epiphyte	Sukna wild life, Sukna Forest, West Bengal	735	June to August	Unknown
2.	*A. rigida* (Buch.- Ham. ex Sm.) P.F. Hunt ANUBH1202	Epiphyte	Near sacred water body, Lamahatta, Tukdah Forest, West Bengal	6255	May to June	Unknown
3.	*Aerides multiflora* Roxb ANUBH1203	Epiphyte	Near Latpanchar Forests, Kurseong, West Bengal	3579	April to May	Unknown
4.	*Arundina graminifolia* (D.Don) Hochr ANUBH1204	Terrestrial	NHPC, NH31A, road side, Kalijhora, West Bengal	745	January to November	Unknown
5.	*Bulbophyllum guttulatum* (Hook.f.) N.P. Balakr. ANUBH1205	Epiphyte	Sittong forest, West Bengal	2736	June to August	Unknown
6.	*B. leopardinum* (Wall.) Lindl ANUBH1206	Epiphyte	Near roadsides of Tista Bazar, West Bengal	701	June to August	Least Concern ver 3.1
7.	*Coelogyne cristata* Lindl. ANUBH1207	Epiphyte	Pankhabari road side, Kurseong, West Bengal	4852	January to April	Unknown
8.	*Cymbidium aloifolium* (L.) Swartz ANUBH1208	Epiphyte	11th Tinchuley Lopchu Road, Tukdah Forest, West Bengal	5006	April to June	Unknown
9.	*Dendrobium aphyllum* (Roxb.) C.E.C. Fisch. ANUBH1209	Epiphyte	NH 10, Teesta riversides, Rangpo Forest, West Bengal	1320	March to May	Least Concern ver 3.1

10.	*D. fimbriatum* Hook. ANUBH1210	Epiphyte	Roadsides of river Rangeet, West Bengal	1890	March to May	Unknown
11.	*D. moschatum* (Buch.-Ham.) Sw. ANUBH1211	Epiphyte	Rangli Forest, West Bengal	1706	May to June	Unknown
12.	*Eria lasiopetala* (Willd) Ormerod. ANUBH1212	Epiphyte	Sukna Forest, West Bengal	790	March to June	Unknown
13.	*E. stricta* Lindl. ANUBH1213	Epiphyte	Near opposite roadside of Mahananda Wild life Sanctuary,West Bengal	1568	February to May	Unknown
14.	*Panisea uniflora* (Lindl.) Lindl ANUBH1214	Epiphyte	NH 10, Birik Forest, West Bengal	862	March to June	Unknown
15.	*Paphiopedilum insigne* (Wall. ex Lindl.) Pfitzer ANUBH1215	Terrestrial	Tukdah Forest, West Bengal	6003	November to December	Endangered B1ab(ii, iii, v) + 2ab(ii, iii, v) ver 3.1
16.	*Papilionanthe teres* (Roxb.) Schltr. ANUBH1216	Epiphyte	Lish forest, West Bengal	1365	May to June	Unknown
17.	*Phaius tankervilleae* (Banks) Blume ANUBH1217	Terrestrial	Mazeok Forest, West Bengal	2132	April to May	Unknown
18.	*Phalaenopsis mannii* Rchb.f ANUBH1218	Epiphyte	Tukdah Forest, West Bengal	6009	April to June	Unknown
19.	*Pholidota articulata* Lindl ANUBH1219	Epiphyte	Mangpong Forest, West Bengal	940	February to June	Unknown
20.	*Rhynchostylis retusa* (L.) Blume ANUBH1220	Epiphyte	Mazeok Forest, West Bengal	2132	March to July	Unknown

(continued)

Table 9.2 (continued)

S. no	Name of the orchid species with voucher specimen number	Habitat	Location in Darjeeling Himalaya		Flowering month	IUCN status (Red list 2018)
			Location	Altitude (in feet)		
21.	*Spathoglottis plicata* Blume ANUBH1221	Terrestrial	NH 10, Birik Forest, West Bengal	843	March to April	Unknown
22.	*Thunia alba* (Lindl.) Rchb. f. ANUBH1222	Terrestrial	Latpanchar Forest, West Bengal	1809	May to August	Unknown
23.	*Vanda coerulea* Griff. ex Lindl. ANUBH1223	Epiphyte	Tukdah Forest, West Bengal	4990	July to December	Unknown
24.	*V. cristata* Wall. ex Lindl. ANUBH1224	Epiphyte	Tukdah Forest, West Bengal	5999	May–June.	Unknown
25.	*V. tessellata* (Roxb.) Hook. ex G. Don ANUBH1225	Epiphyte	Near Latpanchar Forests, Kurseong, West Bengal	3005	May to July	Least Concern ver 3.1

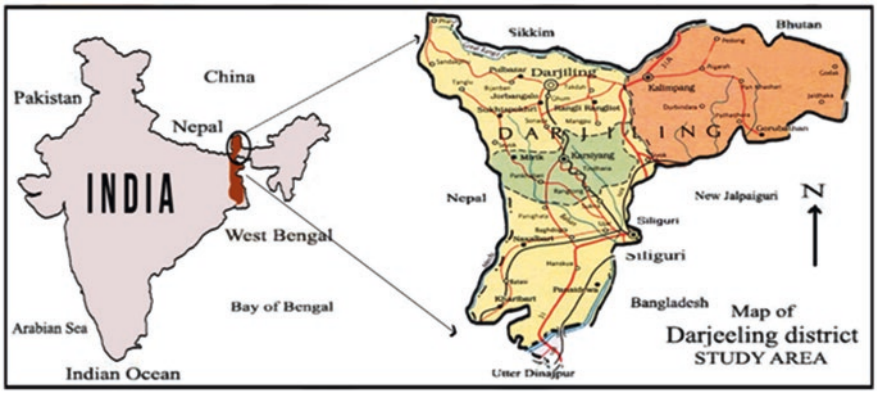

Fig. 9.1 Map of study area (Darjeeling Himalaya)

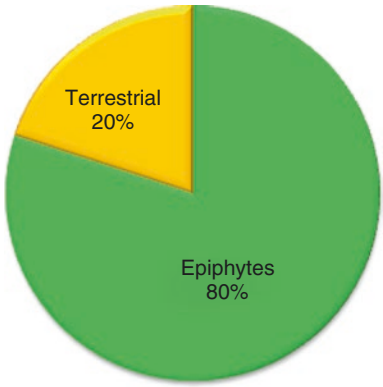

Fig. 9.2 A Pie chart showing the distribution of orchids in their habitats

9.6 Conclusions and Future Perspectives

In our botanical exploration, we observed that few individuals of *Bulbophyllum guttulatum* were noticed on the bark of *Rhus typhina*. Similarly, *Vanda Coerulea* also showed decreasing population trend. Some 25 years back Darjeeling Himalaya was abundant with *V. Coerulea*, but now it has restricted to small patches. Based on our field data and also Udai C. Pradhan's (eminent orchid grower in the region) observations, it was concluded that both *B. guttulatum* and *V. Coerulea* declared as "critical," these may be slipped in to endangered to extinct state in near future. In this scenario we have already started working on micropropagation of these two orchids and attempts to be made to transplant the in vitro derived plantlets to the natural habitat, so as to protect them from the wild.

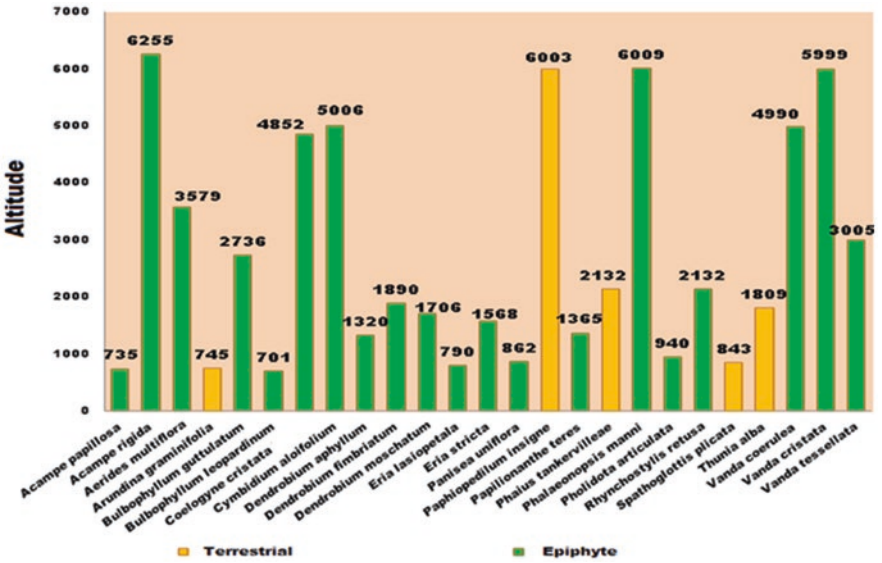

Fig. 9.3 A graph showing the distribution of orchid species with respect to altitudinal variation

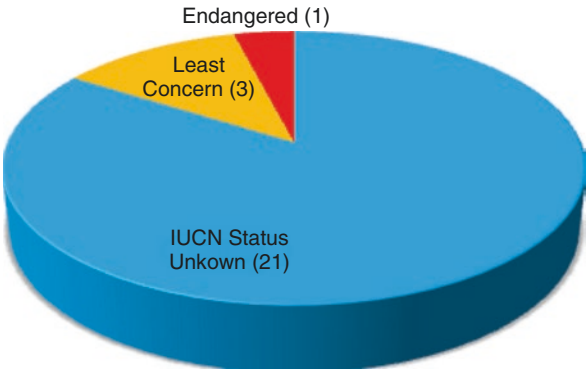

Fig. 9.4 A Pie chart showing IUCN status of the orchids

Fig. 9.5 (**a**) *Acampe papillosa*, (**b**) *A. rigida*, (**c**) *Aerides multiflora*, (**d**) *Arundina graminifolia*, (**e**) Bulbophyllum guttulatum, (**f**) B. leopardinum

Fig. 9.6 (**a**) *Coelogyne cristata*, (**b**) *Cymbidium aloifolium*, (**c**) *Dendrobium aphyllum*, (**d**) *D. fimbriatum*, (**e**) *D. moschatum*, (**f**) *Eria lasiopetala*

Fig. 9.7 (**a**) *E. stricta*, (b) *Panisea uniflora*, (**c**) *Paphiopedilum insigne*, (**d**) *Papilionanthe teres*, (**e**) *Phaius tankervilleae*, (**f**) *Phalaenopsis mannii*

Fig. 9.8 (**a**) *Pholidota articulata*, (**b**) *Rhynchostylis retusa*, (**c**) *Spathoglottis plicata*, (**d**) *Thunia alba*, (**e**) *Vanda coerulea*, (**f**) *V. cristata*

Fig. 9.9 *V. tessellata*

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Orchid Diversity in the Eastern Ghats of Northern Andhra Pradesh, India

10

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Abstract

Northern Andhra Pradesh is located between 81° 06′ to 83° 53′ East and 16° 42′–18° 17′ North in the Eastern Ghats of Andhra Pradesh. Some attempts have been made to study the orchids in this area, and a total of 54 species belonging to 30 genera of orchids were reported. Details of brief description, distributional status and conservation measures were provided in this chapter.

Keywords

Biodiversity · Conservation · Eastern Ghats · Orchids · Northern Andhra Pradesh

10.1 Introduction

Orchidaceae is the second largest family in the flowering plants, and about 28,000 species were distributed in the world (Chase et al. 2003, 2015; Willis 2017; Michael 2018). They are the natives of tropical countries and occur in the humid tropical forests of South and Central America, India, Ceylon, Burma, South China, Brazil, the Philippines, New Guinea and Australia (Rao 1979). Orchids' name is derived from the root tuber which resembles testicle, and they have diversified habit as epiphytes, lithophytes, terrestrial and saprophytes. Epiphytic orchids grow on the main trunk or branches of the trees without any internal connection. The roots have mycorrhizal association with specific fungal species known as symbiosis and have epiphytic roots with a special absorbent tissue known as 'velamen tissue' to absorb moisture from the atmosphere. Orchid's flower exhibits many variations in shape,

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size, colour and posture with showy labellum or lip formed by posterior petal, formation of gynostegium or column, pollen united as pollinia and non-endospermic microseeds.

In India about 1331 species of orchids were reported from various parts of the country, of which 856 are found in the Himalayan region and 320 in South India (De and Medhi 2014). A total of 130 species belonging to 38 genera of orchids were reported in Peninsular India (Jalal and Jayanthi 2012), and 91 endemic orchids were reported from South India (Kumar and Sasidharan 1986). About 190 orchid species belonging to 54 genera were recorded from Eastern Ghats (Gamble and Fischer 1935; Matthew 1984; Henry et al. 1989; Saxena and Brahmam 1995; Pullaiah 1997; Reddy et al. 2001, 2002a, b). From Andhra Pradesh 77 orchid species were reported (Raju et al. 2008). Rao et al. (2009) reported 56 orchids from Eastern Ghats of Andhra Pradesh, 11 from the Talakona sacred grove 11 species (Savitramma 2005), 13 species (Miria et al. 2012) orchids were reported. Ethnobotanical information on orchids (Reddy et al. 2002a, b, 2005) was reported from Andhra Pradesh. Mishra et al. (2008) reported 83 orchid species belonging to 40 genera from Andhra Pradesh. Several workers reported from different districts in the Northren Andhra Pradesh, 27 orchid species belonging to 18 genera from East Godavari district (Rao et al. 1999), 15 orchids belonging to 6 genera from West Godavari district (Rao et al. 1986b), 17 orchids belonging to 12 genera from Srikakulam district (Rao et al. 1986a), 11 orchids belonging to 7 genera from Vizianagaram district (Venkaiah 2004) and 27 orchids belonging to 18 genera from Visakhapatnam district (Subbarao and Kumar 2008).

Orchids are going to be threatened with anthropogenic pressures and destruction in their habitats; thus, there is a decrease in their population. Hence, there is a need to investigate species diversity in orchids and their conservation. Present study focused on diversity of the orchids in the Northern Andhra Pradesh, India.

Northern Andhra Pradesh is part of Eastern Ghats which includes the districts of Srikakulam, Vizianagaram, Visakhapatnam, East Godavari and West Godavari, and it is located between 81° 06′ to 83° 53′ East and 16° 42′ to 18° 17′ North. The Eastern Ghats of this region is rich in biodiversity with good plant resources, enriched fertile soils and good rainfall for the plant growth (Fig. 10.1). The forest types in this region are tropical mixed dry deciduous, tropical moist deciduous, dry deciduous sal, semi-evergreen, dry evergreen, thorny scrub, hilltop and savannah.

Many attempts have been taken to collect orchid species during our field explorations in the study area during the period of 2014–2017 that covered all the seasons. Collected specimens were identified with floras and literature. All the plants were provided in alphabetical order, and details of their current accepted name, habit, distributional status in the study area, etc. were also provided. Some photographs were provided for easy identification of the species from the study area (Figs. 10.2 and 10.3) (Table 10.1).

Fig. 10.1 Map of the study area

10.2 Orchid Diversity in the Eastern Ghats of Andhra Pradesh, India

A total of 54 species belonging to 30 genera of orchids were reported from the study area. Among the 54 orchids, 26 are epiphytes (48.15%), 24 are terrestrial (44.44%), 1 is saprophyte (1.8%) and 3 are epiphytes cum lithophytes (5.5%) (Fig. 10.4). Details of nomenclature and description of all the orchid species are provided below:

***Acampe ochracea* (Lindl.) Hochr.** In New York Bot. 6:270. 1910. *Saccolabium ochraceum* Lindl. In Edw. Bot. 2.1842. FBI 6: 62.1890.

An epiphyte; stem non-bulbous, woody; leaves lorate, 10–20 × 1.5–2.5 cm, keeled, thick, coriaceous; flowers in raceme panicles, yellow with red transverse striae across the perianth segments; capsule fusiform, erect, 4 × 0.5 cm.

***A. praemorsa (Roxb.)* Blatt. & McCann** in J. Bombay Nat. Hist. Soc. 35.495. 1932. *Epidendrum praemorsum* Roxb. Pl. Cor. T. 43.1795 *Saccolabium praemorsum* (Roxb.) Hook. F. Fl. Brit. India. 6:12. 1890. *S. wrightianum* Hook. F. Loc. Cit. *Acampe wrightiana* Lindl. Fol. Orchid. *Acampe.* 2.1853. G.3. 1011.1928.

Fig. 10.2 (**a**) *Acampe praemorsa* (Roxb.) Blatt. & McCann; (**b**) *Cymbidium aloifolium* (L.) Sw.; (**c**) *Dendrobium aphyllum* (Roxb.) C.E.C.Fisch.; (**d**) *Nervilia concolor* (Blume) Schltr.; (**e**) *Nervilia plicata* (Andrews) Schltr. (**f**) *Oberonia ensiformis* (Sm.) Lindl.

An epiphyte; stem woody, 50–60 cm long; leaves lorate, coriaceous, 15–17 cm; flowers yellow barred with red in racemes; capsule fusiform (Fig. 10.2a).

***A. rigida* (Buch. Ham. Ex Sm.) P. F. Hunt**, *Aerides rigida* Buch. Ham. Ex Sm.

An epiphyte herb; leaves lorate, coriaceous, flowers in racemes, capsule fusiform.

***Aerides multiflorum* Roxb.** Pl. Cor. 3:68. T.271. 1820;FBI 6:44. 1890.

An epiphyte; stem 10–25 cm long, crowded; racemes 15–30 cm long; flowers rose purple; spur straight, obtuse, upcurved; capsule sub-clavate pedicelled.

***A. odoratum* Lour. Fl. Cochinch.** 2.525. 1790. FBI 6:47. 1890. G.3.1008, 1928.

An epiphyte, stem stout; leaves oblong, lanceolate, 12–25 × 2–4 cm; raceme 25–30 cm long flowers purple white; spur large, uncinately curved.

Fig. 10.3 (**a**) *Oberonia wightiana* Lindl.; (**b**) *Pholidota pallida* Lindl.; (**c**) *Rhynchostylis retusa* (L.) Blume; (**d**) *Vanda tessellata* (Roxb.) Hook. ex G.Don; (**e**) *Vanda testacea* (Lindl.) Rchb.f.; (**f**) *Vanilla wightiana* Lindl.

***A. ringens* (Lidl.) C Fischer in Bull**. Misc. Inform. 1928. 284. 1928 and G.3:1008. 1928. *Saccolabium ringens* Lindl. *Gen. Sp. Orchid, Pl 221. 1883. Aerides radicosum* A. Rich. In Ann. Sci. Nat. B. Ser. 2.15:65. 18841;FBI 6:46.1890. *A lineare* Hook. F. loc. Cit. FBI 6:47 1890.

An epiphyte with short stems; leaves loriform, thick coriaceous,17–18 × 1–4 cm; racemes simple; flowers white pale pink; capsule fusiform.

***Ascocentrum curvifolium* (Lindl.) Schltr.** in Wlap. Ann. 6:253. 1861. *B densiflorum* Rolfie in Kew Bull. 1892. 139. 1892. *B bisetum* sensu Hook. f. F.B.I. 5:767. 1890. p.p non Lindl.1842.

Table 10.1 Details of diversity and distributional status of orchids in the study area

S. no.	Botanical name	Status	Habit	Distributional status in the study area
1	*Acampe ochracea* (Lindl.) Hochr.	R	EP	Rare in Visakhapatnam district
2	*A. praemorsa* (Roxb.) Blatt. & McCann	C	EP	Common in the study area
3	*A. rigida* (Buch.-Ham. ex Sm.) P.F.Hunt	R	EP	Rare in East Godavari district
4	*Aerides multiflorum* Roxb.	O	EP	Present in the East and West Godavari districts. Endemic in Himalaya to Indo-China
5	*A. odoratum* Reinw. ex Blume	O	EP	Present in Visakhapatnam, East Godavari and West Godavari districts
6	*A. ringens* (Lindl.) C.E.C.Fisch.	R	EP	Rare in West Godavari district
7	*Ascocentrum curvifolium* (Lindl.) Schltr.	R	EP	Rarely seen in Visakhapatnam district
8	*Bulbophyllum cariniflorum* Rchb.f.	R	EP&LT	Present in Visakhapatnam district
9	*B. sterile* (Lam.) Suresh	R	EP	Present in Visakhapatnam and East Godavari districts
10	*Callostylis bambusifolia* (Lindl.) S.C.Chen & J.J.Wood	R	EP	Rare in Visakhapatnam district
11	*Crepidium resupinatum (G.Forst.) Szlach.*	O	TR	Present in Visakhapatnam, East Godavari and West Godavari districts
12	*Cymbidium aloifolium* (L.) Sw.	C	EP	Common in all areas
13	*Dendrobium aphyllum* (Roxb.) C.E.C.Fisch.	C	EP	Common in the study area
14	*D. aqueum* Lindl.	O	EP	Seen on hills of East Godavari district
15	*D. herbaceum* Lindl.	R	EP	Rare in East and West Godavari districts. Endemic to South India
16	*Didymoplexis pallens* Griff.	R	SP	Present in West Godavari district. Endemic to India
17	*Eulophia epidendraea* (J.Koenig ex Retz.) C.E.C.Fisch.	O	TR	Present in Visakhapatnam and Srikakulam districts and endemic to India, Bangladesh and Sri Lanka
18	*E. explanata* Lindl.	R	TR	Rare in Visakhapatnam and East Godavari districts. Endemic to India
19	*E. spectabilis* (Dennst.) Suresh	R	TR	Present on hills of Visakhapatnam district. Endangered
20	*Geodorum densiflorum* (Lam.) Schltr.	C	TR	Common in Visakhapatnam, Srikakulam and Vizianagaram districts
21	*Goodyera procera* (Ker Gawl.) Hook.	O	TR	Found in Visakhapatnam district
22	*Habenaria digitata* Lindl.	R	TR	Rarely seen in East and West Godavari districts

(continued)

Table 10.1 (continued)

S. no.	Botanical name	Status	Habit	Distributional status in the study area
23	*H. furcifera* Lindl.	O	TR	Present in Srikakulam, Vizianagaram and Visakhapatnam districts
24	*H. hollandiana* Santapau	C	TR	Common in the study area
25	*H. longicorniculata* J.Graham	R	TR	Rare in Visakhapatnam district
26	*H. marginata Colebr.*	R	TR	Rare in Visakhapatnam district
27	*H. plantaginea* Lindl.	O	TR	Present in East, West Godavari, Visakhapatnam and Vizianagaram districts. Endemic to India
28	*H. roxburghii* Nicolson	O	TR	Frequent in Northern Andhra Pradesh. Endemic to India
29	*Liparis deflexa* Hook.f.	R	TR	Present in East Godavari district
30	*L. viridiflora* (Blume) Lindl.	R	EP& LT	Found in Visakhapatnam district
31	*Luisia tristis* (G.Forst.) Hook.f.	O	EP	Rarely growing in Srikakulam, Vizianagaram and Visakhapatnam districts
32	*Nervilia concolor* (Blume) Schltr.	R	TR	Present in East Godavari district
33	*N. crociformis* (Zoll. & Moritzi) Seidenf.	R	TR	Present in Visakhapatnam district
34	*N. plicata* (Andrews) Schltr.	R	TR	Present in Visakhapatnam district
35	*Oberonia brunoniana* Wight	O	EP	Present in East Godavari district
36	*O. ensiformis* (Sm.) Lindl.	O	EP	Frequent on trees in North Coastal Andhra Pradesh
37	*O. falconeri* Hook.f.	R	EP	Present in Visakhapatnam district
38	*O. mucronata* (D.Don) Ormerod & Seidenf.	O	EP	Present in Vizianagaram and East Godavari districts
39	*O. wightiana* Lindl.	O	EP	Present in East Godavari and Visakhapatnam districts
40	*Papilionanthe teres* (Roxb.) Schltr.	R	EP	Rare in Visakhapatnam district
41	*Pecteilis gigantea* (Sm.) Raf.	R	TR	Occasional in the hills of East Godavari districts
42	*Peristylus goodyeroides* (D.Don) Lindl.	R	TR	Present in Visakhapatnam district
43	*Peristylus plantagineus* (Lindl.) Lindl.	R	TR	Present in Rampa hills and Maredumilli in East Godavari district
44	*Phalaenopsis deliciosa* Rchb.f.	R	EP	Found in Northern Andhra Pradesh
45	*Pholidota pallida* Lindl.	R	EP& LT	Rare in East Godavari, Srikakulam and Visakhapatnam districts

(continued)

Table 10.1 (continued)

S. no.	Botanical name	Status	Habit	Distributional status in the study area
46	*Polystachya concreta* (Jacq.) Garay & H.R.Sweet	R	EP	Rare in Visakhapatnam district
47	*Pomatocalpa spicatum* Breda, Kuhl & Hasselt	R	EP	Present in Visakhapatnam district
48	*Rhynchostylis retusa (L.) Blume*	O	EP	Rarely seen in Northern Andhra Pradesh
49	*Satyrium nepalense* D.Don	R	TR	Present in Visakhapatnam district
50	*Spiranthes sinensis* (Pers.) Ames	R	TR	Rare in the study area
51	*Tropidia acuminata* Schltr.	R	TR	Rare in East Godavari district
52	*Vanda tessellata* (Roxb.) Hook. ex G.Don	C	EP	Common in the study area
53	*Vanda testacea* (Lindl.) Rchb.f.	O	EP	Occasionally seen in East Godavari, Visakhapatnam and Vizianagaram districts
54	*Vanilla wightiana* Lindl.	R	TR	Present in East Godavari district

C common, *O* occasional, *R* rare, *EP* epiphyte, *TR* terrestrial, *SP* saprophyte, *EP& LT* epiphyte and lithophyte

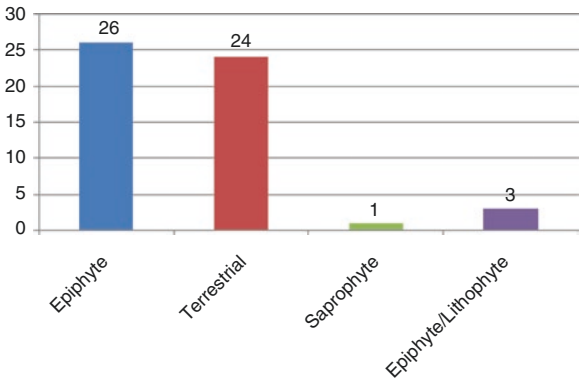

Fig. 10.4 Habit-wise details of orchids in the study area

An epiphytic herb; rhizome creeping; pseudobulbs aggregate; leaves two or rarely three to four, lanceolate; flowers in spikes.

Bulbophyllum cariniflorum **Rchb.f.** in Wlap. Ann. 6:253. 1861. B. densiflorum Rolfe in Kew Bull. 1892: 139. 1892. B. bisetum sensu Hook. Fl. Brit. India 5:767. 1890. p.p. non Lindl. 1892

An epiphytic caespitose herb, rhizome creeping, slender, pseudobulbs aggregate. Leaves two or rarely three to four lanceolate, subsessile; base cuneate; apex acute. Flowers in spikes, yellowish green, lip tongue-shaped; column erect; pollinia four, subglobose.

Present in Visakhapatnam district.

***B. sterile* (Lam.) Suresh.** *Epidendrum sterile* Lam. *Bulbophyllum neilgherrense* Wight (heterotypic synonym).

An epiphytic herb with pseudobulb; leaves two to four; flowers in spikes.

***Callostylis bambusifolia* (Lindl.) S.C.Chen & J.J.Wood**. Soc. 3:61. 1859; FBI 5:805. 1890; Fischer 3:14224(997). 1928.

An epiphyte herb; stem pseudobulbous, robust, 1 m long; leaves narrow, elliptic oblong, 10–20 × 4–8 cm. Racemes axillary, many flowered, 30 cm rusty pubescent; bracts apiculate. Flowers 1.2–1.5 cm long; sepals subequal, broadly elliptic; petals narrower.

***Crepidium resupinatum* (G.Forst.) Szlach.** *Epidendrum resupinatum.* (G. Forst.) *Malaxis rheedii* S W. in Kongl. Vet. Acad. Nya Handl. 21. 235. 1800, *Microstylis versicolor*, *M. rheedii* Wight, Ic. T. 902. 1844. FBI 5:690.

A terrestrial or lithophytic herb. Stem slender, pseudobulbous; leaves broadly ovate or elliptic, thin 6–18 × 2–6 cm; scape 15–25 cm long; flowers green yellow or purple in racemes; sepals ovate; petals narrow, linear; capsule 1 cm long.

***Cymbidium aloifolium* (L.) Sw.** in Nova act Upsal. 2, 6:73. 1799; FBI 6:10, 1890. p.p.; Fischer 3:1434 (1004). 1928. *Epidendrum aloifolium L.,* Sp.Pl. 953. 1753. *E. pendulum* Roxb.,Pl. Corom. 1:35. 1795. *Cymbidium pendulum* (Roxb.) Sw., loc. Cit, *C. erectum* Wight, Ic. T. 1753. 1852. *C. intermedium* Jones in Reinwardtia 9:71, 1974.

A tufted epiphyte; stems fleshy; roots vermiform; leaves linear, lorate, 25–45 × 2–2.5 cm, coriaceous; racemes pendulous, 25–30 cm long; flowers cream coloured; sepals and petals with a purple lip. Capsule pyriform (Fig. 10.2b).

***Dendrobium aphyllum* (Roxb.) C.E.C.Fisch.**, Fl. Pres. Madras 3:1416 (991). 1928. *Limodorum aphyllum* Roxb., Pl. Cor. 1:34.t. 9.1823; FBI 5:738. *Dendrobium pierardii* Roxb. In Hook., Fl.1.t.9. 1823;FBI 5:738. 1890.

An epiphytic herb. Stem slender, pendulous, 1 m long; leaves lanceolate, 3.5–7 × 2.2 cm, acuminate; flowers pale rose; sepals oblong-lanceolate, subacute; spur conical (Fif. 10.2c).

***D. aqueum* Lindl.** Bot. Reg. 6,t.54. 1843;FBI 5:739. 1890; Fischer 3:1417 (991). 1928.

A stout epiphytic herb; stem 30–50 cm long; leafy and yellowish green when young. Leaves ovate-lanceolate, 6–12 × 1.2–4, acuminate. Flowers two to three and cream white; sepals ovate; petals obovate; spur short.

***D. herbaceum* Lindl.** Bot. Reg. 26:69 1840; FBI 5:719. 1890; Fischer 3:1416(990). 1928.

A pendulous epiphyte; stem with many branches, 1 m long; lower part of the stem is naked; leaves linear-lanceolate, 5–10 × 0.6–0.9 cm. Flowers yellow; pedicels filiform; bracts lanceolate; petals linear, obtuse; spur minute. Fruit ellipsoid, tapering at the base.

Didymoplexis pallens Griff. FBI 6:122 King & Pants. Ann, Cal, Viii.t. 346. *Apetalon minutum* Wt. Ic; t.1758.Vol. 3.1459.

A slender saprophytic, leafless tuberous terrestrial herb; stem 7–10 cm long; sheaths lose; racemes terminal four to eight flowered; flowers dull yellowish white; lip stipitate, membranous, transversely oblong; disc papilose.

***Eulophia epidendraea* (J.Koenig ex Retz.) C.E.C.Fisch.** Misc. Inform. 1928: 283. 1928 & Fl. Pres. Madras 3:1434 (1003). 1928. *Sepias epidendrae*a J. Koing in Reitz., Obs. Bot. 6:65. 1791. *Eulophia visens* (Rox.) R. Br. Ex Lindley, Bot. Re. 7 :Sub t. 573. 1821; FBI 6:1 1890. *Limodorum virens* Roxb. Pl. Cor.t.38.1795.

A terrestrial herb; stem pseudobulbous basal sheaths; leaves linear; flowers ash green. Floral bracts ovate, acute; dorsal sepal lanceolate-oblong; lateral lobes of lip small, erect; midlobe broad, oblong round, recurved; anther terminal, two loculed; pollinia two, globose.

***E. explanata* Lindl.** Gen, Sp. Orchids 180. 1832; FBI 6:3. &195. 1890.

A terrestrial herb about 12 cm height; pseudobulb three, ovoid, annulated. Leaves elliptic-lanceolate. Scape lateral, 30 cm long, ten flowered; flowers yellow; pedicle 1 cm long; sepals and petals subequal; sepals spathulate-lanceolate, acute; petals broadly oblong, rounded at apex; lip sessile obscurely three-lobed, shorter than sepals and petals.

***E. spectabilis* (Dennst). Suresh**. *Wolfia spectabilis* Denn. *E. nuda* Lindl. Gen. Sp. Orchid. Pl.180. 1833; FBI 6:5, 1890.

A terrestrial herb about 12 cm height, pseudobulbous, sheaths; leaves linear; margin entire; apex acuminate. Racemes loosely flowered, arising from the base of the stem; peduncle 40–50 cm long. Floral bracts lanceolate, membranous; sepals oblong; petals oblong, obtuse; lip ovate, recurved; lateral lobes erect; spur dark purplish blue; flowers with white lip.

***Geodorum densiflorum* (Lam.) Schltr.** In Feddes Report, 4:259. 1929, G. dilatum sensu. Hook. f.Fl. Brit. Ind. 6:17. 1890.

A terrestrial herb with underground tuberous pseudobulbs; stem sheathed; leaves oblong; lanceolate flowers, white with yellow and pink lip. Capsules pendulous.

***Goodyera procera* (Ker Gawl.) Hook.** Exot, Fl.t. 39.1823;FBI 6:111.1890.Fischer 3:1456(1018) 1928*, Neotlia procera* Ker-Gawl. In Bot. Reg. 8:t639. 1822.

A terrestrial herb; stem erect from a creeping base, 25–40 cm long; roots vermiform; leaves lanceolate,10–18, 2.5 cm; apex acuminate; midrib stout; flowers greenish white in spikes, fragrant.

***Habenaria digitata* Lindl**. Gen.Sp. Pl. 307. 1835. FBI b:134.1890. Fischer 3:1469(1062). 1928.

An erect orchid with leaves sheathing at bases; flowers white in racemes of 10 cm long; sepals greenish white; petals two-partile; spur 1.8 cm.

***H. furcifera*. Lindl.** Gen. Sp. Orchid. Pl. 1835; FBI 6;149. 1890.

An erect herb about 20–30 cm; stem curved with sheaths below the cluster of leaves; leaves variable in shape, elliptic oblong or naturally elliptic, sessile, 8–9 cm × 3.5 to 3.8 cm; spike slender, 35 to 40 cm long; flowers in a cream colour; petals entire; capsule elliptic, ribbed.

***H. hollandiana* Santapau.** 126. 1958. Wt. Ic.t. 1707, 1851, FBI 6:148, G.3.1028, 1928.

A small herb 60 to 80 cm height; leaves clustered in the middle of the stem, elliptic-lanceolate; spike 50 cm long, cylindrical; flowers white.

***H. longicorniculata* J.Graham.** Cat. Bombay, Pl. 202.1839. *H. longicorniculata* A.Rich. Ann. Sci. Nat. Bot, 2, 15, 17t. 3B1841; FBI 6:141. 1890.G.3.1027.1928.

A tuberous terrestrial herb, 40–75 cm high; leaves oblong-lanceolate, variable, 4–10 × 2–3 cm; flowers white, jasmine-scented; spur very long, 10 cm.

***H. marginata* Clobe.** In Hook. Exot. fl. T. 136. 1825; FBI 6:150. 1890; Fischer 3:1471 (1028). 1928.

A terrestrial tuberous herb; leaves sessile, oblong, obtuse, with yellow margins, lanceolate. Flowers yellow, sessile on spikes; bracts lanceolate; lateral sepals broad; dorsal sepal broadly ovate, acute; petals oblong-lanceolate, acute. Fruit capsule, fusiform.

***H. plantaginea* Lindl.** Gen. Sp. Orchid. 323. 1835. FBI 6:141. 1891. G. 3:1027, 1928.

A herb with a short stem and oblong tubers; leaves radical, narrow, oval, 7.5 × 2 cm long; sepals ovate, oblong; petals linear-lanceolate; spur very slender; capsule fusiform.

***H. roxburghii* Nicolson.** in saldanha and Nicolson. Fl. Hassan dt. 834,1876, *Orchis roxburghii* Pers, Syan. Pl, 2:503. 1807 nom. Illeg. (incl. *O. plantaginea* Roxb.) *H. platyphylla* (wild.), Spreng. In L.Syst Veg. (ed.16) 690, 1826,nom. Illg. (incl. *O. plantaginea* Roxb.) FBI 6:140. 1890. G.3;1027,1927.

A terrestrial orchid, 10 cm high with leaves apressed to the ground; leaves elliptic or orbicular, 4–10 × 4–8 cm; flowers white on 15–30 cm scape; sepals broadly oblong; petals linear.

***Liparis deflexa* Hook.f.** *Eulophia spectabilis* In J. Asiat. Soc. Bengal. Pt 2, Nat. Hist. 66:582 1897; Ellis & Swamin. In.j. Bombay. Nat. Hist. Soc.66:233. 1969. *L. flavoviridis* Blatt. & Mc Cnn. In j. Bombay. Nat. Hist. Soc 35:360 1931.

A terrestrial herb; pseudobulb ovate or oblong; leaves two to five lying on soil, sessile, alternate ovate elliptic, lanceolate. Scape with racemes 20 cm long with bracts; dorsal sepal lanceolate-acuminate; pollinia caudicles in pairs; ovary sessile, slightly winged; column elongate; capsule broadly oblong, stalked.

***L. viridiflora* (Blume) Lindl.** Gen. Sp. Orchid. Pl. 31.1830;FBI 5:704. 1890; *Malaxis viridiflora* Blume, Bijdr. 392. 1825. *Liparis longipes* Lindl. In Wall. Pl. As. Rar. 1:31.t. 35. 1830; FBI 5:703. 1890.

An epiphytic herb; pseudobulbs close together in a row. Leaves elliptic-lanceolate 5–12 × 1.5–2.5 cm; Racemes many flowered, 10–15 cm long; pedicels recurved; flowers pale yellowish green; sepals linear-oblong, subacute; petals linear.

***Luisia tristis* (G.Forst.) Hook.f.**. Orchid. Luisia 3.1853. *L. teretifolia* Sensu Hook. f., Fl. Brit. India 6:22. 1890. 1890 non Guad. 1829. *L. truncata* Blatt. & McCann J. Bombay Nat. Hist. Soc. 35:491. 1932.

A tufted epiphytic herb, 15–30 cm; stem terete, sheathed; roots vermiform; leaves green, spotted with purple, 15–16 × 0.5–1 cm, terete. Flowers in short two to three flowered spikes; sepals greenish-yellow; lateral sepal oblong, subacute. Capsules oblong, 3 × 0.8 cm, winged.

***Nervilia concolor* (Blume) Schltr.**. Bot. 422. t. 35, 1887; Fischer 3: 1458(1020). 1928 *Pogonia flabelliformis* Lindl. Gen. Sp. Orchid. 1415, 1840; FBI 6:121. 1890 *P. carinata* (Roxb). Lindl. loc.cit. Hook. F. Loc. Cit.

A terrestrial tuberous herb with one leaf; leaf is 10–15 cm, orbicular or subreniform; base cordate; margin waved, coarsely dentate; flowers 1.5 cm long; sepals and petals green; lip three-lobed; middle lobe ovate; column elongate (Fig. 10.2d).

***N. crociformis* (Zoll. & Moritzi) Seidenf.**. Botanisk. Arkin ellp32:15.1978. *Boltorchis corciformis* Mor in Mortizi syst. Verz. Pl. 2011. 89. 1846. *Pogonia crispate* Bl. Mus. Bot. Lugd. Bot. 1:32. 1849. *Nervilea crispate* (Bl.) Schltr. ex Kranzal in Schum & Lauterb. Fl. Deut. Schultz geb. 240.1901.

A tuberous herb with one leaf, orbicular-cordate, densely velvety pubescent when young and scattered at maturity; scape one flowered; flower terminal; pollinia yellow.

***N. plicata* (Andrews) Schltr.**, in Engl. Bot. Jahrb. Syst. 45. 40. 403. 1911. Gamle Vol 3:1458(1020) 1928. *Arethusa plicata* Andr. Bot rpr.5.t.321. 1803. *Pogonia plicata (*Andr*)* Lindl. Gen. Sp. Orchid.415. 1840, FBI 6:119.1890. *Pogonia biflora* Wight Ic.t 1758. 1851. *Nervelia biflora* (White) schltr.loc.cit.

A tuberous herb, one leaf lying flat on the ground, broadly ovate or suborbicular, obtuse, acute; base cordate 5–10 cm long, dark green mottled with purplish brown; scape two to three flowered, 6–15 cm long; sepals and petals linear-lanceolate, acuminate (Fig. 10.2e).

***Oberonia brunoniana* Wight.** Ic. T. 1622. 1851; FBI 5:681. 1888; Fischer 3; 1406(984). 1928.

A large, handsome, fleshy epiphyte; leaves linear-oblong, 15–35 × 1.8–3 cm, succulent, as long as the inflorescence; scapes 5–20 cm long; flower sessile 10–15 cm long; spikes erect; bracts lanceolate; sepals brown; petals pale yellow. Capsule 6 mm long.

***O. ensiformis* (Sm.) Lindl.**., Fol. Oberon. 8.1859; FBI 6:679. 1888 Fischer 3:1406(984). 1928. *Malaxis ensiformis* J.E. Smith as a direct taxonomic synonym (in Rees Cyclop. 22.1812 No. 14 in the protologue of *O. iridifolia.)*

A tufted, pendulous epiphyte; leaves 15–45 cm long, coriaceous, acuminate; flowers yellow, minute, in dense or interpreted spikes or racemes; sepals and petals reflexed; sepals subequal, ovate; petals ovate. Capsule 0.3 cm and ribbed (Fig. 10.2f).

***O. falconeri* Hook.f.** in Hook. Ic Pl. t.1780. 1880, FBI 5:678. 1890. Gamble. Vol 3. 1406 AP. Fl. 953.

An epiphyte erect or pendulous; leaves lanceolate, oblong, falcate, inflorescence raceme; scape erect; flowers greenish-yellow; numerous bracts hyaline, lanceolate, denticulate, acuminate; sepals pale yellow; petals yellow; lip greenish yellow three-lobed; column yellow.

***O. mucronata* (D.Don) Ormerod & Seidenf.**, *Stelis mucronata* D.Don. *O. denticulate* Wight. Ic. t. 1625. 1851 *Oberonia iridifolia* (Roxb.) Lidl. Gen. Sp. Orchid. 15. 1830; var *denticulatei* (Wight.) Hook.f FBI 5:676 1888, Fisher 3:1406 (933, 1928. *Cymbidium iridifolium* Roxb. Fl., Ind. 3:458. 1832.

A long tufted epiphytic herb; leaves broadly ensiform, 7–20 × 1–2 cm, fleshy. Spikes slender, up to 30 cm long. Flowers yellow, sessile. Capsule up to 0.25 cm.

***O. wightiana* Lindl.** *Oberonia arnottiana* Wight, Ic. 5(1): 3,t. 1698. 1851. *Oberonia wightiana* Lindl. var. *arnottiana* (Wight.) Ansari et al. In J. Econ. Tax. Bot. 3: 118. 1982. *O. wightiana* Sensu Hook.f., Fl. Brit. India 5:683. 1888. P.p. non Lindl., Fischer 3:1407 (894). 1928.

A small tufted epiphyte. Leaves linear-oblong or narrow ensiform, 3–6 × 0.5–1 cm, Scape terete; raceme slender, longer than the leaves; flowers pale yellowish green (Fig. 10.3a).

***Papilionanthe teres* (Roxb.) Schltr.**. In Orchids 9:78. 19.15. *Dendrobium teres* Roxb.Fl. Ind. 3:485. 1832. *Vanda teres* (Roxb.) Lindl. Gen. Sp. Orchid. 217. 1833; FBI 6:49. 1890; Fischer 3:1444(1009). 1928.

An epiphyte; stem slender, 2–3 m long; leaves terete, slender, 8–20 cm long. Racemes two to six flowered, axillary. Flowers white or rose; sepals oblong or obovate; petals larger, subrotund; spur funnel shaped.

***Pecteilis gigantea* (Sm.) Raf.** Fl. Tell. 2:38. 1837. *Orchis gigantea* J. E. Smith, Exot. 2:79.t.100. 1805. *Habenaria susannae* acut. non (L.) R.Br. ex Spreng. 1826; Lindl 1835; Fischer 3:1475 (1031). 1928.

A tall stout, terrestrial herb, 0.5–1.25 m long; stem leafy up to the inflorescence. Leaves ovate-oblong or oblong-lanceolate, 5–9 × 2.5–5 cm; apex acute or acuminate, the upper sheathing, becoming smaller and passing into large leafy bracts above. Racemes three to six flowered. Flowers white, fragrant, very shortly pedicellate; sepals obliquely subquadrately oblong; dorsal shorter, broadly rhomboid; petals linear, acute; lip as long as the lateral sepals; anther very broad and larger; pollinia linear; capsules with pedicel 5 cm long, ellipsoid, beaked.

***Peristylus goodyeroides* (Don) Lindl.** Gen. Sp. Orchid. Pl.299. 1835. G.V. 3. 1475. 1928. *Habenaria goodyeroides* D. Don. Prodr. Fl. Nep.25. 1825. FBI. 6:161 1890 pp; Bot. Bihar & Orissa 1159. 1924.

A tall robust herb; stems ribbed or terete covered with long sheaths at base; leaves appearing; flowers clustered or scattered at the middle of the stem and spike; flowers white; pollinia two; ovary twisted.

***P. plantagineus* Lindl.** Gen. Sp. Orchid. 300. 1835; Fischer 3:1475(1030). 1928. *Habenaria wightii* Trimen, Cat. Pl. Ceylon 91. 1885; FBI 162. 1890.

A terrestrial herb with large, cylindrical-oblong tuber; roots fleshy and fibrous; stems lower portion ensheathed by loose long lanceolate sheaths. Leaves oblong-lanceolate, 12–16 × 6–7 cm; margin entire; apex acute. Racemose spike 30–60 cm long; the flower bearing axis 10–20 cm long; floral bracts 6 mm long. Flowers greenish with white petals and lip; dorsal sepal ovate; lateral sepal longer and oblong; petals very broad; nerves branched; tips recurved; lip as long as sepals, broadly ovate, three-lobed; lobes obtuse; spur very small, globose; column short; base continuous in to the lip; rostellum short.

***Phalaenopsis deliciosa* Rchb.f.** in Amer. Orchid. Soc. Bull 39:1095. 1970. *Aerides decumbens* Griff.,Notul. 3:365. 1851. *Kingiella decumbens* Rolfie in Orchid Rev. 25:197. 1917; Fischer 3:1439 (1006). 1928. *Kingidium decumbens (Griff.) P. F.* Hunt in Knew Bull. 24:97. 1970.

An epiphytic herb; stem stout, 2.5 cm long; leaves 1–4 oblong 6–20 × 2–5 cm; flowers small pale purple; sepals and petals subequal in length.

***Pholidota pallida* Lindl. Edw.** Bot. Reg. 21:subt. 1777. 1836. *Pholidota imbricata* acut. Non Lindl. Bot. Reg.t. 1213. 1828 non 1825; FBI 5:845. 1890; Fischer 3:1431 (1000). 1928.

A pendent epiphyte; pseudobulb aggregated, narrowly ovate-cylindrical, 2.5–6 cm long. Leaf solitary, elliptic-lanceolate or oblanceolate, with short petioles; flowers pink in terminal drooping racemes; bracts pale brown. Capsule ellipsoid (Fig. 10.3b).

Polystachya concreta (Jacq.) Garay & Sweet in Revista Soc. Columb. Orquideol. 9:206. 1974. *Epidendrum concretum* Jacq. Enum. Pl. Carib. 30. 1760. *Polystachya purpurea* Wight, Ic. T.1679. 1851; FBI 6:1437(1005). 1928. *P. wightiana* Reichb. F. in Walp. Ann. 6:640. 1861; FBI 6:21. 1890; Fischer 3:1437 (1005). 1928.

An epiphyte; leaves three to four, narrowly oblong or lanceolate, 5–15 × 1.2–2.5 cm. Scape compressed; rachis angular; flowers small, purple, 2 mm long, racemose or panicled; sepals free; petals much smaller, very narrow.

***Pomatocalpa spicatum* Breda, Kuhl & Hasselt** in Nat. Tijdschr. Ned. Ind. 72:105. 1912. *Cleisostoma mannii* Reichb. f. in flora 55:273. 1872; FBI 6:74. 1896; Fischer 3:1448 (1013). 1928.

An epiphyte; stem 0.5 m long. Leaves distichous, imbricate, linear; apex unequally bluntly two-lobed, 8–20 × 2.5 cm. Racemes rather stout, simple or branched, 5–8 cm long. Flowers yellow flushed with red; sepals and petals ovate; spur saccate.

***Rhynchostylis retusa* (L.) Blume, Bijdr**. 286. T. 49. 1825; FBI 6:32. 1890; Fischer 3:1440 (1007). 1928. *Epidendrum retusum* L. Sp. Pl. 953. 1753.

An epiphyte, 5–25 cm long; lower part leafless with short internodes covered with remaining old leaf sheaths. Leaves linear, 14–35 × 1.5–2 cm, star shaped, recurved; apex unequally lobed, coriaceous. Racemes lateral and drooping. Flowers white tinged with violet and pink; peduncles 8–10 cm long, stout. Capsules obovate, six ridged, 3 × 1.5 cm long.

***Satyrium nepalense* D.Don. Prdr.** 26, 1825 FBI 6:168. 1890 Vol. 3.1476.

A terrestrial herb, unbranched; roots tuberous; leaves few, lanceolate, ovate, oblong, acute; spikes dense, many flowered; flowers white to pink.

***Spiranthes sinensis* (Pers) Amens.** Orchid: 2:53 1908. Vol 3. 1454, *Neotia sinensis* Pers. Syn 2:511. 1807. *Spiranthes australis* (R.Br) Lindl in Bot. Reg. 10. Sub. T. 823. 1814; FBI 6:102. *Neotia australis* R. Br. Prodr. Fl, Nov. Holl. 1810.

A terrestrial, Slender herb with tuberous roots; stems ribbed with hairy sheathing bracts; leaves clustered near the base; spikes 8 cm long, glandular hairy; flowers white, ovary ellipsoid.

***Tropidia acuminata* Schltr.** Orchid. 122. 1859; FBI 6:92. 1890; Fischer 3:1452(1015). *Cnemedia angulosa* Lindl. Gen. Sp. Orchid. Pl. 468. 1840.

A terrestrial herb; stem 20–30 cm long, often branched, sheathed, below the leaves. Leaves two to three, elliptic, broadly ovate, 6–15 × 3.5–6 cm; base rounded or

cordate, acuminate. Flowers white, small, on terminal; peduncle spiky; bracts linear, slender, spreading, 1–1.8 cm long; lateral sepals lanceolate, acuminate, connate nearly to the apex, forming a mentum and enclosing the lip and spur; dorsal sepal narrower; petals ovate-lanceolate; lip oblong, obtuse; spur cylindrical; column short; foot absent; rostellum long.

***Vanda tessellata* (Roxb.) Hook. ex G.Don** in Loud Hort. Brit. 372. 1830. Fisher 3:1445 (1010). 1928. *Epidendrum tassellatum* Roxb. Pl. Cor. T. 42. 1795. *Vanda roxburghii* R. Br. In Bot. Reg. 6: t. 506. 1820;FBI6:52. 1890.

A stout epiphyte with non-pseudobulb stem, 30–60 cm long. Leaves linear-oblong, 15–20 × 1.6–2.5 cm, thick, coriaceous, distichous, strap shaped. Racemes four to ten flowered 15–20 cm long; peduncle stout. Flowers large, 5 cm across, greyish blue; spur conical. Capsules 6–9 cm long, ribbed with short pedicles (Fig. 10.3d).

V. testacea (Lindl.) Reichb. f. Gard. (n.s.) 8:166. 1877. *Aerides testacea* Lindl. Gen. Sp. Orchid. Pl. 238.1833 *(testaceum.) Vanda parviflora* Lindl. In Edwards Bot. Reg. 30 :Misc. 57. 1884; FBI 6:50. 1890; Fischer. (1928).

An epiphyte with nonpseudobulbous stem, 10–20 cm long; roots large, thick and vermiform. Leaves linear lorate, distichous, 10–25 × 1–2 cm coriaceous. Raceme erect, many flowered 5–15 cm long; peduncles stout. Flowers cream yellow with a white, purple or reddish pink lip; spur narrow; capsule 2–3 cm long, pedicellate clavate (Fig. 10.3e).

***Vanilla wightiana* Lindl.** Gen. Sp. Orchid. 436. 1840; FBI 6:90. 1890; Fischer 3:1451 (1015). 1928.

Climbing orchid with a root at each node, abortive leaves or no leaf; stems fleshy, green. Scape arising at the nodes, 2–3 cm long, three to five flowered. Flowers cream coloured with a pale green tinge, 3 cm across; sepals oblong obovate, obtuse or subacute at apex; petals oblong, obtuse at apex; lip trumpet shaped; capsule cylindrical, 15 × 1.2 cm, smooth (Fig. 10.3f).

Among the 30 genera reported, *Habenaria* is the dominant genus with 7 species (23.33%) followed by *Oberoni*a with 5 species (16.66%); 5 genera, *Acampe*, *Aerides*, *Dendrobium*, *Eulophia* and *Nervilia*, are with 3 species (10%); 4 genera, *Bulbophyllum*, *Liparis*, *Peristylus* and *Vanda*, are with 2 species (6.66%); and 19 genera are with single species (3.33%).

Habit-wise analysis reveals that among the 54 species, the highest numbers of orchids were identified as epiphytes with 26 species (48.15%) followed by terrestrial orchids with 24 species (44.44%), both epiphytic and lithophytic conditions observed in 3 species (5.55%) and only one species (1.8%) identified as saprophyte (Fig. 10.4).

On the basis of distribution, among these 54 orchids, the highest numbers of orchids were identified as rare with 32 species (59.25%), followed by occasional with 16 species (29.63%) and common with 6 species (11.11%) in the study area (Fig. 10.5). Maximum rare orchids are found in specific forest pockets like undisturbed areas, higher altitudes and microclimatic zones in the study area. Occurrence

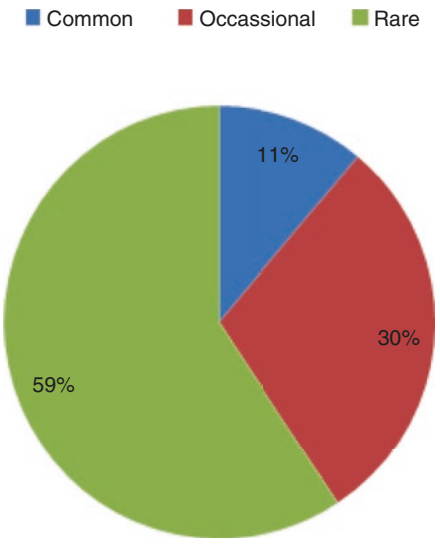

Fig. 10.5 Distributional-wise details of orchids in the study area

of common and occasional orchids from lower elevation to higher elevation indicates that microclimatic zones and undisturbed forest pockets have rich source of orchid diversity. Due to the coffee and pepper cultivation, old trees were projected in the study area, which are good source for orchids.

Terrestrial orchids like *Eulophia spectabilis* and *Nervilia concolor* are endangered species, and *Habenaria longicorniculata* endemic orchid is vulnerable in their conservation status (Raju et al. 2008; Reddy and Reddy 2008). These three species are only distributed in East Godavari and Visakhapatnam districts.

Among the 54 orchids, saprophytic orchid like *Didymoplexis pallens*, epiphytic orchids like *Aerides multiflorum* and *Dendrobium herbaceum* and terrestrial orchids like *Eulophia epidendraea*, *Eulophia explanata*, *Habenaria longicorniculata*, *Habenaria plantaginea* and *Habenaria roxburghii* were endemic species in the different regions of India, and they are rarely or occasionally distributed in the study area.

10.3 Conclusion

The orchids with botanical and economical value, which are habitat-specific growing plants in the moist deciduous and semievergreen forests of Northern Andhra Pradesh, need in situ as well as ex situ conservation for their survival and ecological balance in nature. The major threats are habitat (forest) destruction and illegal harvesting, resulting in diversity loss. For the conservation of orchids, there is a need to protect the microclimatic zones and undisturbed forests. Coffee and pepper cultivation is also a source to conserve old natural trees which are most suitable for conservation of epiphytic orchid diversity in their natural habitats. Some of the orchids are

thought to be vulnerable to the effects of global environmental changes (Fay and Chase 2009; Swarts and Dixon 2009a; Gale et al. 2018). So, the Government of Andhra Pradesh has to take in situ conservation of these forests along with the orchids. The Government of Andhra Pradesh and Biodiversity Board have to take steps to develop botanical gardens in the hilly region of this area for the ex situ conservation of orchids. The present study reveals that East Godavari and Visakhapatnam districts are the more suitable areas for conservation of orchids in their natural habitats. It is also suggested to establish an orchidarium and botanical garden in these two districts for ex situ conservation. Swarts and Dixon (2009b) have given the role of botanical gardens in supporting orchid conservation scientifically and horticulturally. There is a need to micropropagation of rare, endangered and endemic orchids for the ex situ conservation of orchids.

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11 History and Scientific Potential of the Orchid Collection of the Stock Greenhouse of the Main Botanical Garden RAS (Moscow, Russia)

Galina L. Kolomeitseva

Abstract

The history of the creation and development of the orchid collection of the Stock greenhouse of the Tsitsin Main Botanical Garden of the Russian Academy of Sciences is shown. The first orchids were obtained from the botanical gardens and flower firms of Germany in 1948; the number of natural species was 140. A complete list of preserved German orchid species, including 65 species from the 29 genera, is given. Further stages forming of the collection of orchids are described. Now the collection of tropical orchids of the Stock greenhouse includes 1328 species, subspecies, and varieties of orchids, belonging to 250 genera, and 69 species that are included in various categories of IUCN. The main areas of research with living collection plants are listed. These include the inventory and maintenance of an electronic database, the study of orchid adaptation to cultivation in greenhouses (morphology, phenology, symbiotrophism), reproductive biology issues (embryology, artificial pollination, seed morphometry), cryopreservation, and in vitro cultivation.

Keywords

Tropical and subtropical orchids · Botanical garden · Greenhouse · Living collection · Storage · Seed germination · In vitro cultivation · Cryopreservation of seeds

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11.1 Introduction

The Tsitsin Main Botanical Garden of the Russian Academy of Sciences is located in the northeast of Moscow and occupies an area of 328 ha. It was founded in 1945 as a scientific institute of the USSR Academy of Sciences in honor of its 220th anniversary. Less than 1 month before the end of the Second World War, the presidium of the USSR Academy of Sciences adopted the program for the construction of the Main Botanical Garden, which provided for the construction of a large Stock greenhouse for maintaining scientific collections of tropical and subtropical plants (11.1).

The technical design of the Stock greenhouse was developed by the project management Akademstroyproekt. Still remaining metal structures were made by the German company Karl Veigelt and sent from Dresden to Moscow in June 1948 (Kuzmin et al., 2009). In the same year, the construction of the Stock greenhouse began on the territory of the newly formed Main Botanical Garden. There were built foundations and drainage systems and a building for scientific research and personnel, and a metal frame was installed. Further construction of the Stock greenhouse (glazing, installation of heating, lighting, ventilation, water supply, etc.) took place in two stages. The central building and the western half of the greenhouse (three exposition and five collection greenhouses) were built in 1948–1950. The construction of the eastern half (two exposition, five collection, and two water greenhouses) was completed in 1953. The Stock greenhouse was a large greenhouse with a total area of 5725 square meters with a maximum height of 14.5 m and consisted of 19 greenhouses with different temperature regimes. It is still functioning successfully without significant changes.

Before construction, the Tsitsin Main Botanical Garden already had a large collection of orchids brought from Germany in 1946–1948 (Kolomeitseva 2014). These were plants from botanical institutions and private flower-growing firms destroyed during the war, which the botanical group of the USSR Academy of Sciences gathered on the Territoria throughout the Soviet occupation zone and temporarily preserved in the Sanssouci palace and park complex near Potsdam in the greenhouses not destroyed by the bombardments. The restoration of war-torn Soviet botanical gardens and greenhouse complexes was the main task of the botanical group. In addition, the botanical group provided assistance to the affected German botanical institutions and greenhouse farms that could not survive the winter of 1945–1946, without the Soviet side help. For example, the botanical group provided fuel to the botanical gardens of universities in the cities of Halle and Jena, the State gardening school in Pillnitz near Dresden.

Orchids was brought to the base in Sanssouci not only from Germany's state-owned botanical gardens but also from private gardening enterprises such as Nicolai's company (Coswig), E. Richter's company (Crimmitschau), Kruyff's company (Babelsberg), and Frankenstein's company (Holzhausen) (Table 11.1). This interesting historical fact was restored according to archival documents. The greenhouses of the largest Dutch company "Orchideen Flora" (owner Mr. Kruyff) in the suburbs of Potsdam (Babelsberg) during the time of the Potsdam Conference in the summer of 1945 were in the forbidden zone of negotiations between the heads of

Table 11.1 Mobilization of tropical plants by the botanical group of the Academy of Sciences of the USSR in Germany from greenhouses of trading companies (1947)

Name of the greenhouse complex	Assortment of orchids	Total number of orchids
Horticultural firm "Orchideen Flora," Babelsberg	Hybrids of *Cattleya*, *Paphiopedilum*, *Cymbidium*, *Miltonia*	1093
Horticultural firm "Nikolai," Coswig	Hybrids of *Cymbidium*	269
Horticultural firm "Richter," Crimmitschau	*Dendrobium*, *Phalaenopsis*, large assortment of other tropical orchids	679
Horticultural firm "Frankenstein," Holzhausen	Hybrids of Cattleyas, *Rhyncholaelia digbyana*, *Cattleya warscewiczii*	19
Greenhouse complex Sanssouci, Potsdam	A wide range of species and hybrids of tropical orchids	5703

the victorious countries. The entrance to the restricted area was completely blocked; 80–85 thousand samples of orchids were dying in the company's greenhouses without watering. The botanical group of the USSR Academy of Sciences received permission from the highest Soviet command, found the necessary transport, and transported the plants to the base at Sanssouci. In gratitude for saving the plants, the owner of the company, Mr. Kruyff, gave samples of all his orchids, including his famous hybrids *Cattleya* Lindl., *Cymbidium* Sw., and *Paphiopedilum* Pfitz. in the total amount of 1093 pieces (Kuzmin et al. 2009).

The historical lists of German plants that were sent from Sanssouci to Moscow in August 1948 included 528 species and hybrids of orchids. Among them were 164 species and hybrids of *Cattleya*, 1 species and 5 hybrids of *Cymbidium*, 24 species and 2 hybrids of *Dendrobium* Sw., 5 species and 142 hybrids of *Paphiopedilum*, and 4 species and 4 hybrids of *Phalaenopsis* Blume (Kolomeitseva 2014).

In 1948, the Main Botanical Garden of the USSR Academy of Sciences did not have its own greenhouses yet. Therefore, orchids brought from Germany were placed in the greenhouses of the floricultural farm Marfino and then in the greenhouses of the Exhibition of Achievements of National Economy (VDNKh) (Kuzmin et al. 2009; Demidov and Kolomeitseva 2007; Golovkin et al. 2010; Kolomeitseva and Lukyanova 2011; Romanov et al. 2015). In 1950, orchids were moved to the territory of the Main Botanical Garden and kept in a small greenhouse specially built for German plants. And only in 1953, orchids took a permanent place in the three collection greenhouses of the eastern wing of the Stock greenhouse with three temperature regimes (Fig. 11.1).

In addition to a large number of hybrids, there were 140 natural orchid species from 36 genera brought from Germany to Moscow in 1948. 70 years have passed since then. Old specimens of German orchids, preserved to this day, belong to 65 species and varieties from 29 genera (Table 11.2). This is about 5% of the total number of species contained today in the Stock greenhouse of the Main Botanical Garden RAS.

By the mid-1980s, the number of orchid greenhouses increased to four (a greenhouse with a warm temperature regime of 192 m^2 was added) and in 2015 to five (a greenhouse with a cold temperature regime of 193 m^2 was added). At present, the

Fig. 11.1 Stock greenhouse of the Tsitsin Main Botanical Garden of the Russian Academy of Sciences in Moscow

orchid collection occupies 5 greenhouses with a total area of 769 m^2 and contains 8920 samples of orchids (Table 11.3). Today it is one of the largest collections of tropical and subtropical orchids in Russia.

An increase in the collection of orchids during the Soviet Union was carried out by botanical expeditions to different regions of the world: India, Cuba, Vietnam, Africa, Central and South America, etc. A large number of species were collected after the expeditions of 1961 to India (Lapin and Korovin 1961) and 1981–1982 to the countries of the Atlantic and Indian Oceans on the research vessel "Akademik Vernadsky." For example, in 1982, as a result of an expedition to Cuba, the collection of orchids received 49 new species of orchids collected in natural populations and in botanical gardens.

Many interesting species and hybrids of orchids were received in 1961–1987 as a result of the exchange between the botanical gardens of the countries of the socialist community – the GDR, Czechoslovakia, and Poland. In the 1980s the Main Botanical Garden made large purchases of natural species of tropical orchids propagated in floriculture farms in Western Europe (France, 1980, 1986; Federal Republic of Germany, 1984; England, 1987). As a result, the collection was expanded with very interesting species, mainly from the tropics of Asia and America, as well as with rare species of the *Paphiopedilum* and *Phragmipedium*.

Table 11.2 Species composition and condition of orchids from the German collection 70 years later, data 2018 (species names are given based on modern nomenclature)

No	Species	Number of samples	Total number of shoots	Flowering frequency
1	*Angraecum distichum* Lindl.	1	7	Every year (Sep–Nov–Jan)
2	*Brassia verrucosa* Bateman ex Lindl.	17	8–18	Every year (May–July)
3	*Bulbophyllum ambrosia* (Hance) Schltr.	3	More 100	Every year (Oct–Mar)
4	*B. ornatissimum* (Rchb.f.) J.J.Sm.	10	More 30	Every year (autumn, winter)
5	*B. longiflorum* Thouars	2	More 35	Every year (Mar–May)
6	*Cattleya mossiae* C. Parker ex Hook.	3	4–7	Every year (May–July)
7	*C. percivaliana* (Rchb.f.) O'Brien	3	4–6	Every year (Dec–Feb)
8	*C. purpurata* (Lindl. & Paxt.) Rollisson ex Lindl.	1	3–4	Not every year (May, June)
9	*C. trianae* Linden & Rchb.f.	1	5	Every year (Jan–Apr)
10	*C. trianae* var. *alba* L.Linden & Rodigas	1	4	Every year (Jan–Apr)
11	*Coelogyne cristata* Lindl.	36	More 100	Every year (Dec–Mar)
12	*C. fimbriata* Lindl.	9	More 50	Every year (May–Feb)
13	*C. flaccida* Lindl.	10	18–38	Every year (Aug–Sept, Dec–May)
14	*C. tomentosa* Lindl.	12	10–20	Every year (April, May, July–Sept)
15	*C. speciosa* (Blume) Lindl.	10	5–18	Every year (autumn, winter)
16	*Cryptochilus roseus* (Lindl.) S.C. Chen & J.J. Wood	4	10–28	Every year (Jan–Feb)
17	*Cuitlauzina pendula* Lex.	13	5–14	Not every year (spring)
18	*C. pulchella* (Batem.ex Lindl.) Dressler & N.H. Williams	12	6–20	Every year (winter)
19	*Cymbidium lowianum* (Rchb.f.) Rchb.f.	12	3–7	Every year (Jan–May)
20	*Cyrtochilum divaricatum* (Lindl.) Dalström	1	8	Every year (May–July)
21	*C. flexuosum* Kunth	11	10–100	Every year (spring, summer)

(continued)

Table 11.2 (continued)

No	Species	Number of samples	Total number of shoots	Flowering frequency
22	*Dendrobium aphyllum* R.Br.	5	22	Every year (Feb–May)
23	*D. bigibbum* Lindl.	2	3–6	Every year (winter)
24	*D. chrysotoxum* Lindl.	10	20–35	Every year (Feb–July)
25	*D. aphyllum* (Roxb.) C.E.C.Fisch.	7	8–15	Every year (Nov–July)
26	*D. delicatum* (F.M. Bailey) F.M. Bailey	18	From 15 to 100	Every year (Feb–Mar)
27	*D. densiflorum* Lindl.	9	6–13	Every year (Mar–Aug)
28	*D. fimbriatum* Hook.	5	4–10	Every year (Feb–April, Sep–Dec)
29	*D. kingianum* Bidwill ex Lindl.	28	8–30	Every year (Jan–April)
30	*D. lindleyi* Steud.	2	More 50	Every year (Feb–April, July)
31	*D. loddigesii* Rolfe	1	More 50	Every year (Jan–June, Aug)
32	*D. moschatum* (Banks) Sw.	12	5–20	Every year (April–Nov)
33	*D. moschatum* (Willd.) Sw. var. *calceolaria* Veitch	12	5–20	Every year (April–Nov)
34	*D. nobile* Lindl.	8	4–30	Every year (Dec–July)
35	*D. speciosum* Sm.	14	6–22	Every year (Jan–Mar)
36	*Dendrochilum cobbianum* Rchb.f.	11	15–48	Every year (Feb–Mar)
37	*D. glumaceum* Lindl.	8	16–40	Every year (Feb–Mar)
38	*D. latifolium* Lindl.	10	More 50	Every year (Apr–May)
39	*Epidendrum ciliare* L.	5	3–10	Every year (Oct–Feb, May)
40	*Gongora galeata* (Lindl. ex Bosse) Rchb.f.	10	More 50	Every year (May–July)
41	*Guarianthe bowringiana* (O'Brien) Dressler & W.E. Higgins	1	4	Every year (Oct, Nov)
42	*G. skinneri* (Bateman) Dressler & W.E. Higgins	1	4	Every year (Mar–June)
43	*Laelia anceps* Lindl.	10	4–9	Every year (Dec–Feb)

(continued)

Table 11.2 (continued)

No	Species	Number of samples	Total number of shoots	Flowering frequency
44	*L. autumnalis* (Lex.) Lindl.	1	3	Not every year (Dec, Jan)
45	*Lycaste aromatica* (Graham) Lindl.	2	4–8	Every year (Mar–July)
46	*Maxillaria densa* Lindl.	10	More 100	Every year (Jan, Feb)
47	*M. porphyrostele* Rchb.f.	12	More 60	Every year (Dec–Feb)
48	*M. tenuifolia* Lindl.	2	More 100	Every year (Mar–July)
49	*Oncidium auriculatum* (Rolfe) M.W. Chase & N.H. Williams	1	5	Every year (May–July)
50	*O. ornithorhynchum* Kunth	1	7–14	Every year (Oct–Feb)
51	*O. sphacelatum* Lindl.	10	More 100	Every year (Mar–June)
52	*Paphiopedilum insigne* (Wall. ex Lindl.) Pfitz.	27	67	Every year (Dec–Feb)
53	*P. insigne* var. *sanderae* (Rchb.f.) Pfitz.	7	28	Every year (Nov–Jan)
54	*Papilionanthe teres* (Roxb.) Schltr.	1	1	Does not bloom
55	*Phalaenopsis schilleriana* Rchb.f. (Fig. 11.2)	2	2	Every year (Jan–Mar)
56	*Pholidota imbricata* Hook.	3	2, 10, 14	Every year (April, May, Sept–Jan)
57	*Prosthechea cochleata* (L.) W.E. Higgins	1	4	Every year (Jan–Mar, June–Aug)
58	*Rhyncholaelia glauca* (Lindl.) Schltr.	2	5, 12	Every year (Jan–Mar)
59	*Rossioglossum grande* (Lindl.) Garay & G.C. Kenn.	4	3–14	Every year (Aug–Nov)
60	*Sobralia macrantha* Lindl.	6	More 50	Every year (May–Aug)
61	*Stanhopea oculata* (Lodd.) Lindl.	1	3	Not every year (July–Oct)
62	*S. tigrina* Bateman ex Lindl.	5	4–10	Every year (June–Sep)
63	*Trichocentrum splendidum* (A.Rich. ex Duch.) M.W. Chase & N.H. Williams	5	4–10	Every year (Jan–Mar)
64	*Vanda tricolor* Lindl.	1	2	Not every year (spring, summer)
65	*V. tricolor* var. *suavis* (Lindl.) Rchb.f.	12	1–5	Every year (spring, summer, autumn)

Fig. 11.2 *Phalaenopsis schilleriana* from the German collection. This sample is over 70 years old. It blooms regularly, but does not form fertile seeds after artificial pollination

At the beginning of the 1990s, due to the restructuring and change of the socio-political system, which caused a collapse of the economy and a significant deterioration in the financing of science, botanical expeditions were almost ceased; the flow of seed and planting material for replenishing the collection through exchanges and purchases decreased. In spite of this in 1994, a small batch of orchids was purchased in the Netherlands (the company "Floralia").

The period 2010–2012 was the only time when there was a negative trend in the increase of orchid collection in the Stock greenhouse. Lowering the temperature in greenhouses in the winter of 2009–2010 with repeated shutdowns of heating in the cold period led to a significant loss of orchids, which lasted throughout the subsequent growing season, from spring 2010 to winter 2011. During this time, the collection lost 77 species of orchids, including 2 species of African *Aeranthes* Lindl., 4 species of *Catasetum* Rich. ex Kunth, 17 species and subspecies of *Cattleya* Lindl. from Central and South America, 5 species of *Encyclia* Hook., brought in 1982–1986 from about Cuba, and 14 species of *Oncidium* Sw., purchased mainly in Germany (1984) and in the United Kingdom (1987). The massive loss of

Table 11.3 Temperature conditions of five orchid greenhouses and the distribution of some genera (data 2018)

№ greenhouse, area (m^2)	Temperature conditions	Number of plants	The main genera and the number of their species in the collection
№ 6	Cool	1876	*Bulbophyllum* Thou.(81 species)
96	14–18 ° C		*Dendrobium* (37 species)
	night average		*Liparis* Rich. (31 species)
			Lycaste Lindl. (6 species)
			Paphiopedilum (33 species)
			Phragmipedium Rolfe (5 species)
			Pleione D.Don (7 species)
			Sobralia Ruiz & Pav. (5 species) etc.
№ 8	Cold	1035	*Maxillaria* Ruiz & Pav. (24 species)
96	10–14 ° C		*Cymbidium* (26 species)
	night average		*Dracula* Luer (10 species)
			Epidendrum L. (6 species)
			Masdevallia Ruiz & Pav. (20 species)
			Scaphosepalum Pfitz. (6 species) etc.
№ 10	Warm	2064	*Angraecum* Bory (5 species + 4 primary hybrids)
192	18–24 ° C		*Calanthe* Ker-Gawl. (23 species)
	night average		*Pescatoria* Rchb.f. (10 species)
			Phalaenopsis Blume (16 species)
			Renanthera Lour. (4 species)
			Stanhopea Frost. ex Hook.(7 species)
			Vanda Jones ex R.Br. (6 species) etc.
№ 12	Warm	2895	*Brassia* R.Br. (6 species)
192	18–24 ° C		*Cattleya* (25 species)
	night average		*Cleisostoma* Blume (16 species)
			Coelogyne Lindl. (37 species)
			Dendrobium (80 species)
			Epidendrum (10 species)
			Guarianthe Dressler & W.E.Higgins
			(4 species)
			Pholidota Lindl. ex Hook. (17 species) etc.
№ 16	Cold	1050	*Cymbidium* (40 hybrids) etc.
193	10–14 ° C		
	night average		
Total		8920	

heat-loving African and South American orchids has determined a further downward trend in the proportion of species from tropical America and an increase in the proportion of species from tropical Asia.

In 1990–2017 the collection of orchid was expanded due to regular expeditions of the Joint Russian-Vietnamese Science and Technological Tropical Centre and individual expeditions of the Garden's employees to the tropics of different continents.

Many interesting orchid species have been derived from South Africa, Namibia, Argentina, China, Thailand, Laos, and Vietnam. Especially interesting are species from Kalimantan, Sulawesi, New Caledonia, etc. Many of these plants have not bloomed yet and therefore so far identified only to the genus. However, it is already clear that the newly obtained taxa will fill up the group of island tropical species well.

Botanical gardens and universities of Russia (Botanical Garden of Lomonosov Moscow State University, Botanical Garden of Lobachevsky Nizhny Novgorod State University, Botanical Garden of Komarov Botanical Institute RAS, and others) also assisted in replenishing the collection of orchids. During the last 5 years, many rare orchid species were obtained from the collection of the Botanical Garden of the University of Nizhny Novgorod, including seedlings of the African orchid *Disa uniflora* P.J. Bergius, as well as several new paphs, oncidiums, and dendrobiums.

Thanks to amateur orchid growers, the collection is replenished with new species of orchids regularly. For example, in 2015–2017, amateur A. Romanko gave the Stock greenhouse a collection of cold-loving and moisture-loving orchids from Oncidium/Odontoglossum alliance, including species from the genera *Cyrtochilum* Kunth, *Oncidium* Sw., *Rhynchostele* Rchb.f., and *Caucaea* Schltr.

In 2014–2018 several dozens of American tropics orchid species were purchased through the gardening firms of Ecuador and Argentina, including species from the sub-tribe Pleurothallidinae (*Dracula* Luer, *Masdevallia* Ruiz and Pav., *Pleurothallis* R.Br., *Scaphyglottis* Poeppi and Endl.) and Zygopetalinae (*Chaubardia* Rchb.f., *Chaubardiella* Garay, *Cochleanthes* Raf., *Pescatoria* Rchb.f.).

Currently, the Stock greenhouse cultivates 1328 species, subspecies, and varieties orchids, belonging to 250 genera. The most fully represented are the genera *Bulbophyllum* (81 species), *Calanthe* (23 species and subspecies), *Coelogyne* (37 species), *Cymbidium* (26 species), *Dendrobium* (117 species), and *Paphiopedilum* (33 species and forms). Most of these genera originate from Asian species diversity center. The endemic orchid flora of Vietnam is most fully represented in collection. There are 92 genera and 404 species of orchids from the flora of Vietnam; of them 38 species are endemic. Another 12 species are subendemic, confined to several Indo-Chinese centers of endemism. The uniqueness of the Vietnamese collection of the Stock greenhouse is determined by the long duration of accumulation material (over 30 years of cooperation with the Tropical Centre), the good representativeness of the tribal complexes, the introduction of orchid flora from all floristic provinces of Vietnam, and the presence of several clones of individual species (Kolomeitseva et al. 2015).

Great attention is paid to the conservation of rare or almost extinct orchid species in nature. For example, it was thought that the endemic of Vietnam *Bulbophyllum hiepii* Aver. is completely extinct after the complete destruction of its habitats. However, two samples of this unique species are preserved in the Stock greenhouse of the Main Botanical Garden since 1980. These plants were found in South Vietnam in the province of Gia Lai-Kon Tum during an expedition of employees of the Institute of Ecology and Evolution named after A.N. Severtsov RAS and transferred to us. This happened long before the scientific description of the species, which was made in 1992 by the Russian botanist L.V. Averyanov using a different sample.

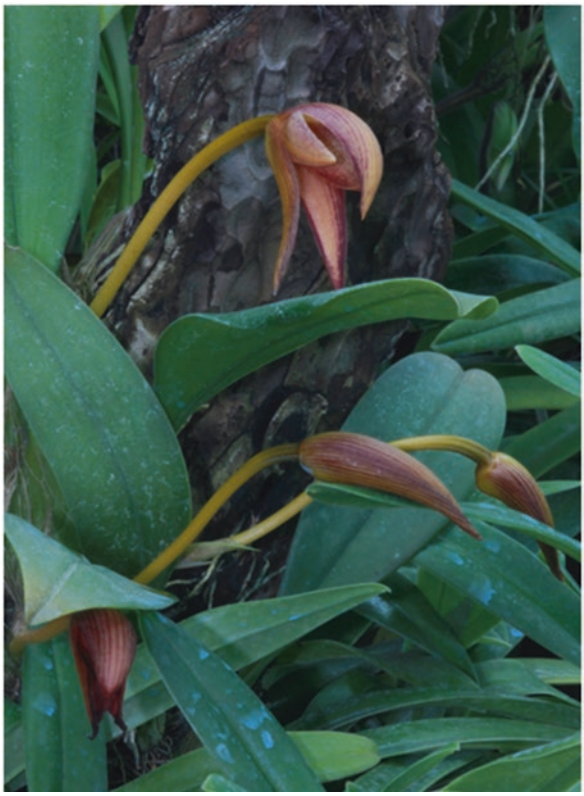

Fig. 11.3 *Bulbophyllum hiepii* – very rare or already extinct orchid in natural habitats. This specimen from the wet mountain forests of Vietnam (800–2600 m above sea level). Shoots with single-leaf pseudobulbs and thick rhizomes. One flower arises from the base of the pseudobulb, a pedicel 8–10 cm tall. Flowers brown-orange with purple longitudinal stripes, never fully open. Triangular sepals 5 cm long with pointed ends, petals are little smaller. Lip is yellow in the middle and with small purple dots on the edges. Blooms in January–February. Grows in a block culture in a greenhouse with a cold temperature regime

Prior to this description, our plants for 12 years were kept as samples, defined only up to the level of the section *Sestochilos* (Breda) Benth. et Hook.f. Several years ago, one of the samples was vegetatively divided into two species. Each of the two copies of *B. hiepii* has more than 20 shoot sand blooms every year, but it has not been possible to receive germinating seeds (Fig. 11.3).

There are 69 species in the collection of orchids in the Stock greenhouse that are included in various categories of IUCN. Nine species of the genus *Paphiopedilum* (*P. delenatii* Guill., *P. exul* (Ridley) Rolfe, *P. fowliei* Birk, *P. gratrixianum* (Masters) Guill., *P. helenae* Aver., *P. primulinum* M. Wood et P. Taylor, *P. sukhakulii* Schoser et Senghas, *P. tranlienianum* O.Gruss & H.Perner, *P. urbanianum* Fowlie) have the status of the critical threat of complete extinction in the wild (critically endangered or CR). The full ontogenesis cycle of species of the genus *Paphiopedilum* is 6–12 years, natural seed renewal is severely limited, and populations are constantly depleted due to unreasonable commercial exploitation and human activities. Of the 69 rare species available, 44 species regularly blossom in the Stock greenhouse. All adult orchids included in the IUCN Red List after artificial pollination are able to form seeds suitable for embryological research, experimentation, long-term storage at low temperatures, and seed germination in vitro.

Seeds of many species of tropical orchids can easily be obtained in greenhouse conditions using artificial pollination. In vitro culture allows you to multiply these orchids in large quantities. For reproduction of species that are not capable to tie full-fledged seeds, we use the methods of vegetative propagation. Thus, the orchid collection of the Stock greenhouse has the function of a donor, which donates seedlings and adult plants to botanical gardens in Russia and other countries for free of cost.

The live collection of orchids of the Main Botanical Garden serves for a preservation of rare taxa and is also actively used in scientific and educational work based on a study of various aspects of orchid biology (embryology, morphology, ecology, reproductive biology, symbiotrophism, etc.). Scientific workers of the laboratory of tropical plants regularly published monographs, dissertations, and numerous scientific articles. Students of universities use collection of orchids for writing qualifying and scientific works on the biology of orchids. Today, researches in the orchid collection are conducted in the following directions.

11.2 Problems of the Nomenclature of the Orchidaceae in Systematic Living Collections

During the nineteenth and twentieth centuries, decrease and integration of low-rank taxa in the orchid family occurred repeatedly. The unification of genera into genera complexes is typical for many large genera of Asian and American tropical orchids. Impressive revisions of the intrageneric boundaries have undergone major orchid genera, such as *Dendrobium*, *Encyclia* Hook., *Epidendrum*, *Eria* Lindl., *Maxillaria*, *Odontoglossum* Kunth, *Oncidium* Sw., etc. Taking into account all modern taxonomic changes cause problems in the definition, labeling, and movement of living plants within systematic collections. Therefore, today information has been collected on the synonymy of collection species for the creation of a special database in which the search can be conducted not only by priority names but also by synonyms. At present, a computer base of plants of the Stock greenhouse is being created; most of the orchids from the collection have inventory labels with the inventory number, the adopted name of the plant, and the species range, source, and year of collection shown. These data are loaded into a special computer program and easily read from labels using a scanner.

11.3 Adaptation of Orchids to Cultivation in Greenhouse

When orchids are transferred from their natural habitats to greenhouse cultivation conditions, they withstand a number of stressful influences that can change their morpho-structure, phenology, or sometimes a type of metabolism. The morphological diversity and specialization of the shoots and roots of collected orchids have been the object of our study for many years. Using the example of greenhouse plants in the orchid family, an architectural model with alternating deterministic and non-deterministic shoots (Kolomeitseva 2005, 2007), as well as anatomical

differences of aerial and substrate roots (Zalukaeva 1995), was described for the first time.

11.4 Reproductive Biology

Orchid embryogenesis in the Stock greenhouse was first studied in the mid-20-th century. Embryologist V.A. Poddubnaya-Arnoldi was the first in the Soviet Union to begin work on orchid embryology and cultivation seedling in vitro. Three species of orchids (*Calanthe* Veitchii, *Dendrobium nobile*, *Paphiopedilum insigne*) were used as experimental objects. The features of the development of the embryo sac and embryo were revealed and published as a drawings. The seedlings of these orchids in vitro were obtained in 1957–59. The research results were reflected in numerous publications (Poddubnaya-Arnoldi 1964, 1976); they are still in demand and are actively cited in the world scientific literature (Arditti 1992; Batygina et al. 2003). Currently, embryological research targets are orchids from the tribes Malaxideae and Sobralieae (Kolomeitseva et al. 2017). The study of various stages of pollination and the development of germinal sacks and embryos is carried out using confocal microscopy techniques.

Various aspects of the adaptation of orchid seeds to the functions of dissemination, the reclamation of the new habitats, the survival in adverse periods, and the use of biotic partners for pollination, settlement, and germination are being studied. Microstructure data of the seed coat of more than 150 species of tropical orchids and orchids in European Russia (Kolomeitseva et al. 2012) were obtained. The most interesting results of this work were correlations found between the seed structure of some seed types (Limodorum-type, Calanthe-type, Bletia-type) (according to the classification of seed types of Dressler 1993) and the ecological belonging of the species. For example, a comparative morphological analysis of seeds of 20 species from the genus *Paphiopedilum* (seeds of the Limodorum-type) showed that large spindle-shaped seeds with large air cavities inside the seed coat are typical for terrestrial species from the sections *Parvisepalum* (Karasawa et Saito) Cribb (subgenus *Brachipetalum*) and *Barbata* (subgenus *Paphiopedilum*). At the same time were found epiphytes from sections of *Cochlopetalum* Hallier ex Pfitz. and *Pardalopetalum* Hallier ex Pfitz. (subgenus *Paphiopedilum*) have an almost granular form seeds with a small amount of free space inside (Kolomeitseva et al. 2012).

11.5 Cryobank of Orchid Seeds

The creation of a cryobank of orchid seeds stored at liquid nitrogen temperature (−196° C) began in 2000 on the basis of the Institute of Plant Physiology named after K.A. Timiryazev RAS. Orchid seeds from our collection (*Angraecum magdalenae* Schltr. et Feddes, *Trichopilia tortilis* Lindl., *Prosthechea cochleata* (L.) W.E.Higgins, *Calanthe vestita* var. *rubro-oculata* Paxt., *Calanthe* Gorey, *Bratonia*

hybr.) were first stored in liquid nitrogen (Nikishina et al. 2001; Popova et al. 2003). These samples have been stored there for 18 years. Cryobank is regularly replenished with new samples, and today it contains seeds more of 100 species from the Stock greenhouse collection. In all samples, moisture was determined before being put into liquid nitrogen. It turned out that the seeds of some species of tropical orchids can tolerate prolonged freezing at 24% humidity (Nikishina et al. 2001). For each batch of seeds incorporated in the cryobank, special passports are created. In passports there are data on the systematic position, range, and status of the rarity of the species; the duration of fruit ripening; the nature of pollination in greenhouse conditions; the time of put in a cryobank; the duration of pre-storage; moisture and the germination before freezing; type of seeds (according to the method Barthlott and Ziegler 1981; Dressler 1993); and seed morphometry. Each passport has a photography taken with an electron microscope.

11.6 Symbiotrophism

The study of some aspects of orchid symbiotrophism in greenhouse conditions has been conducted since 1990 (Zalukaeva 1990; Tsavkelova et al. 2001, 2003a, b, c, 2016). The species composition of bacteria and fungi entering into an associative relationship with the aerial and substrate roots of greenhouse orchids was determined, and the stimulating effect of microbiological associates on the germination of orchid seeds in vitro culture was determined. It is shown that the symbiotic interaction of orchids and some bacteria in vitro culture is stable and can be maintained during the entire cultivation period, until the seedling is transferred from culture to greenhouse conditions on ordinary substrates. This study confirms the ability of some bacteria to stimulate the germination of orchid seeds in vitro culture to the protocorm phase and form normally developed seedlings.

A large and representative collection of live orchids of the Stock greenhouse of the Main Botanical Garden is in demand not only by research assistants but also taxonomists, morphologists, cytologists, and microbiologists. Every year, many people from Russia and other countries visit the orchid collection. Volunteers help to take care of plants. Artists paint pictures or are inspired by the flowers of orchids to create new patterns on fabrics. Blooming orchids are regularly exhibited at trade shows. Scientific study and popularization of knowledge about these unusual and beautiful plants among the population are necessary components of their successful preservation ex situ.

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Status of Orchid Diversity, Conservation, and Research and Development in Arunachal Pradesh: An Overview

12

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Abstract

Arunachal Pradesh is one of the biodiversity-rich states and is considered as one of the "Biodiversity Hot Spots" in India. One of the important biodiversity elements is that of orchids with about 550 species in 132 genera. Out of them, as many as 376 are epiphytes including some lithophytes, 157 species are terrestrials, and 17 are mycotrophs (saprophytes). While *Dendrobium* has the maximum number of species (56 species) followed by *Bulbophyllum* with 55, the genera *Satyrium* and *Thunia* are represented by single species each. Besides, out of 404 species found endemic in various phytogeographical regions of India, 31 species are found endemic in Arunachal Pradesh only. In this paper, an overview of diversity and habitat distribution of orchids in various phytoclimatic zones and forest types which have been characterized by the elevation, temperature, forest type with major tree species, and microclimate that influence the occurrence of various orchid species has been presented. Recognizing the impact of development in the state, efforts in conserving the orchids ex situ and in situ by establishing orchid sanctuaries and centers provide an exemplary approach toward conservation of orchids. Research and development on orchids carried out for the last 48 years in exploring the orchid flora; conservation of germplasm in various orchid centers of the state by rescuing orchids from felled trees and disturbed forest areas; development of herbarium; breeding and development of hybrids; aseptic culture and micropropagation of ornamental and rare, endangered, and threatened (RET) species of orchids; and transfer of cultivation and farming technology through training the entrepreneurs and women self-help groups have also been presented for the development of a vibrant orchid industry in the state.

Keywords

Orchid diversity · Status in India · Research and development · Arunachal Pradesh

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12.1 Introduction

Arunachal Pradesh, the then North-East Frontier Agency (NEFA), is strategically situated in the extreme northeast part of Indian Himalayan region with international border with Bhutan in the west, China in the north and northeast, and Myanmar in the east and nationally with the states of Assam in the south and Nagaland in the southeast part of India. This territory was less known until independence of India (1947) and was also referred to as "Hidden Land," "Elusive Frontiers," and "Land of the Dawn-lit Mountains," mainly because of inaccessibility due to rugged terrain of rising hills from 100 m to 7000 m MSL, from tropical humid valleys to subtropical, temperate, and snow-capped alpine hills, crisscrossed with several rivers and rivulets, with varying forest types rich in biological diversity – flora and fauna – and of course receiving the first morning sun rays on Indian soil (Hegde 2002a, b). In the northeast region, Arunachal Pradesh with an area of about 83,743 km^2. has the highest forest cover in India and the least populated with about 12.6 lakhs as per latest Arunachal Pradesh government portals. Although native people practice slash and burn type of agriculture traditionally, there has not been much effect on growth and extent of forest cover in the state mainly because of higher rain fall and humidity favoring regeneration quickly. The communities living in and around the forests largely depend upon forest resources for their shelter, food, and fodder. And, the life of communities revolves around the forests in the state. With the opening up of North-East Frontier Agency (NEFA) for various developmental and other welfare programs of the people of this unique territory and attainment of statehood renamed as Arunachal Pradesh, the inaccessible forests were opened up for development of townships, infrastructure, roads, agriculture, horticulture and plantations, besides other supplemental crops, and hydroelectrical projects impacting the biodiversity and livelihood issues.

The state is one of the "Biodiversity Hot Spots" in India, harboring more than 33% of the total Indian flora with unique taxa and large number of genetic resources making it a "cradle of speciation" (Hegde 2002a, b) and center of origin of a number of cultivated plants. Besides timber, bamboos, canes, and medicinal plants, orchids are found to be important elements of this biodiversity with about 550 species in 132 genera of orchids distributed in various phytoclimatic zones and forest types of this territory (Hegde 1984; Hegde et al. 2017).

12.2 Broad Phytoclimatic Zones and Forest Types

Based on the altitudinal variations from 100 m to 7000 MSL, resulting in varying temperature, sunlight, humidity, rainfall, and vegetation types, accompanied by varying microclimate from hot humid valleys to the cool climatic situations, the following four orchid zones could be recognized in which various orchid species find their habitat. Type of soil with humus, presence of mycorrhiza, density, and type of vegetation with various tree species (phorophytes) have significant influence in the occurrence and distribution of orchids (Hegde 1985) in Arunachal Pradesh.

I. **Tropical Evergreen Rain Forest Zone** (100–900 MSL)

Warm humid climate; high rainfall in summer months; cool in winter with moderate humidity; tall trees, evergreen forests with mixed deciduous trees in some pockets. While some orchids prefer branches of tall trees on top canopy, others are found on the lower main branches of tree trunks indicating their habitat requirements such as light and shade conditions. Dominant tree species are *Castanopsis tribuloides*, *Terminalia myriocarpa*, *Canarium resiniferum*, *Artocarpus chaplasha*, *Altingia excelsa*, etc.

Important orchid genera found: *Acampe*, *Acanthephippium*, *Aerides*, *Agrostophyllum*, *Apostasia*, *Arundina*, *Biermannia*, *Bulbophyllum*, *Calanthe*, *Cleisocentron* (Fig. 12.1), *Coelogyne*, *Cymbidium*, *Dendrobium*, *Diplomeris*, *Eria*, *Gastrochilus*, *Geodorum*, *Goodyera*, *Habenaria*, *Liparis*, *Luisia*, *Micropera*, *Nervilia*, *Oberonia*, *Papilionanthe*, *Paphiopedilum*, *Pecteilis*, *Phaius*, *Phalaenopsis*, *Pholidota*, *Rhynchostylis*, *Spathoglottis*, *Spiranthes*, *Thrixspermum*, *Vanda*, *Zeuxine*, etc. *Diplomeris hirsuta* and *Paphiopedilum venustum* are rarely found in the foothill area on forest floor. Dominant ground orchids are *Arundina graminifolia* and *Goodyera procera* found on disturbed areas of forest floor.

II. **Subtropical Forest Zone** (900–1800 MSL)

Characteristically lesser rains, humid, foggy cooler conditions; south or north of the hills, determine the habitat distribution of various orchid species in the following two types of forest vegetation. Dominant tree species are *Schima wallichii*, *Castanopsis indica*, *Quercus* species, etc. All these trees are heavily moss covered, making the habitat congenial for the growth and proliferation of orchids. Dominant orchid genera found are *Agrostophyllum*, *Anthogonium*, *Arachnis*, *Bulbophyllum*, *Calanthe*, *Ceratostylis*, *Cleisostoma*, *Coelogyne*, *Cryptochilus*, *Cymbidium*, *Dendrobium*, *Eria*, *Galeola*, *Gastrochilus*, *Liparis*, *Luisia*, *Monomeria*, *Nervilia*, *Oberonia*, *Otochilus*, *Phaius*, *Pholidota*, *Phreatia*, *Pleione*, *Satyrium*, *Thelasis*, *Thunia*, *Uncifera*, and *Vanda.*

Fig 12.1 *Cleisocentron trichromum*

(i). ***Mixed wet forest belt:*** Cool, wet, and humid climate. Some of the epiphytes like *Coelogyne*, *Cymbidium*, *Dendrobium*, and *Eria* are frequently seen and dominate the tree trunks, while ground flora is often seen with *Anthogonium gracile* in sunny spots and *Phaius flavus* and *P. mishmensis* in humus-rich shady forest floor. A number of saprophytes/myco-heterotrophs such as *Epipogium sessanum*, *Eulophia zollingeri*, *Galeola lindleyana*, etc. are seen on humus-rich forest floor. Epipogiums when in bloom strike the eyes with their white pigment less flowering spikes on the forest floor and are mostly ephemerals (Hegde 1981a, b, Hegde and Rao 1982a, b c, d).

(ii). ***Mixed or pinus (partially dry) forest belt****:* Cool climate with varying humidity – partially dry in winter and highly humid in summer. Dominant tree species are *Alnus*, *Pinus*, *Quercus*, and *Rhododendron*. Prominent orchid genera seen in this area are *Calanthe*, *Coelogyne*, *Cymbidium* (Fig. 12.2a), *Dendrobium* (Fig. 12.2b), *Gastrochilus*, *Phaius*, and *Vanda. Galeola lindleyana* and *G. falconeri* are the two giant saprophytic orchids seen in this belt. One of the rare and endangered species of *Paphiopedilum fairrieanum* (Fig. 12.2c) is also found in some pockets of this belt.

III. ***Temperate Forest Zone*** (1800–3500 MSL)

Cool foggy climate with frost in winter; receives moderate rainfall; experiences brief and light snowfall in peak winter on ridges of hill ranges. Dominant tree species are *Pinus*, *Quercus*, *Alnus*, and *Rhododendron*. Dominant orchid genera are *Bulbophyllum*, *Cephalanthera*, *Coelogyne*, *Cymbidium*, *Dendrobium*, *Galeola*, *Gastrochilus*, *Herminium*, *Panisea*, *Paphiopedilum*, and *Satyrium*.

IV. **Alpine Forest Zone** (3500–7000 MSL)

This zone mostly remains covered with snow for about 4–6 months in winter. Only terrestrial orchids belonging to the genera, viz., *Dienia*, *Malaxis*, *Ponerorchis*, *Satyrium*, *Platanthera*, and *Herminium* are found here. *Herminium longilobatum* Hegde et Rao is a new species described (Hegde and Rao 1982c).

Further, distribution of orchids also varies from western parts of Arunachal to that of eastern part very significantly. For example, *Paphiopedilum fairrieanum* found in western part of Arunachal is not found in eastern part of the state. Similarly, *Vanda coerulea* (Fig. 12.2d) found in eastern districts, Changlang and Tirap, are not found in western districts, viz., West Kameng and Tawang, as the phytoclimatic conditions significantly vary.

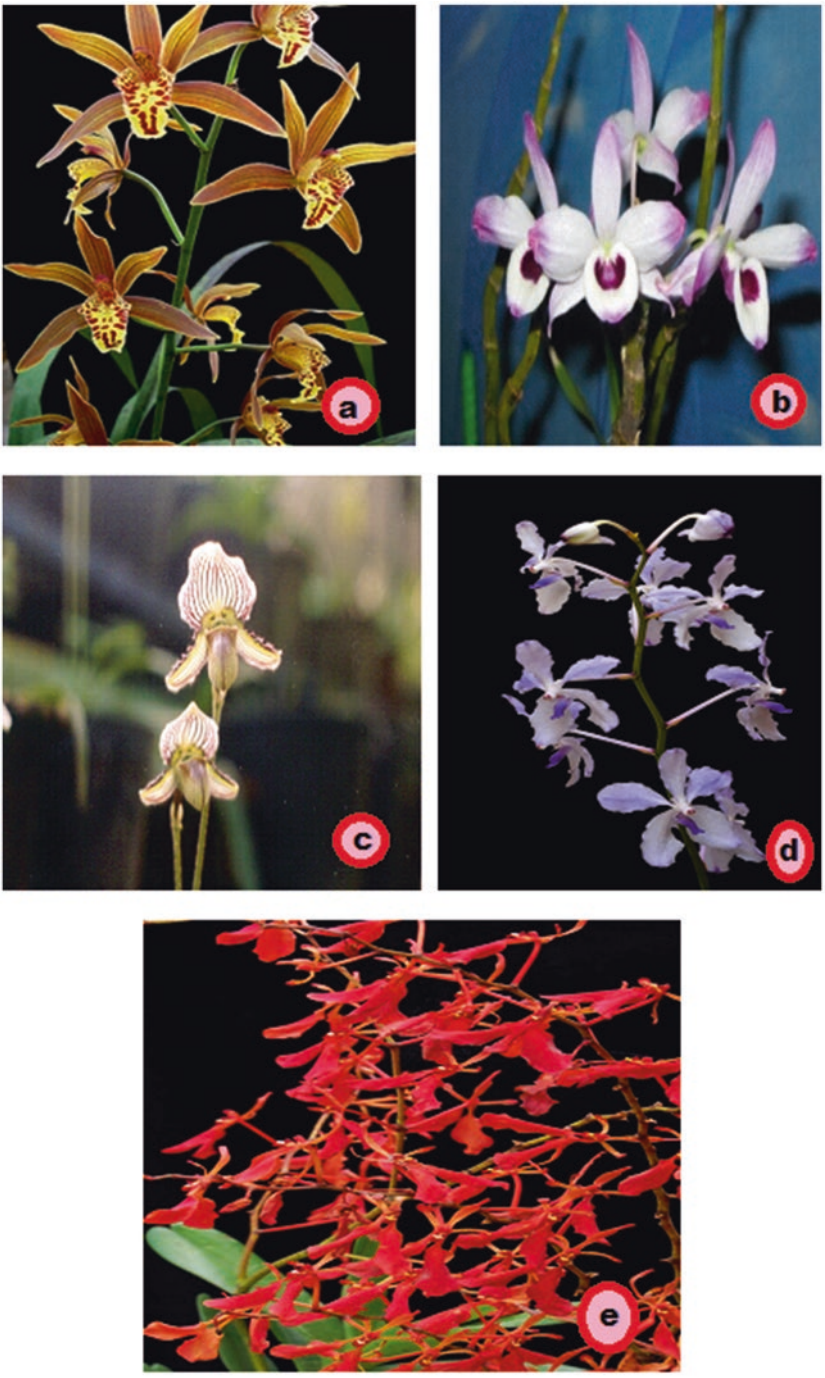

Fig 12.2 (**a**) *Cymbidium iridioides*, (**b**) *Dendrobium nobile*, (**c**) *Paphiopedilum fairrieanum*, (**d**) *Vanda coerulea*, (**e**) *Renanthera imschootiana*

12.3 Orchid Diversity

Orchids are known for their diversity of habit and habitat. In habit, they are either terrestrial (e.g., *Calanthe masuca*) growing in the soil or epiphytes (*Dendrobium*, *Vanda*, etc.) growing on tree trunks. Sometimes, they are found growing on moss-covered rocks, called lithophytes. Terrestrial orchids are either leafy with fibrous, rhizomatous, or tuberous roots or leafless saprophytes, also called myco-heterotrophs in which orchids engage themselves in mutualistic mycorrhizal relationships. Most of the orchids – terrestrial or epiphytes – are found growing as sympodial, forming a stem cluster at the base. Some epiphytes belonging to Vandoideae orchids are monopodial, in which stem does not branch out. It is important to note that all orchids have symbiotic relationship established right from the seed germination stage in roots.

Vegetative and floral structures are also highly modified from species to species and genus to genus which have attracted the attention of orchidologists the world over for the last two centuries. While both vegetative and floral structures are important in the classification of orchids, lip or labellum characters are diagnostic in deciding the names of orchids at the species level. Thus today, about 29,285 species in about 850 genera have been reported the world over (Shashidhar 2019).

In India, there are about 1350 species in 186 genera reported so far distributed in various phytogeographical regions (Hegde 1997, 2016, 2019). Out of that, Northeast Himalayan region alone contributes about 876 species in 151 genera (Kataki et al. 1984; Hegde 1980, 1981a, b, 1987a, b, 1988, 2000, 2001, 2005). In Arunachal Pradesh, more than 600 species have been reported by various workers so far (Hegde 1981a, b, 1984, 1985, 1987a, b, 1989, 1993a, b, 1997, 2016; Chowdhury 1998; Rao 2010; Hegde and Rao 1982a, b, c, d, 1983a, b, 1984a, b, 1985a, b, 1987; Joseph et al. 1982; Hegde and Rao 1984a, b, 1987, 1989; Rao and Hegde 1988; Rao et al. 1988). However, based on the herbarium and live evidence, as many as 550 species in 132 genera have been confirmed based on the revised study conducted at the State Forest Research Institute, Arunachal Pradesh (Hegde et al. 2017).

12.4 Orchid Diversity Analysis

From the analysis of 550 species in 132 genera found in Arunachal Pradesh, 376 are epiphytes including some lithophytes, 157 species are terrestrials, and 17 are mycotrophs (saprophytes). While *Dendrobium* has the maximum number of species (56 species) followed by *Bulbophyllum* with 55, the genera *Satyrium* and *Thunia* are represented by single species each. The following table depicts the top five genera with species richness in a descending order (Table 12.1).

Further, out of about 404 endemic species distributed in various phytogeographical regions of India (Hegde 2016), as many as 31 species are found only in Arunachal Pradesh (Hegde et al. 2017) which speaks of uniqueness of orchid flora and needs to be conserved. It is also significant to note that the rare, endangered, and threatened (RET) species of orchids protected under Wildlife Protection Act of

Table 12.1 Orchid genera in Arunachal Pradesh, India

S.No	Epiphytes		Terrestrials		Mycotrophs	
	Genus	Number of species	Genus	Number of species	Genus	Number of species
1.	*Dendrobium*	56	*Calanthe*	16	*Galeola*	3
2.	*Bulbophyllum*	55	*Goodyera*	12	*Epipogium*	2
3.	*Oberonia*	22	*Zeuxine*	08	*Gastrodia*	2
4.	*Coelogyne*	20	*Platanthera*	07	*Eulophia zollingeri*	1
5.	*Liparis*	16	*Cheirostylis*	05 + 01 var.	*Cymbidium macrorhizon*	1

Government of India and red listed under IUCN, viz., *Paphiopedilum fairrieanum* (Fig. 12.2c), *Vanda coerulea* (Fig. 12.2d), *Renanthera imschootiana* (Fig. 12.2e), and *P. venustum*, also find their habitat in Arunachal Pradesh. However, one species of *Paphiopedilum wardii* reported from Lohit Valley of Arunachal could not be found in its habitat in spite of several field investigations so far.

It is worthy to note that 550 species in 132 genera belonging to the **family Orchidaceae** are represented in two **subfamilies**, viz., **I. Cypripedioideae** and **II. Orchidoideae** under the phylogenetic system of classification proposed by Dressler and Dodson (1960). Each **subfamily** is divided into tribes as follows.

I. **Subfamily Cypripedioideae:** Tribe 1. Apostasieae; Tribe 2. Cypripedieae

Tribe Apostasieae has a genus *Apostasia* which is considered to be most primitive in the family Orchidaceae. In Arunachal Pradesh, the genus *Apostasia* is represented by two species – *A. odorata* Blume and *A. wallichii* R. Br. (Hegde 1981a, b).

Tribe Cypripedieae is represented by the genera *Cypripedium* and *Paphiopedilum.* While *Cypripedium* is represented by a single species, viz., *C. tibeticum* King ex Rolfe, *Paphiopedilum* is represented by three species, viz., *P. fairrieanum* (Lindl.) Stein, *P. venustum* (Wall ex. Sims) Pfitzer, and *P. wardii* Summerh. All the species are rare, endangered, and threatened and therefore protected under Wildlife Protection Act of GOI.

II. **Subfamily Orchidoideae: Tribe 1. Neottieae; 2. Orchideae; 3. Epidendreae**

Tribe Neottieae has 2 sub-tribes, tribe Orchideae has 3 sub-tribes, and tribe Epidendreae has 13 sub-tribes. The first 12 sub-tribes under the tribe Epidendreae are sympodial, while the 13th sub-tribe Sarcanthinae is monopodial in habit.

[It is worthy to note that Dressler (1981) has proposed separate subfamily for *Apostasia* as **Apostasiodeae** and for the genera under tribe Neottieae to be placed under a separate subfamily – **Neottioideae –** This warrants further study in respect of Indian species].

Each sub-tribe is further classified into genera and species based on the vegetative and floral characters, besides other minute details like color, size, and shape of sepals, petals, and lip, anther/pollinial structure and number, stigmatic structure, mentum and spur, etc., thus giving rise to a number of species diversity in the family Orchidaceae. In fact, structure of column, stigmatic surface, anther, and pollinia besides the shape and color of spur and lip facilitate pollination by various species of insects which are attracted toward the flowers of orchids and corresponding mechanism to pollinate effectively in a deceptive manner to achieve pollination, fertilization, and subsequent seed formation and proliferation (Hegde 1984).

12.5 Conservation

Realizing the abundance of orchids in Arunachal Pradesh and their importance and potential in floriculture for establishing a vibrant orchid cultivation and trade industry to supplement the economy of tribal inhabitants, Government of Arunachal Pradesh established Orchid Research Center at Tipi in Kameng District. Initial survey and collection revealed that although the orchid resources are abundant, there is a need for conserving the resources and developing the same in a sustainable manner by conserving the species in situ and ex situ. Accordingly, Sessa Orchid Sanctuary (Fig. 12.3) was established in the year 1979 in about 85 ha area at Doimara Reserve Forest of Kameng District for the purpose of orchid conservation, propagation, and proliferation (Hegde 1980) which was subsequently extended for 100 km^2 area in 1982 (Hegde 1986). Since orchids are specialized in their habit, either as saprophyte, terrestrial, or epiphyte and with varying habitat, requiring specific environmental requirements of light, humidity, temperature, pH of soil and host tree, mycorrhiza, phorophyte or specific host, and other microclimatic conditions, besides the availability of pollinators, all care was taken to introduce them in

Fig 12.3 A view of Sessa Orchid Sanctuary in Arunachal Pradesh

Fig 12.4 (**a**) *Epipogium sessanum* (**b**) *Cymbidium grandiflorum*

appropriate locations in the sanctuary for their growth and proliferation in as natural manner as possible. Accordingly, the concept and procedure of maintenance of orchid flora as per their habitat requirement were prepared for guidance of the officers entrusted with the responsibility of maintaining the sanctuary.

Subsequent survey and study of the area have yielded five new species, viz., *Biermannia jainiana* Hegde and Rao, *Cleisostoma tricallosum* Hegde and Rao, *Epipogium sessanum* Hegde and Rao (Fig. 12.4a), *Eria connata* Joseph, Rao, and Hegde, and *Gastrodia arunachalensis* Hegde and Rao (Rao et al. 1991; Rao 2010), 6 saprophytes, 28 leafy terrestrials, and about 140 epiphytic orchids in this sanctuary. It is significant to note that nearly 32% of subtropical orchids found in Arunachal Pradesh are found in this sanctuary, besides the presence of 15 endemic species of the Eastern Himalayas. Among them, *Bulbophyllum*, *Coelogyne*, *Cymbidium* (Fig. 12.4b), *Dendrobium*, and *Eria* are dominant genera with maximum number of species. *P. fairrieanum* at the peak of the hills (2800–3100 m MSL) and *P. venustum* (Hegde 1991a, b) at the foothill ranges (250–1000 m MSL) of the sanctuary find their habitat here which are under protected RET species.

In order to conserve the orchids ex situ, occurring in various parts of the state, chains of orchid centers at various elevations and climatic conditions of Arunachal Pradesh have been established at Tipi, Dirang, Itanagar, Jenging, Roing, and Deomali. About 300 species have been cultivated and propagated in these centers as a measure of ex situ conservation and to create awareness among the local inhabitants on the importance of orchids and impart training in orchid cultivation as a supplemental crop. Such efforts should be made by all the orchid-rich states of our country to save orchids in their natural habitat for future generations and development of our orchid industry.

12.6 Research and Development

To conserve, cultivate, propagate, and transfer the technique of orchid growing and farming to the people of Arunachal Pradesh and other northeast Indian states, a number of research and development programs have been carried out – especially establishment of aseptic laboratory for culturing orchid seeds and mericloning of selected orchid tissues, establishment of orchid herbarium for taxonomical studies and germplasm collection, breeding, and training of entrepreneurs and women self-help groups (Hegde 1987a, b, 1995a, b, 1998, 1999, 2000, 2001; Hegde and Ingalhalli 1985; Hegde et al. 1990, 2017; Sinha and Hegde 1997a, b). As a result of interspecific and intergeneric hybridization program involving native species and exotic hybrids, 16 different hybrids have been produced, and the following 5 have been registered at the Royal Horticulture Society, London, successfully (Hegde 1990a, b; Hegde et al. 2017): these are (1) Renades "Arunoday," (2) *Cymbidium* "Sessa Green Beauty," (3) *Ascocenda* "Tipi Blue Boy," (4) Arachnocentron "Tipi Blue Boy," and (5) *Esmeralda* "Millennium Dawn."

Similarly, extensive experiments have been conducted in standardizing protocols for aseptic seed germination and tissue culture from epical meristem, nodal tissues, leaf, and root segments of selected orchid species and hybrids (Hegde et al. 1988; Sinha and Hegde 1999; Sinha SK and Hegde SN 1999; Shadang et al., 2007). Besides, ethnobotanical usage and beliefs of the native inhabitants in the traditional system of medicine have also been recorded (Hegde 1984; Hegde and Ingalhalli 1988; Hegde et al. 2017).

Recognizing the ideal agroclimatic situations prevailing in Arunachal Pradesh Hill region and considering the market demand for orchid cut flowers in various colors, established *Cymbidium* hybrid clones are chosen for farming. Seedlings of selected clones were distributed to the selected entrepreneurs and women self-help groups in Hapoli, Ziro, Bomdila, and Dirang areas. All growers were trained in the farm at Tipi and Sessa and also at Hapoli in an Entrepreneurs Cymbidium Farm promoted by Orchid Research Center, SFRI. After 2.5 years, when blooming started, cut flowers were sent to market (Hegde 1987a, b, 1999). It was realized transportation cost by air to Calcutta turned out to be higher than the market value because of distance and nonavailability of subsidy for transport and other infrastructure of cold storage and cargo facilities. Once these problems are sorted out, further growth of this sector would pick up as a supplemental crop developing a vibrant orchid industry in the northeast region.

12.7 Conclusion

Arunachal Pradesh with more than 550 species in 132 genera occurring in various phytoclimatic conditions deserves to be called a "Paradise of Orchids" in India. It is significant to note that as many as 31 species are found to be endemic in Arunachal Pradesh only. The very fact that the Orchid Flora of Arunachal Pradesh consists of 376 epiphytes including some lithophytes, 157 species of terrestrials, and 17

mycotrophs (saprophytes) speak of its diversity and also point toward rich bioresource which could be utilized for the development of a vibrant orchid-based floriculture to supplement the economy of the people of the state. The very fact that a number of orchids found here are ornamental especially those belonging to the genera *Cymbidium*, *Dendrobium*, *Paphiopedilum*, *Renanthera*, and *Vanda* speaks of the potential for the development of cut flower and pot orchid trade in the state.

At the same time, it is pertinent to mention that every effort should be made to conserve this invaluable orchid resource both in situ and ex situ for future benevolence in a sustainable manner by adopting biotechnological approaches.

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Part III

Anatomy and Physiology

Orchid Mycorrhizal Fungi: Structure, Function, and Diversity

13

Kullaiyan Sathiyadash, Thangavelu Muthukumar, Velusamy Karthikeyan, and Kuppu Rajendran

Abstract

All orchids are mycoheterotrophic during seed germination and early stages of seedling development. Nevertheless, this dependency on the mycobiont extended into adulthood in many green photosynthetic orchids and is termed as mixotrophy. The fungal hyphae colonize orchids early during seed germination and protocorm development and form highly coiled structures called pelotons. Conventional studies mostly focused on orchid mycorrhizal fungi (OMF) that are saprophytic, but later the role of both ectomycorrhizal and parasitic fungi in orchid mycorrhizal symbiosis were recognized. Although there is enough evidence to believe that OMF is not host-specific, there are also indications which suggest the possible existence of physiological compatibility in orchid-fungal interaction. Current advances in molecular techniques have enabled us to untangle the diversity of fungi involved in the symbiosis and have helped to overcome the bottlenecks associated with the traditional identification of the fungal taxa using morphological characters. OMF symbiosis is shown to assure orchid survival in habitats vulnerable to stressful conditions or habitats with resource limitations. Further, the OMF has been shown to play a key role in the rehabilitation of threatened orchid species in their natural habitats. In spite of this, there is a large gap in our understanding of the fungal diversity associated with the tropical epiphytic and lithophytic orchid taxa.

Keywords

Orchidaceae · Peloton · Nutrient uptake · Growth promotion · Specificity · Seedling recruitment

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13.1 Introduction

Orchidaceae is one of the largest angiospermic plant families with roughly around 28,484 species (Govaerts et al. 2017). A majority of the orchids are narrowly distributed in a particular region (Lozano et al. 1996) and are highly affected by habitat clearance compared to other plant species (Jacquemyn et al. 2007). The mycorrhizal fungi symbiotically associate with orchids and serve as an important source of nutrients and water to the orchids (Zettler 1997a, b). The specialization in pollination and mycorrhizal association is linked to the size and diversity of taxa in Orchidaceae (Stewart and Kane 2007; Cozzolino and Widmer 2005; Taylor et al. 2003). The floral beauty and attraction of orchids directly contribute to their mass decline in the wild. But, mycorrhizal associations have enabled orchids to survive in diverse habitats around the world. In nature, orchid seeds fail to germinate in the absence of mycorrhizal fungi (Fig. 13.1). *In vitro* culture of orchids was successful using both seeds (Knudson 1922) and explants (Arditti 1984). Many studies have focused on asymbiotic propagation of orchids from seeds (Arditti 1967; Sheehan 1983). As symbiotic propagation and restoration of orchid population in the wild are either totally neglected or not much emphasized, Rasmussen (1995) suggested that a worldwide support and importance should be given to investigations involving symbiotic seed germination. Orchids are economically important as Vanillin from *Vanilla planifolia* is used as flavoring agent for food and drink, and tissues of *Gastrodia* are used in the preparations of natural medicine (Griesbach 2000). Anthropogenic activities including theft of attractive individuals have driven many of the aesthetic orchid species in nature to extinction. Therefore, conservation efforts for these orchid species should involve a thorough understanding of their biology (Dearnaley 2007). In this chapter we discuss not only the structure, diversity, and significance of the mycorrhizal fungi in orchid lifecycle, but also the importance of these fungi in orchid conservation.

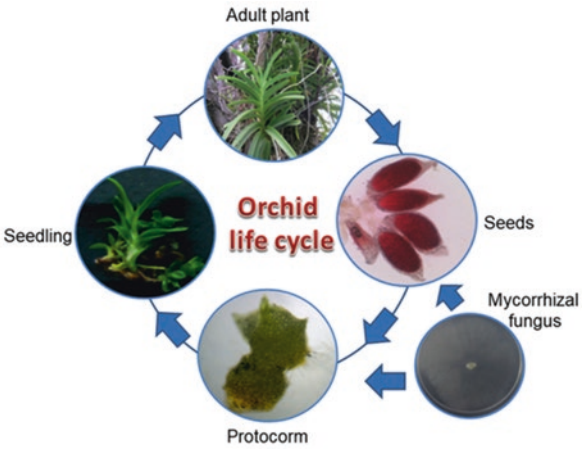

Fig. 13.1 Different stages in the lifecycle of an orchid. Mycorrhizal fungi are mandatory for seed germination and seedling development. Adult green photosynthetic orchid can acquire nutrients through mycorrhizal fungi and even supplement carbon through mixotrophy

13.2 Types of Orchid Roots and Velamen

The root system of orchid life-forms differs in several aspects (Rasmussen 1995). The roots of epiphytic and lithophytic orchids are ecologically similar as the roots are exposed to light and air (Fig. 13.2). The aerial roots of epiphytic and lithophytic orchids are perennial, photosynthetic with a fairly constant growth throughout the year (Dressler 1993; Muthukumar and Shenbagam 2018). In contrast, roots of terrestrial orchids are usually non-photosynthetic, have a limited life span of up to 3 years, and show seasonal changes in growth and architecture (Bayman and Otero 2006).

Roots of terrestrial orchids grow in soil or litter, and certain terrestrial orchids have two distinct types of roots, i.e., mycorrhizal and non-mycorrhizal (Rasmussen 1995). Non-mycorrhizal roots have more xylem and higher amyloplast than mycorrhizal roots (Bayman and Otero 2006). Nevertheless, most of the terrestrial orchids possess mycorrhizal fungi in their roots even in the adult stage of their life cycle (Rasmussen 1995). Compared to the obligate mycorrhizal nature of the terrestrial orchids, roots of most of the epiphytic and lithophytic orchids are facultatively mycorrhizal with frequency of colonization exhibiting variation to certain extent (Zelmer et al. 1996; Bayman et al. 1997; Otero et al. 2002; Rasmussen 2002).

Orchids have multiple epidermis consisting of one to several layers of thin-walled cells called velamen (Porembski and Barthlott 1988). The velamen helps the roots to trap water and nutrients (Dressler 1990; Rasmussen 1995). Roots of epiphytic orchids have more layers of velamen than terrestrial roots (Dressler 1990). In addition, epiphytic orchids growing in exposed or dry microenvironment like the *Acampe* tend to have multilayered velamen compared to those like *Bulbophyllum* which grow in more humid microenvironment (Muthukumar and Kowsalya 2017; Muthukumar and Shenbagam 2018). Based on their observations of orchids from exposed habitats of dry and humid environments, Sanford and Adanlawo (1973) suggested that the size of velamen may be indicative of the prevailing environmental factors such as moisture and temperature of the habitats. Oliveira and Sajo (1999) indicated that the velamen cells with suberized and lignified thickenings provided mechanical support to avoid cellular collapse during dehydration.

13.3 Nature of the Endophytic Fungi

In nature, orchid roots are colonized by diverse group of fungi, some of which may not be of true mycorrhizal in nature (Warcup 1981; Bayman et al. 1997). The endophytic orchid mycorrhizal fungi (OMF) of true mycorrhizal nature should have the potential to stimulate seed germination, enhance protocorms, and/or early seedling development and subsequently improve the growth and reproduction of the adult plants (Liu et al. 2010). OMF forms pelotons in root cortical cells and in seeds and the limitation of the OMF colonization to suspensor cells in the embryo and the epidermal hairs in germinating seeds clearly shows that the colonization process is dictated by the orchid and the symbiosis is adapted to this control (Hadley 1982).

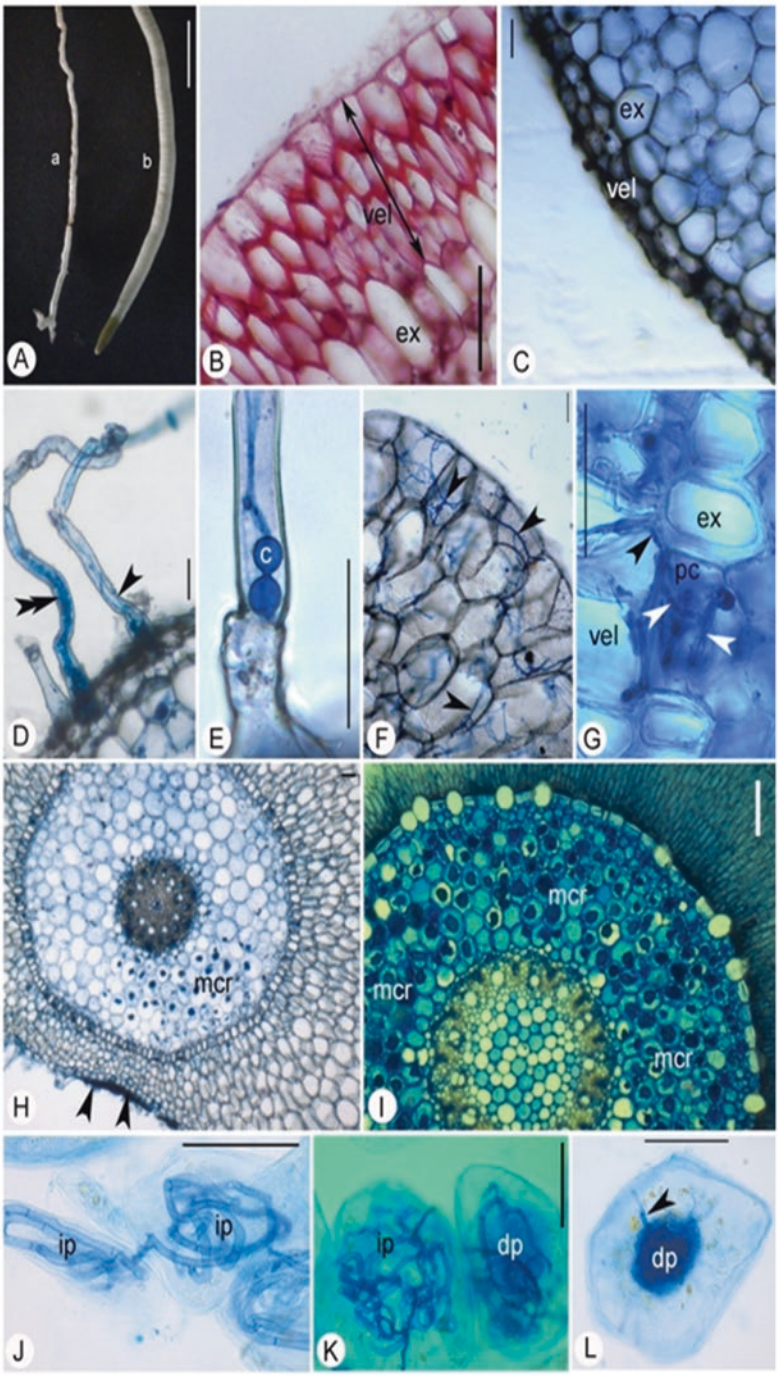

Fig. 13.2 (**a**)–(**l**) Morphology and mycorrhizal anatomy of orchid roots. (**a**), Velamentous roots of *Dendrobium* sp. (*a*) and *Acampe praemorsa* (*b*); (**b**), Velamen (*vel*) and exodermis (*ex*) in aerial roots of *A. praemorsa*; (**c**), Bilayered velamen (*vel*) and exodermis (*ex*) in *Spathoglottis plicata*;

The one way, through which the orchids obtain nutrients from the fungus, is through the digestion of the fungal hyphae. In orchids, the digestion of the pelotons takes place either by tolypophagy or phytophagy. In tolypophagy the complete digestion of pelotons occurs, whereas in phytophagy the fungal cell contents are released into the plant fungus interface through the lysis of the fungal tips (Burgeff 1959). Therefore, at any given point of time, orchid roots contain different proportions of intact and lysed pelotons (Fig. 13.2). Varying levels of intact to lysed or lysing pelotons have been reported in several orchids growing in the Western Ghats of south India (Muthukumar and Sathiyadash 2009; Muthukumar et al. 2011; Sathiyadash et al. 2012). Some components derived during this hyphal digestion play the role of fungal elicitors, which make easier the further colonization of the OMF (Liu et al. 2010). Further, the fungal elicitors of OMF have been shown to promote the development of protocorms and the development of seedling growth in orchids (Dong et al. 2008; Zhao and Liu 2008).

Several fungi that exhibit differential responses as parasites and pathogens are also shown to be symbionts of orchids. For example, *Ceratobasidium* stimulate the germination and seedling growth of *Ionopsis* sp. (Otero et al. 2004), but the same fungal isolate was shown to be pathogenic to *Dendrobium mericlones* (Porras-Alfaro 2004). Similarly, studies have also shown that the fungal symbionts of orchids could cause root rots in pea, soy bean, pine and spruce (Hietala et al. 2001; Yang et al. 2005). *Thanatephorus* isolated from the orchid *Pterostylis acuminata* was moderately pathogenic on lettuce and severely pathogenic on cauliflower and radish (Carling et al. 1999). *Rhizoctonia solani* which act as mycorrhizal symbionts in *V. planifolia* and *Vanilla phaeantha* also cause pathogenic lesions on the same root (Alconero 1969).

13.4 Orchid Mcyorrhizal Fungi (OMF)

13.4.1 Colonization Patterns of OMF in Orchid Roots

The fungal colonization patterns significantly differ both in epiphytic or lithophytic and ground orchids. In all these types the fungus enters the root primarily through the root hairs and occasionally directly through the epidermal cells (Sathiyadash et al.

Fig. 13.2 (continued) (**d**), Root hairs of *S. plicata* with fungal hyphae (black single arrow head) and moniliform cells (black double arrow head); (**e**) Chlamydospore-like cells (*c*) in root hair of *S. plicata*; (**f**), Fungal hyphae (black arrow heads) transversing the velamen in *Dendrobium* sp.; (**g**), Fungal hyphae (black arrow head) in the velamen (*vel*) and passage cell (*pc*) of the exodermis (*ex*) (white arrow heads) in *Dendrobium* sp. root; (**h**), Transverse section (T.S) of *Dendrobium* sp. substrate root with patchy mycorrhizal colonized region (*mcr*). The root portion attached to the substrate is indicated by black arrow heads; (**i**), T.S. of *Eulophia epidendraea* terrestrial root with diffused mycorrhizal colonized regions (*mcr*) throughout the root cortex; (**j**), Intact pelotons (*ip*) in cortical cells of *Luisia pulniana*; (**k**), Intact (*ip*) and degenerating (*dp*) pelotons in *Vanda spathulata*; (**l**), Degenerated peloton (*dp*) with fungal hyphal remnants (black arrow head) in cortical cell of *L. pulniana*. Scale bars: A = 2 cm; B–L = 50 μm

2012). In epiphytic forms, root portions that are only attached to the substrate like the tree bark contain mycorrhizal colonization and those that are not attached to any substrate are free from fungal colonization (Fig. 13.2). Nevertheless, the free aerial root upon entry into soil develops mycorrhizal colonization (Muthukumar and Kowsalya 2017). The fungal colonization therefore is patchy in epiphytic and lithophytic orchids, whereas it is diffused throughout in ground orchids (Sathiyadash et al. 2012; Muthukumar and Kowsalya 2017). The fungal hyphae transverse the velamen tissue and enter the cortex through the passage cells of the exodermis. From the exodermis the fungus spreads through the root cortex intracellularly forming a highly coiled structure called pelotons (Fig. 13.2). The size of the pelotons varies with the orchid species and is determined by the cortical cell size as evidenced by a strong correlation between the peloton and cortical cell dimensions (Sathiyadash et al. 2012). The nucleus of the cortical cells is pushed to the periphery in the invaded cells and like in other mycorrhizal types, the fungus never trespass the endodermis. In the cells of the root cortex and in root hairs chlamydospore-like structures or moniliform cells are present (Sathiyadash et al. 2012).

The relationship between orchid and the mycorrhizal fungi is unique in the plant kingdom. Orchid mycorrhizae may at times involve mycobionts that are pathogenic to other plant species as shown above, suggesting the orchid's specific ability to neutralize the possible virulence pathways of the pathogens (Watkinson 2002). These factors render orchids as an excellent model to study plant-fungal interactions. The establishment of the symbiotic interaction between the mycorrhizal fungi and the process of fungal colonization encompasses a wide range of similarities and differences with pathogenesis. This knowledge could provide an idea in developing new strategies to overcome or reduce the severity of the pathogenic interactions in non-orchidaceous plant species (Watkinson 2002). In the symbiosis between the orchid and the fungi, orchid is a dominant partner which helps to predict the evolution of plant-fungal interactions.

An increased survival of the orchid seedlings was often possible when the seedlings are mycorrhizal (Anderson 1991). Seeds of certain orchids like *Encyclia tampensis, Liparis liliifolia,* and *Taeniophyllum obtusum* germinate only in the presence of OMF under *in vitro* conditions (Irawati 1993; Rasmussen and Whigham 1998; Zettler et al. 1999). Likewise, protocorms of *Eulophia alta* develop more rapidly when mycorrhizal under *in vitro* conditions (Johnson et al. 2007). Symbiotically raised orchid seedlings can serve as both plant material as well as the source for mycorrhizal inoculums in conservation efforts (Batty et al. 2006).

13.4.2 Nutrient Transfer by OMF

Most of the orchids depend on mycorrhizal partners for their nutrients during seed germination and early developmental stages (Rasmussen 1995; Cameron et al. 2006; Rasmussen and Rasmussen 2009; Wu et al. 2013) (Fig. 13.1). The limited food resources in the seeds and the inefficiency of orchids to acquire nutrients from the substrates render orchids highly dependent on mycorrhizal fungi (Leake 1994).

The OMF transfer nutrients from different substrates to their symbiotic orchid host (Dearnaley and Cameron 2017). Numerous studies have demonstrated the mycorrhizal fungi mediated enhancement of nutrient acquisition by orchids in the native ecosystems (Alexander 2007; Smith and Read 2008 and references there in). In mixotrophic orchids, OMF transfer carbon (C), nitrogen (N), phosphorus (P), and other minerals to the adult photosynthesizing green plants (Zimmer et al. 2007). In epiphytic orchids, the OMF mycelium decompose organic matter such as bark and make available the nutrients to the orchids (Zhang et al. 2018). Nevertheless, at times the transfer of nutrients could be unidirectional (Hadley 1989), bidirectional (Cameron et al. 2006) or even mycorrhizal independent (Purves and Hadley 1975).

Carbon Transfer Most of the adult green orchids are autotrophic and fulfill their C requirement by photosynthesis. In addition to these, the orchid might also supplement its C requirement through the transfer organic C from the OMF (Gebauer and Meyer 2003). No difference in the C fixation or distribution was evident among the mycorrhizal and non-mycorrhizal *Goodyera repens* plants exposed to $^{14}CO_2$ (Hadley and Purves 1974). Moreover, the fungal mycelium originating from the colonized roots had no measurable radioactivity. So it could be inferred that *Rhizoctonia goodyerae-repentis* fail to utilize the photosynthates from the host orchids (Hadley and Purves 1974). The absence of C translocation from the host to the fungus could be either due to the inability of the fungus to obtain the host metabolites, or may also be due to the adequate amount of C in the growing media (Hadley and Purves 1974). In *Ceratobasidium cornigerum*, ^{14}C supplied as glucose peaked in the external mycelium within 48 hours, while its accumulation took several days in *G. repens* protocorms (Hadley 1984). Further, when the protocorms, plantlets and plants of *G. repens* were fed with ^{14}C through the external mycelium of the fungus, the plants failed to take up the C even under stressed conditions (Alexander and Hadley 1985). This suggests that the translocation of C movement mostly was unidirectional from the fungus to the host and the translocation of C ceased when the orchids reached certain stage of development (Alexander and Hadley 1985). In contrast to these, the C uptake rate by mycorrhizal *Dactylorhiza purpurella* and *Cymbidium* protocorms was higher than non-mycorrhizal protocorms (Hadley 1984). In the same study, the C uptake by the mycorrhizal *G. repens* protocorms was 50% higher than the non-mycorrhizal protocorms (Hadley 1984). In addition to the mycorrhizal mycelium of *Ceratobasidium cornigerum* transferring C to roots, rhizomes and green shoots of the *G. repens* plants, the C fed to shoots as $^{14}CO_2$ was readily assimilated and transferred to the rhizome and onwards to the extending external mycelium of the OMF (Cameron et al. 2006). This confirms the bidirectional flow of C and the true mutualistic nature of OMF.

Nitrogen Transfer The plants absorb the N in the form of NO_3^- and NH_4^+ (Zhang et al. 2018). Both terrestrial and epiphytic orchid life forms absorb NO_3^- and NH_4^+ from the substrates, but terrestrial orchids tend to absorb more NO_3^- than NH_4^+. In addition, NO_3^- concentration plays a critical role in flower and flower bud formation in *Cymbidium sinense*, whereas NH_4^+ concentration fails to produce any such effects

(Pan and Chen 1994). This clearly shows the orchids preference for different N forms. The inability of the orchids to utilize organic N emphasizes the importance of the mycorrhizal symbiosis in orchid seed germination and development of the seedlings in the natural habitats (Dijk and Eck 1995). In addition, the N nutrition of orchids gets even more complicated as the N sources vary depending on the orchids' requirement and life stages (Dijk and Eck 1995). The mycorrhizal seedlings of *Cymbidium goeringii* also differed in its uptake of N forms from the soil at different depths due to the variation in the distribution patterns of the *Rhizoctonia* spp., hyphae in the soil. Further, the fungal hyphae present in the upper and deeper layers of the soil are responsible for NH_4^+ and organic N uptake respectively (Wu et al. 2013). The transfer of N from the fungus to the orchid was first demonstrated by Cameron et al. (2006) in *G. repens*. Significant amounts of glycine labeled N (^{15}N) was transferred to the roots (2%) and shoots (20%) of the terrestrial orchid *G. repens* by the mycorrhizal fungus (Cameron et al. 2006). This suggests that the rate N transfer from the fungus to the plant is likely to be dependent on the activity and nature of the plant-fungal interfaces in the roots (Cameron et al. 2006).

The foliar concentrations of isotope labeled N (^{15}N) both in green (*Cephalanthera damasonium*) and achlorophyllous (*Dactylorhiza sambucina*) orchids growing in forest and grassland sites in Europe was significantly higher than the co-occurring herbs and trees suggesting the significance of the fungal symbiosis (Gebauer and Meyer 2003). Earlier studies have demonstrated an improved N nutrition in certain autotrophic plants, when glycine and other amino compounds were provided to its mycorrhizal fungal partner (Taylor et al. 2004).

Phosphorus Transfer The P uptake of adult *G. repens* plants colonized by *R. goodyerae-repentis* and grown in different P levels was 100 times more than its non-mycorrhizal counterparts because of OMF mediated P uptake like in other mycorrhizal systems (Alexander and Hadley 1984). Radioactivity could be detected in the protocorms of *D. purpurella* inoculated with orchid endophytes and fed with radioactive P orthophosphate suggesting the transfer of P from the fungus to the host plant (Smith 1966). The translocation of ^{32}P via the fungal hyphae occurs up to a distance of 9 cm from the root, with optimum distance being 2–3 cm (Alexander and Hadley 1984). However, the density of the fungal hyphae decreases with an increasing distance leading to a delay of phosphate reaching the nearest root (Alexander and Hadley 1984). Although the lack of a significant positive correlation between root length and ^{32}P content in mycorrhizal *G. repens* (Alexander and Hadley 1984) casts doubt on the role of OMF in P nutrition, later studies have shown that OMF mediated P transfer. For example, the external mycelium of *C. cornigerum* colonizing *G. repens* was capable of assimilating and transporting the ^{33}P orthophosphate into the plant (Cameron et al. 2007). Further, 7 days after ^{33}P exposure, 6.3% of the 10% of the P transferred over diffusion barrier were detected in the shoots of *G. repens* indicating that the OMF could uptake and translocate significant amount of P to its partner (Cameron et al. 2007).

13.4.3 Plant Growth Stimulation by OMF

Colonization by OMF enhances both the orchids vegetative and reproductive growth, ex vitro survival rates, induces early flowering, improves flower quality, and reduces disease severity in the seedlings (Chang 2008). Similarly, all the *Acampe praemorsa in vitro* raised seedlings inoculated with OMF survived under *ex vitro* conditions (Sathiyadash et al. 2013). Colonization of roots by *Rhizoctonia* sp., increases plant height, biomass, root formation, and root length in *Cymbidium kanran* (Lee et al. 2003), *Cymbidium goeringii* (Wu et al. 2013), and *Cymbidium* sp. (Wu et al. 2010). However, colonization by *Tulasnella repens* only enhanced the plant biomass in these orchids. Similar effects were also reported for *Dendrobium nobile*, *D. loddigesii* (Zaiqi and Yin 2008), *Dendrobium officinale* (Yang et al. 2008a), *Haemaria discolor* (Chang and Chou 2001), and *Anoectochilus formosanus* (Chang and Chou 2007) colonized by different OMF. Orchids like *Anoectochilus roxburghii* (Dan et al. 2012a), *Dendrobium candidum* and *D. nobile* colonized by *Epulorhiza* sp., *Mycena dendrobii*, *Moniliopsis* sp., *Gliocladium* sp., *Mycena anoectochila* were taller and had higher biomass (Dan et al. 2012b). *Cymbidium* colonized by six strains of unidentified OMF endophytes, as well as *Doritaenopsis* and *Phalaenopsis* colonized by *Rhizoctonia* and *Ceratobasidium* had higher biomass than un-inoculated seedlings (Fang et al. 2008; Wu et al. 2009, 2011). The *in vitro* raised *Guarianthe skinneri* when acclimatized with *Trichoderma harzianum* were taller and had more leaf and shoot numbers (Gutierrez-Miceli et al. 2008). Ex vitro raised *Cattleya aurantiaca* and *Brassavola nodasa* colonized by *Epulorhiza* were taller and heavier than their non-mycorrhizal counterparts (Ovando et al. 2005). Colonization of *Dendrobium officinale* roots by *Mycena* sp., increased plant height, biomass, and the number of new buds by two- four folds (Zhang et al. 2012). Further, increment in plant height and biomass are often considered as reliable parameters for the successful establishment of the symbiotic relationship (Jin et al. 2009a, b).

13.4.4 Phytohormone Production by OMF

The beneficial plant growth promoting microorganisms produce phytohormones, which are utilized by the host plants and it is one of the mechanisms for plant growth promotion by the microorganisms (Van Loon 2007; Shoresh et al. 2010). The bioactive compounds like indole-acetic acid (IAA), gibberellic acid (GA), and naphthalene acetic acid (NAA) produced by OMF are suggested to stimulate the development of *D. candidum* and *D. nobile* plantlets (Dan et al. 2012b). Gibberellic acid and NAA synthesized by OMF can also promote the elongation of stems and roots of *D. huoshanense* (Zhang et al. 1999). Isolates of *Tulasnella*, *Epulorhiza* and an unusual orchid endophyte *Colletotrichum gloeosporioides* has been shown to produce IAA under *in vitro* culture conditions (Robinson et al. 1998; Chung et al. 2003). The significant amounts of IAA detected both in the fungal mycelium as well as in the culture medium may be transferred to the orchid hosts which may influence the colonization process (Barroso et al. 1986). In addition, the detection of the presence of indole-3-ethanol

(IEt) in the *Ophrys lutea* culture medium indicates its synthesis and this IEt could act as a precursor for IAA synthesis in the host orchids (Barroso et al. 1986).

Earlier studies have shown that OMF are capable of secreting gibberellins, heteroauxin, dormin, zeatin and zeatin riboside (Wu and Zheng 1994; Wu et al. 2002) and these plant hormones were shown to improve the growth of orchids (Yang et al. 2008a, b). In *Gastrodia elata*, seed germination and cell differentiation process are stimulated by plant hormones or fungal metabolic products (Guo and Xu 1990; Xu 1993). In *Dendrobium hancockii,* the germination of seeds was stimulated by the presence of OMF extracts obtained from the protocorms of other orchids like *Liparis nervosa* and *G. elata* (Guo and Xu 1990). *Trichoderma* sp., can either synthesis phytohormones or alter the internal phytohormone homeostasis of the host plant (Shoresh et al. 2010; Salas-Marina et al. 2011). Extracted compounds and mixtures of fungi, filter concentrates, mycelia extracts, and hydrolytic products of cell walls, peptides and proteins are shown to act as fungal elicitors (Smith 1996; Hahn 1996).

13.4.5 Role of OMF in Disease Resistance

One of the major bottlenecks in commercial orchid cultivation is the disease outbreak, as bacteria, fungi or viruses affect the quality of plants by leaving brown spots or scars, and when the disease is not controlled or eradicated it may cause huge economic loss in orchid production (Wu et al. 2011). Soft rot diseases caused by *Erwinia* spp. is the most devastating disease in commercial orchid production (Liau et al. 2003). *Phalaenopsis* inoculated with *R. solani* and *Ceratobasidium* strains reduced the severity of soft rot, and a correlation also exists between disease symptom reduction and plant growth (Wu et al. 2011). In *Vanilla*, colonization by *Ceratobasidium* was shown to control the root rot caused by *Fusarium* (Bayman et al. 2011). OMF directly inhibit the pathogens through competition for space or nutrients. Further, OMF provides growth promoters or systemic-induced resistance to strengthen the plant defense mechanisms (Burns and Benson 2000; González et al. 2002). The improved nutritional status of orchids due to OMF makes them less susceptible to pathogens (Bayman et al. 2011).

Ceratobasidium isolated from orchids could also protect other type of plants from pathogens (Bayman et al. 2011). Hypovirulent and non-pathogenic *Ceratobasidium* present in soil protect the seedlings of cucumber and other crops from damping-off disease (Sneh et al. 2004; Ichielevich-Auster et al. 1985). *Ceratobasidium* isolated from *Cranichis* sp., and *Maxillaria* sp., decreased the severity of sheath blight in rice caused by *R. solani* (Mosquera-Espinosa et al. 2013).

13.5 OMF Diversity

The presence of compatible mycobionts and different environmental factors plays a major role in the growth and recruitment of orchids in nature (McCormick et al. 2004). The unique feature of orchidaceae as well as its wide diversity may be

attributed to its distinctive relationship with the mycorrhizal fungi (Zettler et al. 2004). The OMF diversity could be separated based on the ecology and photosynthetic ability of the orchid hosts (Taylor et al. 2002). However, information is limited on the role of fungal diversity in orchid distribution, population size, and genetic diversity (McCormick et al. 2004).

13.5.1 OMF Diversity in Terrestrial Photosynthetic Orchids

Terrestrial photosynthetic orchids are generally colonized by fungi belonging to five groups such as Heterobasidiomycetes, Hericianae, Hymenocaetanae, Thelephoranae, and Agaricanae (Rasmussen 2002) (Table 13.1 and Fig. 13.3). Earlier records mainly focused on the saprophytic fungi involved in the mycorrhizal association of orchids, but later both ectomycorrhizal (ECM) and parasitic fungi were also shown to associate with orchids in a mutualistic manner (Rasmussen 2002). Isolation of the ECM fungi belonging to Russulaceae from *Corallorhiza* spp. (Taylor and Bruns 1997) and the colonization of field-grown seedlings of *Corallorhiza trifida* with *Thelephora–Tomentella* complex of Thelephoriaceae, suggests that the ECM fungi can symbiotically associate with orchids (McKendrick et al. 2000). The ECM fungal genera associating with the non-photosynthetic *Lecanorchis* spp. include *Lactarius*, *Russula*, Atheliaceae, and *Sebacina*, of which *Lactarius* and *Russula* were found to be dominant (Okayama et al. 2012). Colonization of the achlorophyllous *Epipogium roseum* by the saprotrophic members of Coprinaceae suggest that the decaying wood materials would be used as a large and persistent C source for the growth of this orchid (Yamato et al. 2005). Mycobionts of *Hexalectris* spp. were found to be members of Sebacinaceae, Ceratobasidiaceae, Russulaceae, and Thelephoraceae (Kennedy et al. 2011). It is hypothesized that achlorophyllous orchids obligately associated with fungi have access to a large and persistent C source supplied by ECM fungi (Taylor and Bruns 1997; Leake 2005), as these fungi are likely to have access to such C supply (Kennedy et al. 2011).

An investigation on the mycobionts of *Caladenia* yielded 103 fungal isolates, most of which belonged to *Sebacina vermifera* and one isolate belonged to *Tulasnella calospora*. In an allied *Caladenia* species, 94% of the 33 isolates belonged to *S. vermifera* and a few to *T. calospora*, indicating that these orchids were associated with specific OMF (Warcup 1971). Further, investigations on mycobionts of terrestrial orchids have resulted in the discovery of new fungal taxa. For example, two new mycorrhizal endophytes, *Ceratorhiza pernacatena* and *Epulorhiza calendulina*, were characterized and identified from the roots of *Platanthera praeclara* and *Amerorachis rotundifolia*, respectively, from Canada (Zelmer and Currah 1995). A phylogenetic analysis of the mycorrhizal diversity from a single peloton of *Disabracteata* (a South African orchid) and *Pyrorchis nigricans* showed the presence of the fungi belonging to distinct groups of the *Rhizoctonia* alliance, like *Epulorhiza*, *Ceratobasidium,* and *Sebacina* (Bonnardeaux et al. 2007). Nevertheless, composite peloton formation by multiple endophytes within a cell further complicates the nutritional basis of OMF relationships

Table 13.1 The diversity of fungal taxa involved in mycorrhizal association with different orchid life-forms

Orchid life forms Mycorrhizal fungal taxa	Orchid taxa	References
Epiphytes		
Ceratobasidium cornigerum	*Sarcochilus* sp., *Thrixspermum congestum*	Roberts (1999)
C. endophytica	*Cymbidium sinensis*	Lee and You (2000)
Ceratobasidium sp.	*Taeniophyllum obtusum*	Irawati (1993)
	Campylocentrum fasciola, Campylocentrum filiforme, Ionopsis satyrioides, Ionopsis utricularioides, Tolumnia variegata, Oeceoclades maculata, Oncidium altissimum	Otero et al. (2002)
	I. utricularioides	Otero et al. (2004)
	Sarcochilus weinthalii	Graham and Dearnaley (2012)
	Vanda thwaitesii	Decruse et al. (2018)
	Rhynchostylis retusa, Aerides multiflorum	Hossain et al. (2013)
	Dendrophylax lindenii	Mújica et al. (2018)
Ceratobasidium sp., *Rhizoctonia* sp.	*Erythrodes plantaginea*	Otero et al. (2002)
C. sphaerosporum	*Pomatocalpa macphersonii*	Warcup and Talbot (1971)
	Robiquetia wassellii	Warcup and Talbot (1971)
Ceratorhiza sp.	*Isochilus linearis*, *Oncidium flexuosum*, *Oncidium varicosum*, *Maxillaria marginata*	Pereira et al. (2005)
Epulorhiza calendulina	*Spathoglottis plicata*	Ma et al. (2003)
	Epidendrum rigidum, Polystachya concreta	Pereira et al. (2003)
E. epiphytica	*E. rigidum*, *P. concreta*	Pereira et al. (2005)
E. repens, Tulasnella violea, Trichosporiella multisporum	*Dendrobium friedericksianum*	Khamchatra et al. (2016)
Epulorhiza sp.	*Arachnis* sp., *Arundina graminifolia, Dendrobium crumenatum*, *Diplocaulobium* sp., *Oncidium nanum*, *Vanda* sp.	Ma et al. (2003)
	Coelogyne nervosa	Sathiyadash et al. (2014)
Epulorhiza sp., *Tulanella calopsora*, uncultured *Tulasnella*, *Pluteus seticeps, Ceratobasidium* sp.	*Dendrobium officinale, Dendrobium fimbriatum*	Xing et al. (2013)
Rhizoctonia butinii	*Cyrtochilum myanthum*	Novotna et al. (2018)

(continued)

Table 13.1 (continued)

Orchid life forms Mycorrhizal fungal taxa	Orchid taxa	References
Rhizoctonia sp.	*Tolumnia variegata*	Otero et al. (2004)
	P. nutans, Pterostylis longifolia, Pterostylis obtusa	Bougoure et al. (2005)
	Gastrochilus acaulis, P. concreta, Nervilia prainiana	Senthilkumar (2003)
Rhizoctonia zeae	*Vanda coerulea*	Aggarwal et al. (2012)
Thanatephorus sp.	*Acampe praemorsa*	Senthilkumar et al. (2000)
Tremella sp.	*Cymbidium bicolor*	Downing et al. (2017)
Tulasnella calospora	*Laelia autumnalis*	Beltrán-Nambo et al. (2018)
	Cymbidium tracyanum, Dendrobium crystallinum	Nontachaiyapoom et al. (2010)
T. deliquescens	*Diuris, Acianthus, Caladenia, Thelymitra, Lyperanthes* spp., *Dendrobium dicuphum*	Warcup and Talbot (1980)
	Dendrobium sp.	Roberts (1999)
T. irregularis	*D. dicuphum*	Warcup and Talbot (1980)
	Encyclia tampensis	Zettler et al. (2013)
T. pinicola	*Dendrobium* sp.	Warcup and Talbot (1967)
Tulasnella sp.	*Stelis superbiens*	Novotna et al. (2018)
	Stelis concinna, Stelis hallii, Stelis superbiens	Suarez et al. (2006)
	Pleurothallis lilijae	
	Dendrobium speciosum	Boddington and Dearnaley (2008)
	Cymbidium lowianum	Nontachaiyapoom et al. (2010)
	Cymbidium tracyanum	Athipunyakom et al. (2004b)
	C. macranthos	Shimura et al. (2009)
Uncultured *Tulasnella* sp.	*Cymbidium bicolor*	Downing et al. (2017)
Tulasnella violea	*Dendrobium friedericksianum*	Nontachaiyapoom et al. (2010)
Hemiepiphyte		
Scleroderma areolatum	*Vanilla planifolia, Vanilla pompona, Vanilla insignis*	González-Chávez et al. (2018)
Lithophyte		
Uncultured *Tulasnella* sp.	*Paphiopedilum dianthum, Paphiopedilum hirsutissimum*	Downing et al. (2017)

(continued)

Table 13.1 (continued)

Orchid life forms Mycorrhizal fungal taxa	Orchid taxa	References
Terrestrial		
Armillaria jezoensis	*Galeola septentrionalis*	Cha and Igarashi (1996)
Armillaria mellea	*Gastrodia elata*	Lan et al.(1994)
Atheliaceae	*Lecanorchis flavicans* var. *acutiloba*	Okayama et al. (2012)
Atheliaceae, *Russula* sp.	*Lecanorchis trachycaula*	Okayama et al. (2012)
Ceratobasidium bicorne	*Prasophyllum macrostachyum*	Warcup and Talbot (1980)
C. cornigerum	*Acianthus reniformis*	Warcup and Talbot (1971)
	Canadian orchids	Currah et al. (1987)
	Platanthera obtusata	Currah et al. (1990)
	Pterostylis sp., *Prasophyllum* sp.	Roberts (1999)
C. cornigerum, Rhizoctonia repens, Ceratobasidium globisporum, Thanatephorus cucumeris, Epulorhiza sp., *Tulasnella* sp.	*Habenaria dentata*, *Eulophia flava, Spiranthes hongkongensis*	Shan et al. (2002)
Ceratobasidium sp., *Sebacina* sp., *Rhizoctonia* sp.	*Anacamptis morio*	Waud et al. (2016)
C. globisporum	*Calanthe triplicata*	Roberts (1999)
C. pseudocor- nigerum	*Pterostylis mutica*	Warcup and Talbot (1980)
Ceratobasidium sp.	*Gymnadenia conopsea*	Stark et al. (2009)
	Pterostylis sanguina, *P. recurva*	Bonnardeaux et al. (2007)
	Dactylorhiza hatagirea	Aggarwal and Zettler (2010)
	Paphiopedilum hirsutissimum	Downing et al. (2017)
Ceratobasidium sp., *Leptodontidium* sp., *Phialophora* sp., *Tulasnella* sp.	*Platanthera chlorantha*	Bidartondo et al. (2004)
Ceratobasidium sp., *Leptodontidium* sp., *Sebacina* sp., *Tulasnella* sp.	*Epipactis palustris*	Bidartondo et al. (2004)
Ceratobasidium sp., *Russula* sp., uncultured *Tricholoma* sp.	*P. nutans*	Irwin et al. (2007)
Ceratobasidium sp., *Sebacina* sp.	*Epipactis helleborine*	Bidartondo et al. (2004)
Ceratobasidium sp., *Sebacina vermifera*	*Cypripedium californicum*	Shefferson et al. (2005)
Ceratobasidium sp., *Tulasnella* sp.	*Dactylorhiza majalis*	Bidartondo et al. (2004)

(continued)

Table 13.1 (continued)

Orchid life forms Mycorrhizal fungal taxa	Orchid taxa	References
Ceratobasidium spp.	*Coppensia doniana*	Valadares et al. (2011)
Ceratobasidum albasitensis	*Dactylorhiza baltica*	Shefferson et al. (2008)
	D. baltica	Shefferson et al. (2008)
Ceratorhiza cerealis	*Goodyera procera*	Athipunyakom et al. (2004b)
Ceratorhiza goodyera-repentis	*Ludisia discolor, G. procera*	Athipunyakom et al. (2004b)
Ceratorhiza pernacatena	*Platanthera praeclara*	Zelmer and Currah (1995)
	Calanthe rubens	Athipunyakom et al. (2004b)
Ceratorhiza ramicola	*Paphiopedilum exul*	Athipunyakom et al. (2004b)
	G. procera	Athipunyakom et al. (2004b)
Coprinus spp.	*Epipogium roseum*	Yamato et al. (2005)
Cortinarius, *Hymenogaster*, *Inocybe*, *Thelephora* sp., *Tomentella* sp.	*Cephalanthera damasonium*	Bidartondo et al. (2004)
Epacris pulchella, *Russula mustelina*, *Gymnopus luxurians*	*Erythrorchis cassythoides*	Dearnaley (2006)
Epulorhiza anaticula	*Coeloglossum viride*, *Platanthera obtusata*, *Platanthera hyperborea*	Currah et al. (1990)
E. calendulina	*Amerorchis rotundifolia*	Zelmer and Currah (1995)
	Paphiopedilum concolor	Athipunyakom et al. (2004a, b)
Epulorhiza repens	*Microtis parviflora*	Perkins et al. (1995)
	Oeceoclades maculata	Pereira et al. (2005)
	Spiranthes brevilabris	Johnson et al. (2007)
	Calanthe rosea, Cymbidium sinense, Paphiopedilum concolor, P. exul, P. villosum, Spathoglottis plicata	Athipunyakom et al. (2004a, b)
Epulorhiza sp.	*Eulophia alta*	Johnson et al. (2007)
	Eulophia spectabilis, Pecteilis susannae, Paphiopedilum bellatulum, Spathoglottis affinis	Chutima et al. (2011)

(continued)

Table 13.1 (continued)

Orchid life forms Mycorrhizal fungal taxa	Orchid taxa	References
	C. sinensis, Paphiopedilum callosum, Paphiopedilum charlesworthii, P. exul, Paphiopedilum sukhakulii, P. villosum, Cymbidium (hydrid).	Nontachaiyapoom et al. (2010)
Epulorhiza sp., and *Ceratorhiza* sp.	*P. praeclara*	Sharma et al. (2003)
Erythromyces crocicreas	*Galeola altissima*	Umata (1995)
	Erythrorchis ochobiense	Umata (1998)
Gloeotulasnella sp.	*E. spectabilis*	Chutima et al. (2011)
Inocybe sp., *Leptodontidium* sp., *Phialophora,Sebacinoid* sp., *Tuber* sp., *Tulasnella* sp.,*Wilcoxina* sp.	*Epipactis atrorubens*	Bidartondo et al. (2004)
Lactarius sp.	*Lecanorchis japonica* var. *kiiensis, Lecanorchis kiusiana* var. *suginoana, Lecanorchis virella*	Okayama et al. (2012)
Lactarius sp., and *Russula* sp.	*L. japonica* var. *japonica, L. japonica* var. *kiiensis, L. japonica* var. *hokurikuens, Lecanorchis nigricans*	Okayama et al. (2012)
Leptodontidium sp., *Phialophora* sp., *Tomentella* sp.	*Cephalanthera rubra*	Bidartondo et al. (2004)
Moniliopsis anomala	*Coeloglossum viride, Platanthera hyperborea*	Currah et al. (1990)
Mycena orchidicola	*C. sinense*	Fan et al. (1996)
M. osmundicola	*Gastrodia elata*	Lan et al. (1996)
Oliveonia pauxilla	*G. elata*	Warcup (1975)
Phaeosphaeria phragmiticola	*Acianthus exsertus, A. pusillus*	Bougoure et al. (2005)
Phialophora graminicola	*A. achalensis*	Sebastián et al. (2014)
Phialophora sp.	*Thelymitra crinita*	Bonnardeaux et al. (2007)
Rhizoctonia globularis	*G. procera*, *S. plicata*	Athipunyakom et al. (2004a)
R. repens	*Cymbidium goeringii*	Lee et al. (1998)
	Aerides rosea, Phalaenopsis manii	Saha and Rao (2006)
R. solani	*Phaius tankervilleae*	Saha and Rao (2006)
	Chloraea riojana	Fracchia et al. (2016)

(continued)

Table 13.1 (continued)

Orchid life forms Mycorrhizal fungal taxa	Orchid taxa	References
Rhizoctonia sp.	*Aa achalensis*	Sebastián et al. (2014)
	S. plicata	Athipunyakom et al. (2004a)
Rhizoctonia sp., *Ceratobasidium* sp., Helotiales sp., *Naevola minutissima*	*Dactylorhiza sambucina*	Pellegrino and Bellusci (2009)
Rhizoctonia sp., *Thanatephorus cucumeris, Tulasnella danica*	*Pyrorchis nigricans*	Bonnardeaux et al. (2007)
Rhizoctonia sp., unidentified Tulasnellaceae and Ceratobasidiaceae, *Rhizoctonia* sp., *Sebacina* sp., *Thanatephorus* sp.	*Orchis mascula*	Waud et al. (2016)
Rhizoctonia sp.1	*Paphiopedilum niveum*	Athipunyakom et al. (2004b)
Rhizoctonia sp.2	*P. exul*	Athipunyakom et al. (2004b)
Russula sardonia	*Cypripedium parviflorum*	Shefferson et al. (2005)
Russula sp.	*Limodorum abortivum, L. brulloi, L. trabutianum*	Girlanda et al. (2006)
	Corallorhiza sp.	Whitridge and Southworth (2005)
Russula spp.	*Dipodium variegatum*	Bougoure and Dearnaley (2005)
Russulaceae spp.	*Corallorhiza maculata, Corallorhiza mertensiana*	Taylor and Bruns (1999)
Sebacina sp.	*Hexalectris spicata, Hexalectris spicata* var. *arizonica, Hexalectris revoluta*	Taylor et al. (2003)
	S. plicata	Athipunyakom et al. (2004a)
	Neottia nidus-avis	Bidartondo et al. (2004)
	Lecanorchis flavicans var. *flavicans*	Okayama et al. (2012)
Sebacina spp.	*Stigmatodactylus sikokianus*	Yagame and Yamato (2008)
Sebacina spp., *Ceratobasidium* sp., *Cortinarius* sp., *Thelephoraceae*	*Hexalectris* spp.	Kennedy et al. (2011)
Sebacina vermifera	*Caladenia carnea*	Bougoure et al. (2005)

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Table 13.1 (continued)

Orchid life forms Mycorrhizal fungal taxa	Orchid taxa	References
	Caladenia falcata, *Microtis media*	Bonnardeaux et al. (2007)
Serendipita vermifera	*Acianthus* spp., *Caladenia* spp., *Elythranthera* spp., *Eriochilus* spp., *Glossodia major, Microtis* spp., *Prasophyllum* spp.	Roberts (1999)
Sistotrema sp.	*Platanthera obtusata, Piperiya unalascensis*	Currah et al. (1990)
	Paphiopedilum godefroyae	Athipunyakom et al. (2004b)
Steccherinum ochraceum	*Microtis parviflora*	Bougoure et al. (2005)
Thanatephorus cucumeris	*A. achalensis*	Sebastián et al. (2014)
	Prasophyllum odoratum, Pterostylis foliata	Roberts (1999)
	Goodyera oblongifolia	Shefferson et al. (2005)
	Rhizanthella gardneri	Warcup (1991)
Thanatephorus ochraceus	*Calypso bulbosa*	Currah (1987)
	C. viride, Orchis mascula	Warcup and Talbot (1967)
T. sterigmaticus	*Thelymitra antennifera*	Warcup and Talbot (1967)
Thelephoraceae sp.	*Cephalanthera austinae*	Taylor and Bruns (1997)
Thelephoraceous ectomycorrhiza	*Cypripedium candidum, Cypripedium fasciculatum, Cypripedium montanum*	Shefferson et al. (2005)
Tomentella sp., *Ceratobasidium* sp., and *Tuber* sp.	*Cephalanthera damasonium*	Julou et al. (2005)
Trichosporiella multisporum	*Paphiopedilum niveum*	Athipunyakom et al. (2004b)
Tuber sp. and *Russula* sp.	*Epipactis microphylla*	Selosse et al. (2004)
Tuber sp. and *Sebacina* sp.	*E. microphylla*	Selosse et al. (2004)
Tulansella sp.	*Paphiopedilum micranthum, Paphiopedilum armeniacum, Paphiopedilum dianthum, Cypripedium flavum, Cypripedium guttatum, Cypripedium tibeticum*	Yuan et al. (2010)
Tulasnella asymmetrica	*Cymbidium* (hydrid)	Nontachaiyapoom et al. (2010)
T. calospora	*Prasophyllum giganteum, Diuris magnifica*	Bonnardeaux et al. (2007)

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Table 13.1 (continued)

Orchid life forms Mycorrhizal fungal taxa	Orchid taxa	References
	Paphiopedilum sukhakulii, P. villosum	Nontachaiyapoom et al. (2010)
	Chloraea collicensis, Chloraea gavilu	Pereira et al. (2014)
T. cruciata	*Acianthus caudatus, Thelymitra* spp.	Warcup and Talbot (1971)
T. deliquescens	*Dactylorhiza purpurella*	Roberts (1999)
T. irregularis	*Cymbidium* (hydrid), *Paphiopedilum charlesworthii*	Nontachaiyapoom et al. (2010)
	Thelymitra luteocilium	Warcup and Talbot (1967)
T. pruinosa	*Cymbidium* (hydrid)	Nontachaiyapoom et al. (2010)
T. repens	*Cymbidium goeringii*	Lee and You (2000)
Rhizoctonia sp., *Ceratobasidium* sp., *Sebacina* sp., unidentified Ceratobasidiaceae and Tulasnellaaceae	*Gymnadenia conopsea*	Waud et al. (2016)
Tulasnella sp.	*Goodyera pubescens, Liparis lilifolia, Tipularia discolor*	McCormick et al. (2004)
	Piperia sp.	Whitridge and Southworth (2005)
	E. spectabilis, S. affinis	Chutima et al. (2011)
Tulasnella sp., and *Ceratobasidium* sp.	*Liparis loeselii*	Illyes et al. (2005)
Tulasnella sp., *Epulorhiza* sp., *Nectria mauritiicola*, *Leptontidium orchidicola*	*Disa bracteata*	Bonnardeaux et al. (2007)
Tulasnella sp., *Sebacina* sp., *Russula* sp., *Lactarius* sp., uncultured *Tomentella* sp.	*Gymnadenia conopsea*	Stark et al. (2009)
Tulasnella sp., and *Thanatephorus* sp.	*Neuwiedia veratrifolia*	Kristiansen et al. (2004)
Tulasnella violea	*Thelymitra aristata*	Warcup and Talbot (1971)
	P. villosum	Nontachaiyapoom et al. (2010)
Uncultured Tulasnellaceae	*Gymnadenia conopsea*	Stark et al. (2009)
Uncultured *Ceratobasidium* sp.	*P. nutans*	Irwin et al. (2007)
Uncultured *Tulasnella* sp.	*Cymbidium* (hydrid), *P. villosum*	Nontachaiyapoom et al. (2010)
	Geodorum eulophioides	Downing et al. (2017)
Uncultured Basidiomycetes, *Tomentella*	*Paphiopedilum dianthum*	Downing et al. (2017)
Unnamed Tulasnellaceae	*Epipactis atrorubens*	Shefferson et al. (2008)

(continued)

Table 13.1 (continued)

Orchid life forms Mycorrhizal fungal taxa	Orchid taxa	References
Unnamed Tulasnellaceae	*Orchis militaris*	Shefferson et al. (2008)
Tulasnellaceae	*Epidendrum* sp., *Stelis superbiens*, *Elleanthus* sp., *Elleanthus virgatus, Maxillaria* sp., *Epidendrum lacustre*	Herrera et al. (2018)
Waitea circinata	*Paphiopedilum niveum*	Athipunyakom et al. (2004b)
Wilcoxina sp.	*Epipactis distans*	Bidartondo et al. (2004)

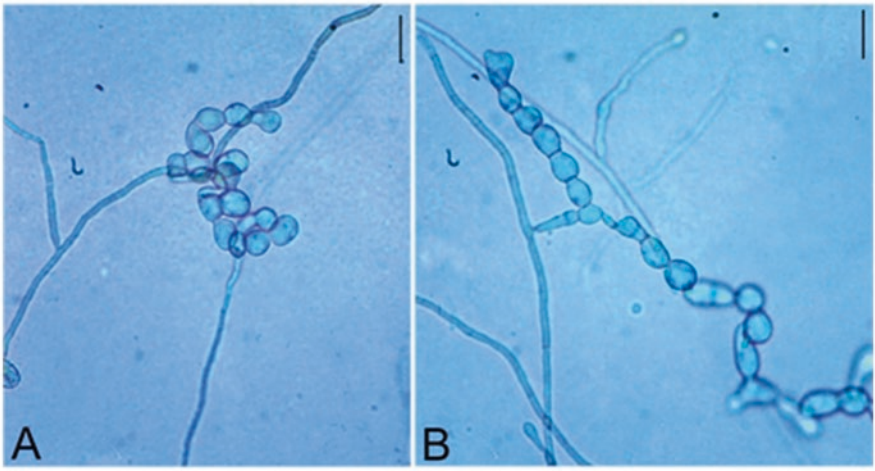

Fig. 13.3 (**a**)–(**b**) Morphology of orchid mycorrhizal fungi isolated from roots of the terrestrial orchid *Tropidia thwaitesii* and cultured in potato dextrose agar medium. (**a**), Hyphae and moniliform cells of *Tulasnella* (**a**) and *Euphlorhiza* (**b**). Scale bars = 20 μm

(Rasmussen 2002). Further, the simultaneous presence of both intact and degraded patches of pelotons in root cortex may represent individual colonization events as adjacent patches may be formed by different fungi (Zelmer et al. 1996). If the nutritional concept is considered over the lifetime of an orchid, different types of fungi could be involved in utilizing different substrates during different periods (Rasmussen 2002). For example, the development of *G. elata* seedlings associated with *Mycena osmundicola* was dependent on its successive association with the parasitic *Armllaria mellea* (Xu and Mu 1990).

The saprotrophs were possibly over represented in previous studies on OMF diversity because of their ability to establish and grow in pure cultures more readily (Rasmussen 2002). Nevertheless, the development of DNA based approaches has

widened the range of endophytic fungi with different nutritional strategies associating with orchids (Rasmussen 2002). In the view of Rasmussen (2002), majority of orchid mycobionts are basidiomycetes. In contrast, 78% of the photosynthetic and non-photosynthetic roots of *Epipactis microphylla* were found to be colonized by the *Tuber* sp., with the remaining roots also being colonized by other ascomycetes and a few basidiomycetes fungi (Selosse et al. 2004). The other potential ascomycetes mycorrhizal endophytes of *Epipactis* spp. in addition to *Tuber* sp., include *Wilcoxina* and *Phialophora* (Bidartondo et al. 2004).

The diversity of OMF associated with photosynthetic terrestrial orchids is generally low due to their high host-mycobiont specificity (McCormick et al. 2004). A phylogenetic analysis of the mycorrhizal association in *Cypripedium* elucidated that, most of the *Cypripedium* fungi formed narrow clades within the family Tulasnellaceae, whereas other root endophytes from Sebacinaceae, Ceratobasidiaceae, and *Phialophora* rarely support the high specificity in photosynthetic orchids (Shefferson et al. 2005). Narrow mycorrhizal specificity was seen in *Stigmatodactylus sikokianus* for *Sebacina* spp. even when the orchid roots were collected from different geographical regions (Yagame and Yamato 2008). The frequent colonization of roots in *Paphiopedilum micranthum*, *Paphiopedilum ameniacum*, *Paphiopedilum dianthum*, *Cypripedium flavum*, *Cypripedium guttatum,* and *Cypripedium tibeticum*, mainly by members of Tulasnellaceae, is a clear proof for the narrow phylogenetic range of mycorrhizal taxa in terrestrial photosynthetic orchids (Yuan et al. 2010). Further, the diverse OMF isolates colonizing these orchids belonged to the four discrete groups of the genus *Epulorhiza*. However, *P. nigricans* was colonized by the widest diversity of fungi including isolates of *Epulorhiza* and *Ceratobasidium* suggesting that this orchid could potentially benefit from this diverse association (Bonnardeaux et al. 2007).

The simultaneous colonization of *D. officinale* by *Tulasnella* spp., *Epulorhiza* sp., and *Plateus seticeps* and association of *Dendrobium fimbriatum* with *Tulasnella* sp., in certain orchid populations (Xing et al. 2013) suggest that co-occurring orchid species could benefit from different mycorrhizal partners (Waterman et al. 2011). However, this needs further confirmation as the small sample size of *Dendrobium* could have led to an underestimation of the fungal diversity (Xing et al. 2013). Twenty seven isolates of *Tulasnella calospora*, *Tulasnella pruinosa*, *Tulasnella asymmetrica*, *Tulasnella irregularis*, *Epulorhiza* sp., *Tulasnella violea,* and uncultured *Tulasnella* were associated with the orchid genera *Paphiopedilum*, *Dendrobium,* and *Cymbidium* (Nontachaiyapoom et al. 2010). In a study on the mycorrhizal diversity of *Coppensia doniana*, all the isolated OMF belonged to the genus *Ceratobasidium* which explains the wide potential habitats of this orchid, as these fungi can gain energy from organic matter in soil, litter, tree barks, and rock surfaces (Valadares et al. 2011).

The diverse group of fungal associates, such as typical mycorrhizal fungi from Tulasnellaceae, Ceratobasidiaceae, and ECM taxa of Pezizales colonizing the terrestrial orchid *Gymnadenia conopsea* contributes to its ability to colonize different habitat types (Stark et al. 2009). Edaphic conditions are also known to play an important role in the distribution of OMF colonizing roots of terrestrial orchids. For

example, *Tulasnella prima* is the major mycobiont colonizing roots of several *Chiloglottis* species growing in soil and sphagnum hammocks over a range of more than 1000 km in southeastern Australia (Ruibal et al. 2017). In contrast *Tulasnella sphagneti* associated with *Chiloglottis* is rather restricted to the wet conditions of alpine sphagnum hammocks (Ruibal et al. 2017).

13.5.2 OMF Diversity Epiphytic Photosynthetic Orchid

In epiphytic orchids, low levels of patchy mycorrhizal colonization could be seen in roots attached to substratum (Bermudes and Benzing 1989; Pereira et al. 2005). Twenty-four *Epulorhiza* isolates were obtained from the roots and protocorms of *Oncidium*, *Vanda*, *Arachnis*, *Dendrobium*, *Arundina*, *Diplocaulobium,* and *Spathoglottis* examined from different sites in Singapore (Ma et al. 2003). The Andean epiphytic orchids like *Stelis concinna*, *Stelis hallii, Stelis superbiens,* and *Pleurothallis lilijae* were found to be colonized by *Tulasnella* spp. based on the septal ultrastructure, whereas the molecular analysis of the roots yielded seven distinct *Tulasnella* clades (Suarez et al. 2006). All the *Tulasnella* sequences were distinct from the already known sequences of mycobionts in certain terrestrial orchids and this indicated the adaptation of these fungi to tree stems and also its importance in orchid growth (Suarez et al. 2006).

Twenty-six isolates of OMF were identified from the tropical epiphytic orchids which formed two fungal lineages, related to *Ceratobasidium* spp., of which majority of orchids hosted more than one fungal lineage suggesting the variation in mycobiont association within related orchid species (Otero et al. 2002). Mycobionts from *Tolumnia variegata* clade with four fungal lineages of which only one lineage include fungi from *Ionopsis utricularioides* (Otero et al. 2002) and in a later study the same orchid was seen colonized with *Ceratobasidium* suggesting that *I. utricularioides* is specialized in effectively exploiting a specific fungal clade (Otero et al. 2004). The broad mycobiont specificity of *I. utricularioides* explains its survival in a broad geographical range and also its large population size (Otero et al. 2004). The rare epiphytic orchid *Sarcochilus weinthalii* was seen associated with single species of *Ceratobasidium* and direct sequencing of colonized root fragments and culture dependent methods also indicated its narrow specificity (Graham and Dearnaley 2012). In China, four epiphytic endemic orchids (*Laelia autumnalis*, *Laelia speciosa*, *Euchile citrina*, and *Prosthechea squalida*) harbored 71 isolates of fungal endophytes belonging to 20 genera of basidiomycetes and ascomycetes indicating the rich diversity of OMF association in these orchid hosts (Beltrán-Nambo et al. 2018). In south Ecuador, the epiphytic orchids colonized by 115 fungal isolates of 49 fungal operational taxonomic units (OTUs,) including four mycorrhizal OTUs belonging to Ceratobasidiaceae and Tulasnellaceae revealed high diversity of fungi colonized with orchid roots (Novotna et al. 2018).

13.6 Taxonomy of OMF

In the past, majority of the peloton forming fungi were classified as a member of anamorphic form-genus *Rhizoctonia* based on morphological characteristics (Athipunyakom et al. 2004b). The genus concept in *Rhizoctonia* was first established by De Candolle (1815). Earlier the form-genus *Rhizoctonia* consisted of a heterogenous assemblage of filamentous fungal taxa that failed to produce sexual spores and only share limited common features in their anamorphic stage (Garcia et al. 2006). However, new morphological criteria include nuclear number, septal nature, and parenthesome perforation, as well as teleomorphic stage for OMF (Moore 1987). Based on these, three new genera such as *Ceratorhiza*, *Epulorhiza*, and *Moniliopsis* were separated from *Rhizoctonia*. Ma et al. (2003) characterized the *Rhizoctonia* isolates using different media and colony hyphal characteristics, and identified them as species belonging to *Epulorhiza*. Basidial morphology was considered to be a reliable criterion for characterizing *Rhizoctonia* species at morphospecies level (Warcup and Talbot 1966, 1971, 1980), as fungal isolates from orchid roots only occasionally forms fruiting bodies in pure culture (Currah et al. 1987, 1990; Milligan and Williams 1988). In addition, the septal ultrastructure characteristics are also considered important for distinguishing the *Rhizoctonia* taxa (Khan and Kimbrough 1982; Marchisio et al. 1985; Currah and Sherburne 1992).

A system of anastomosis grouping based on hyphal fusion was widely adopted until last decade, as the basis for recognizing groups and taxa among the several fungi that constitute form-genus (Sneh et al. 1991). Currently, there are several accepted classifications based on the anastomosis group concept for both multinucleate (*Ceratobasidium*) taxa within the *Rhizoctonia* species complex (Carling 1996). *Rhizoctonia*-like mycobionts from Australian orchids though formed five anastomosing groups; the molecular sequencing showed that most of the anastomosing groups were monophyletic (Ramsay et al. 1987). Therefore, hyphal anastomosis behavior may not be the best indicator of evolutionary relationships between different intraspecific groups (Vilgalys and Cubeta 1994).

Later, the developments of biochemical methods were thought to be better to infer the phylogenetic relationships of fungi in *Rhizoctonia* species complex (Jabaji-Hare 1996). The biochemical methods for characterizing *Rhizoctonia* species include soluble protein patterns, zymograms and isoenzyme profiles which were employed to identify and study the genetic relationships among members of the form-genus (Jabaji-Hare 1996). Damaj et al. (1993) studied the relationship between binucleate *Rhizoctonia* isolates by isoenzyme electrophoresis. Grouping and identification of binucleate *Rhizoctonia* by pectic zymograms distinguished five zymographic groups in *Ceratobasidium cornigerum* (MacNish et al. 1993).

As induction of teleomorphic stages under laboratory conditions has been difficult for *Rhizoctonia*, their characterization is primarily based on the comparison of a limited number of anamorphic features and cytological probes (Moore 1987; Andersen and Stalpers 1994; Roberts 1999). Recently, molecular identification of fungi involving polymerase chain reaction (PCR) amplification of the nuclear ribosomal internal transcribed spacer (ITS) has revolutionized the identification of OMF

(Currah et al. 1995; Gardes and Bruns 1993; Redecker 2000; Vralstad et al. 2002). Internal transcribed spacer region is the most effective single loci for the identification of fungi from species to genus levels (Bruns 2001; Seifert et al. 2007). Gardes and Bruns (1993) suggested that ITS region has certain advantages in fungi, as the whole ITS region has 600–800 bp and could be easily amplified with universal primers (White et al. 1990). Amplification of ITS gene from small, dilute or highly degraded samples is easy because of its multicopy nature of the rDNA repeats. Earlier studies (Gardes and Bruns 1991; Chen et al. 1992; Lee and Taylor 1992) demonstrated that the ITS region is often variable among morphologically distinct fungal species. Gardes and Bruns (1993) also developed a taxon specific primer for amplification of ITS rRNA gene. Based on these developments, the presence of *Tulasnella* and *Laccaria* in roots of *Dactylorhiza majalis* was detected by amplifying the mitochondrial large subunit (Mt-Lt) RNA gene (Kristiansen et al. 2001). The endophytes colonizing *Neuwiedia vetrifolia* roots were identified as belonging to Tulasnellales and Ceratobasidiales by amplification and sequencing of the mitochondrial ribosomal large subunit DNA (Kristiansen et al. 2004). The OMF endophytes of *Acianthus*, *Caladania,* and *Pterostylis* were identified using ITS-restriction fragment length polymorphism (RFLP) analysis from Queensland (Bougoure et al. 2005). Pereira et al. (2005) suggested that random amplification of polymorphic DNA (RAPD) analysis might reveal higher polymorphism between *Epulorhiza epiphytica* and *Epulorhiza repens* than found in the PCR-RFLP analysis. Furthermore, RAPD and morphological analysis indicated a degree of relatedness among the *Ceratorhiza* isolates obtained from the roots of different *Oncidium* species (Pereira et al. 2005).

Though, the presence of *Epulorhiza* in the roots of several tropical orchids was confirmed by Ma et al. (2003) using amplification of rDNA repeat from 3′ end of the 18S rDNA gene and 5′ end of the 28S rDNA gene, the use of PCR based approaches for the amplification of OMF endophyte has certain limitations. For example, taxa belonging to Tulasnellaceae are difficult to characterize using standard PCR primer sets in spite of their common presence in orchid roots. This shortfall arises from the high evolution of the nuclear ribosomal operon (Binder et al. 2005; Moncalvo et al. 2006) and subsequent mutations of the conserved regions to which the primers hybridize (Taylor et al. 2002). Electron microscopic examination of the fungal pelotons of several epiphytic Pleurothalline orchids growing in the Andes revealed the predominance of the fungi belonging to Tulasnellaceae (Suarez et al. 2006). Nevertheless, only few of these fungi could be amplified using standard primers. But, true mycorrhizal symbionts of these orchids started to emerge only when nested PCR and several *Tulasnella* specific primers were used. Therefore, Taylor and McCormick (2008) developed a new set of selective primers with certain advantages like, low amplification of plant sequences and improved amplification of all tested Basidiomycota nuclear ribosomal DNA, including *Tulasnella*. Since then, specific primers have been routinely used to identify the mycobionts of orchids like *Cypripedium*, *Papilopedium*, *Dactylorhiza,* and *Dendrobium*. These types of sequencing analyses have revealed the association of a wide range of fungi belonging to Tulasnellaceae, Pluteaceae, and Ceratobasidiaceae with orchids (Shimura et al. 2009; Yuan et al. 2010; Valadares et al. 2011; Pellegrino and Bellusci 2009;

Xing et al. 2013). The various mycorrhizal fungi isolated from the orchid roots along with their origin are shown in Table 13.1.

13.7 Symbiotic Seed Germination

13.7.1 Fungal Preference

Orchids have been well known for their exclusive association with fungi. Generally, orchids have minute seeds with a very small embryo without an endosperm. Therefore association with fungi is a prerequisite for orchid seed germination, as the germinating seeds are incapable of obtaining nutrients successfully independent of fungi (Arditti 1992; Uetake et al. 1992). Bernard (1899) first described the role of mycobionts in orchid seed germination. Different isolates of fungi are known to differ in their potential to stimulate the germination of orchid seeds which tempted Warcup (1973) to suggest that the most efficient fungal isolates need not have its origin from the same host. This suggestion was also supported by the study of Stewart and Zettler (2002) where the terrestrial orchid *Habenaria macroceratitis* was non-specific in its requirement for the fungal mycobiont. Nevertheless, Stewart and Kane (2006) in a later study speculated that *H. macroceratitis* could have a certain degree of fungal preference as the germination percentage of the seeds was higher in the presence of fungi isolated from orchid populations from which the seeds were collected compared to those isolated from other orchid populations. The existence of fungal preference is also evident in another study (Stewart and Zettler 2002) where *Epulorhiza* strains isolated from *Habenaria quinqueseta* promoted seed germination of *Habenaria repens* better than those isolated from *Epidendrum conspseum*, *Spiranthes brevilabris,* and *H. macroceratitis*. In the same study, Stewart and Zettler (2002) also showed that the *in vitro* raised seedlings of *H. repens* growing in different potting media and colonized by *Epulorhiza* strains isolated from *S. brevilabris* survived better compared to those colonized with *Epulorhiza* strains isolated from *Epidendrum conopseum*. Similarly, *Cymbidium aloifolium* inoculated with OMF *Ceratobasidium* strain RR isolated from distant orchid taxa (*Rhynchostylis retusa*) promoted the number, length, leaf thickness, root number and length, and biomass (Hossain et al. 2013). In addition, *Epulorhiza* sp., isolated from terrestrial orchid *Eulophia epidendraea* promoted the seed germination and seedling development of epiphytic endemic orchid *Coelogyne nervosa* (Sathiyadash et al. 2014). In a recent study *Ceratobasidium* species isolated from the roots of adult ghost orchid *Dendrophylax lindenii* was able to promote seed germination and seedling development of the same orchid (Mújica et al. 2018).

13.7.2 Fungal Specificity

An understanding on the specificity of OMF-orchid interactions is of crucial importance both for ecology and conservation of orchids. Seed germination, protocorm development, and seedling growth are stimulated by digestion of orchid mycobionts

and subsequent uptake of the released nutrients by the immature orchid embryo (Clements 1988; Rasmussen 1995). Widely distributed orchids are expected to be either general in their preferences for mycorrhizal fungi or could be specific in associating with a broadly distributed fungus, as in many mutualistic relationships (Bascompte et al. 2003; Vazquez and Aizen 2003). In contrast, a narrow OMF specificity could be the reason for rarity and vulnerability of certain orchid species. Orchid-mycobiont specificity was considered controversial for many years. Earlier researchers thought that the orchid-fungus relationship to be quite natural and non-specific both under *in vitro* and *in situ* conditions (Knudson 1922; Curtis 1939; Hadley 1970; Masuhara and Katsuya 1989; Masuhara et al. 1993).

To assess the orchid-mycobiont specificity under *in vitro* conditions, symbiotic seed germination techniques are often considered to be more useful (Dixon 1987; Zettler 1997a, b; Stewart and Kane 2006). The seed germination efficiency of terrestrial orchids are often low (Stewart et al. 2003; Zettler et al. 2005; Stewart and Kane 2006) compared to asymbiotic seed germination for the same taxa. This low seed germination efficiency is often attributed to the existence of certain degree of orchid-mycobiont specificity as the mycobionts colonizing terrestrial orchids during seed germination and later stages could be entirely different (Stewart and Kane 2007).

Spiranthes brevilabris although non-specific for mycobionts, fungal isolates Econ 242 (*Epidendrum magnoliae*) and Sbrev-266 (*S. brevilabris*) induced seed germination and development of the seedlings better than the several isolates tested (Stewart et al. 2003). *Spiranthes cernua* seeds successfully developed into leaf bearing stage when colonized by the mycobionts originating from *Platanthera ciliaris* suggesting that the mycobiont specificity could rarely be species-specific (Zettler and McInnis 1993). In contrast, *in vitro* seed germination of *Bipinnula fimbriata* showed the lack of specificity for mycobionts, as the seed germination was stimulated by all the *Rhizoctonia*-like mycobionts isolated from the adult orchids in its habitat (Steinfort et al. 2010). Similarly, *Ceratobasidium* isolated from *Chloraea crispa* of different habitat promoted protocorm development in *B. fimbriata* (Steinfort et al. 2010). Hadley (1970) also observed that the four isolates of *Tulasnella calospora* originating from wide geographical regions exhibited almost similar patterns of symbiotic response in *Coeloglossum*, *Dactylorhiza*, *Goodyera*, *Cymbidium*, *Epidendrum*, *Laeliocattleya,* and *Spathoglottis* (Hadley 1970). Nevertheless, *Thanatephorus orchidicola* originating from *Dactylorhiza elata* and *Coeloglossum viride* failed to form symbiosis with any of the orchids tested (Hadley 1970). As the mycobionts isolated from particular host was equally symbiotic with other orchid hosts, further, the orchid-fungus relationship does not appear to be also completely random, as certain orchids tend to be more receptive to certain endophytes than others (Hadley 1970).

Isolates of *Sebacina vermifera* and *Tulasnella calospora* originating from *Caladenia* and *Diuris* stimulated seed germination of the same host expressing the existence of a genus level specificity (Warcup 1971). Similarly, *Ceratobasidium* sp., (strain VT3) isolated from epiphytic orchid *Vanda thwaitesii* efficiently promoted seed germination, protocorm, leaf, and up to root formation stage in same taxa (Decruse et al. 2018). In addition, the three co-occurring orchid species *Anacamptis morio*, *Gymnadenia conopsea,* and *Orchis mascula* shared a low number of OMF

OTUs, explaining the high specificity in OMF associations (Waud et al. 2016). Further, although *S. vermifera* stimulated the seed germination of *Cladenia*, *Glossodia* and *Elythranthera* (sub tribe Caladeniinae) failed to stimulat seed germination in *Eriochilus*, *Leporella*, *Acianthus* sp., *Microtis,* and *Parsophyllum,* thereby exhibiting specificity at subtribe level (Warcup 1981). The failure of the mycobionts isolated from *Spiranthes floridana* to support the seed germination and seedling development of *S. brevilabris* suggests that the genus *Spiranthes* failed to share the mycobionts during seed germination (Stewart and Kane 2007). Shefferson et al. (2005) also observed high mycobiont specificity at genus level occurring in the terrestrial orchid *Cypripedium*. Similar observation was made in the non-photosynthetic *Hexalectris* spp. colonized by *S. vermifera* (Taylor et al. 2003).

13.7.3 Cold Treatment

A delay in germination due to polymorphism may be an inherent characteristic of orchid seeds of temperate origin preventing the simultaneous germination of the seeds (Baskin and Baskin 1998). In natural habitats, seeds of *Platanthera praeclara* germinate during the first spring following dehiscence of capsule and seed dispersal, but further development of the protocorms were delayed until exposed to one or more winters (Sharma et al. 2003). So seeds subjected to cold treatment appear to be necessary to break seed dormancy in orchids.

The duration of the cold stratification may vary within a genus and may be species specific. Terrestrial orchids may require 3–9 months of cold stratification which may be species specific (Rasmussen 1995). Seeds of *P. leucophaea* require a cold stratification of 2–11 months for germination and the protocorms required chilling to transform into leaf-bearing seedlings (Stoutamire 1996; Zettler et al. 2001). In addition, cold stratification for 4 months combined with colonization by *Ceratorhiza* or *Ceratorhiza* and *Epulorhiza* resulted in higher percentage of protocorms advancing to the next stage (stage 3, top shaped protocorm and appearance of leaf) and proceed further. However, cold treatment for 6 months along with mycorrhization resulted in higher percentage seedlings transforming from stage 3 to 4 (elongation of the leaf promordium and protocorm enlargement) (Sharma et al. 2003). Higher seed germination percentage was observed in *Cypripedium macranthos* inoculated with mycorrhizal fungi after cold treatment (Shimura and Koda 2005).

13.7.4 Light

Orchid seeds possess a hydrophobic testa which maintains the seeds even the soil surface and exposing them to sunlight (Stewart and Kane 2006). Light exposure often initiates nutrient mobilization and therefore may not be directly involved in seed germination (Rasmussen and Rasmussen 1991). The effect of light on orchid seed germination may be genus or species specific (Stewart and Kane 2006). Initiation of photosynthesis occurred in *in vitro* raised *Habenaria repens* seedlings

when exposed to light for 1 week. Similarly, exposure of *Habenaria quinqueseta* seeds to 33 days light increased its germination percentage (Stewart and Zettler 2002). Seedlings of *Platanthera praeclara* in stage 5 (formation of root initial) developed their first green leaf only after exposure to illumination for 30 days. The leaves grew up to 8 cm upon exposure to light for 50 days (Sharma et al. 2003). Transfer of *H. macroceratitis* seeds from dark to a 15 h photoperiod enhanced seed germination and early protocorm development (Stewart and Zettler 2002). The influence of photoperiod on seed germination is evident from the fact that exposure of *H. macroceratitis* seeds to 16–24 h of photoperiod increased the seed germination than those exposed to continuous dark. Further, a 16 h photoperiod appear to be optimum for symbiotic seed germination of *H. macroceratitis* (Stewart and Kane 2006). In contrast, protocorm development tends to peak under dark conditions (Stewart and Kane 2006). However, protocorms of *Eulophia alata* developed into plantlets only on exposure to 16 hour photoperiod (Johnson et al. 2007). Takahashi et al. (2000) failed to find any significant effect of light on the germination of *Habenaria radiata* seeds exposed to either continued darkness or light conditions.

13.8 Role of OMF Specificity in Orchid Rarity

All orchids depend on mycorrhizal fungi during seed germination and adulthood in the wild (Porras-Alfaro and Bayman 2007). As most of the orchid seeds are microscopic (Kull 2002), they lack stored nutrients to support germination (Rasmussen 2002). When the orchid seeds are colonized by compatible fungi, the germination of seed is initiated by utilizing the fungal sugars and this myco-heterotrophic condition is retained into adulthood in many orchids (Gill 1989). Therefore, mycorrhizal associations are a prerequisite for seed germination and seedling growth of orchids in natural habitats.

The rarity of many orchid species around the globe could be attributed to the decline in the occurrence of mycorrhizal fungi, as most of the orchids appear to have certain degree of specificity for certain mycobionts at the time of germination and during later life stages (Stewart and Kane 2007). A study on the specificity and preference of mycorrhizal associations in two species of the genus *Dendrobium* suggested that *D. officinale* associate with a wide range of basidiomycetes, while *D. fimbriatum* had a high degree of specificity toward *Tulasnella* (Xing et al. 2013). The rare status of the Florida terrestrial orchid *Spiranthes brevilabris* could be due to the high mycobiont specificity of this orchid (Stewart and Kane 2007). Tropical epiphytic orchids found that *I. utricularioides* was highly specialized in effectively utilizing a specific fungal clade, *Ceratobasidium* (Otero et al. 2004). In contrast to these, the wide distribution of *S. cernua* can be partially associated with its low-species specificity (Zettler and McInnis 1993).

The orchid-fungal specificity may be at genus or even at species levels. In the terrestrial orchid *Cypripedium*, high mycobiont specificity occurs at generic level (Shefferson et al. 2005). Of the seven species of *Cypripedium* examined, five species had mycobionts belonging to Tulansnellaceae (Shefferson et al. 2005). *Dactylorhiza majalis* predominantly associated with Tulasnellaceae showed

occasional colonization by members of the genus *Laccaria* (Kristiansen et al. 2001). Irrespective of the nutritional mode, genus level specificity was seen in three varieties of non-photosynthetic orchid *Hexalectris spicata* with Sebacinaceae, whereas, species-specific mycobiont specificity was also reported in *Hexalectris* (Taylor and Bruns 1999). The mycorrhizal fungi may limit orchid distribution if they had genus or species specific for certain mycorrhizal fungi.

Fungal specificity is also influenced by certain factors like growth conditions, variety, and life stage of orchids. The fungi that can associate with an orchid in natural habitat may not necessarily germinate orchid seeds *in vitro* (Masuhara and Katsuya 1994). The fungal specificity in natural conditions is termed as ecological specificity and *in vitro* as potential specificity. Fungi may also differ in their ability to grow under different ecological conditions. A study on the tropical orchids, *Tolumnia variegata* and *I. utricularioides,* showed that orchid species with overlapping habitat preferences may differ in mycorrhizal specificity which could influence their distribution (Otero et al. 2004). The varied mycorrhizal diversity and specificity among the different taxa of *Lecanorchis* could be due to the diverse climatic conditions in which they exist (Okayama et al. 2012). Therefore, the patchy distribution of orchids in nature may be due to the presence or absence of the specific mycorrhizal fungi essential for their survival.

The mycorrhizal fungi colonizing the protocorm and the adult roots might not be the same. For example, Milligan and Williams (1988) found that *Epulorhiza* sp., initiated germination and *E. repens* was seen associated in the later stages of *Microtis parviflora.* Pre-infection with one fungus appears not to preclude colonization of a second endophyte. Even though adult *Tipularia discolor* had multiple fungal partners at the adult stage, its protocorm stage is more fungal specific associating with only taxa belonging to Tulasnellaceae (McCormick et al. 2004). But in *Liparis liliifolia*, both protocorms and adult plants fail to associate with multiple mycobionts (McCormick et al. 2012). Bidartondo and Read (2008) hypothesized from their studies on *Cephalanthera* and *Epipactis* that the mycobiont specificity is high during early stages of seedling development compared to more promiscuous germination and mature stages of plants life cycle. Isolation of four distinct types of fungi from *H. spicata* var. *spicata* and *H. spicata* var. a*rizonica* gives evidence for the contribution of mycobiont specificity to the evolutionary diversification in orchids (Taylor et al. 2003). But the orchid *Drakaea* which is widely distributed in different environments, in spite of its preference for *Tulasnella* sp. (Phillips et al. 2011), raises the question of host-fungus specificity within the Orchidaceae which has been a point of contention for many years.

13.9 Importance of OMF in Conservation and Restoration of Orchids

Threat to the survival of orchids in nature is alarming, in spite of the significant advances in our understanding of the ecology of orchids (Gale et al. 2018). It is important to note that only 3.3% of the global natural orchid flora has been subjected to IUCN Red List assessments and 56.5% of these are under one or the other

threat categories (Govaerts et al. 2017; IUCN 2017). Some of the common threats to natural populations of orchids include deforestation, expanding agricultural and forest plantations, and unsustainable exploitation of plants for food, medicine, and ornamental purposes (Gale et al. 2018). Reintroduction of orchids into their natural habitats is a strategy often adopted in the conservation of the threatened species (Vallee et al. 1997). Although orchid seedlings from asymbiotic process have been transferred to ex vitro conditions, they rarely survive due to the biological and ecological specificities of the taxa. The long term survival of orchids in managed or restricted habitats require the presence of appropriate fungi for seedling recruitment and plant nutritional support (Zettler and Piskin 2011).

Asymbiotically raised economically important endangered orchid *Vanda coerulea* exhibited a high mortality (>90%) when introduced into their natural habitat (Aggarwal et al. 2012). But, a successful protocol for the reintroduction of *V. coerulea* seedlings into their natural habitat was developed using *Rhizoctonia* isolated from the same orchid. Anderson (1991) found a 100% survival for all the symbiotic seedlings of *Spiranthes magnicamporum* upon their transfer to soil compared to the 5% survival for the seedlings raised asymbiotically (Anderson 1991). Successful application of symbiotic seed germination using *Ceratobasidium* for reintroduction of endangered terrestrial orchid, *Dactylorhiza hatagirea* has been reported (Aggarwal and Zettler 2010). The reintroduction of endangered orchids, *S. brevilabris* and *H. repens* was highly successful when mycorrhizal with *Epulorhiza* sp. (Stewart et al. 2003). So the symbiotic technique has practical merit for the conservation of highly important and endangered orchids.

Ecological changes at designated orchid locales may destroy the target mycorrhizal species and the compatible fungi may not be present at the site if the orchid itself is not present. So the symbiotically associated seedlings can serve as both plant material and a source of mycorrhizal inoculum for reintroduction efforts. Reintroduction of orchid seedlings from different habitats could be detrimental as this could alter the gene pool of the resident orchids which could eventually initiate anthesis (Zettler et al. 2005). In a recent study, the success of the out planted *in vitro* raised *Cypripedium calceolus* seedlings in the conservation programme in England was shown to be dependent on establishment of mycorrhizal symbiosis by the introduced plants and the presence of suitable mycorrhizal fungi at the introduced sites (Fay et al. 2018).

13.10 Conclusion

OMF colonize orchids and make the orchids survive better in various habitats. Terrestrial orchids show high diversity of OMF fungi, likewise epiphytic orchids are colonized by diverse groups of fungi. OMF is a crucial factor in the reintroduction of orchids and restoration of orchid populations in their natural habitats. So, identification of OMF diversity at specific sites provides the potential OMF which stimulates seed germination and seedling recruitment. This should be used for orchid conservation program in particular habitats.

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Orchid Seed Ultrastructure: Ecological and Taxonomic Implications with Reference to Epidendroideae (Orchidaceae)

14

J. Ramudu, S. M. Khasim, and G. Ramesh

Abstract

Ultrastructure of orchid seed belonging to subfamily Epidendroideae (Orchidaceae) has been discussed. Orchid seeds are tiny and microscopic and produced lakhs in a single pod. In *Cycnoches ventricosum* it contains four million seeds. Since orchid seeds are non-endospermic, very few seeds germinate successfully and give rise to mature plant. The present paper deals with quantitative data related to the length and width of seed and embryo, percentage of airspace and number of testa cells. It is evident from the present study that *Cymbidium* spp. showed higher values of seed volume/embryo volume compared to the vandoid genera whereby higher percentage of airspace had been recorded in cymbidiums. Hence *Cymbidium* deeds are more buoyant and widely distributed throughout Indo-Malayan region. Based on seed morphometry, *Pholidota* is closely allied to *Coelogyne.* Similarly *Oberonia* and *Malaxis* show close affinity with *Vanda*.

Keywords

Orchid seed · SEM studies · Epidendroideae · Functional adaptations · Taxonomic implications

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14.1 Introduction

The Orchidaceae is one of the largest families of flowering plants. It comprises about 28,484 species all over the world (Govaerts et al. 2017). In India, with 1350 species, it represents the second largest flowering plant family and contributes about 10% of Indian flora (Kumar and Manilal 1994; Jalal and Jayathi 2012). Orchid seeds are light in weight and the tiniest among the seeds produced by flowering plants, and these are non-endospermic and vary considerably in their size, shape, morphology and colour, and minute details and morphological characters have got tremendous ecological significance. In majority of orchid species, seed size shows variation from 300 to 800 μm (Molvray and Kores 1995). Orchid seeds exhibit a wide range of diversity in their sizes (ranging from 0.1 mm in *Oberonia* to 6 mm in *Epidendrum*) and shapes but also complexity of light weight seed coat architecture and hierarchical surface sculpturing (Barthlott 2014). The taxonomic significance of the seed characteristics was first reported by Clifford and Smith (1969). Besides, serving as taxonomic markers, the morphological characters of seeds can be used to deduce phylogenetic relationship (Barthlott 1976) and to identify their involvement in hybrid genotypes (Arditti et al. 1979). The present paper deals with an overview on seed ultrastructure with reference to subfamily Epidendroideae (Orchidaceae).

Orchid seeds show the remarkable diversity not only in their sizes (from 0.1 mm in *Oberonia* to 6 mm in *Epidendrum*) and shapes but also in the complexity of light weight seed architecture and surface sculpturing (Barthlott et al. 2014). Verma et al. (2014) studied the seed physical characteristics of 32 Western Himalayan orchids (threatened) using light and scanning electron microscopy; they stressed the importance of seed characteristics in elucidating the taxonomic and phylogenetic interrelationships. Seed morphology has got importance in delineation of species within the genus and also delineation of subgeneric groups (Matthews and Levins 1986; Ness 1989; Vij et al. 1992; Larry 1995; Augustine et al. 2001). Molvray and Kores (1995) also reported that the orchid seed varies in shape from filiform to fusiform, clavate to ellipsoidal and oftenly prominently winged. Barthlott and Ziegler (1981) worked elaborately on the seed coat structure of orchids. In their study, they have recognized 20 different seed types by taking varying seed characteristics.

The taxonomic importance of seed characteristics was first pointed out by Clifford and Smith (1969); later Dressler (1981) proposed several classifications based on conventional micromorphological characters. Matthews and Levins (1986) and Larry (1995) opined that seed micromorphology serves as a source of systematic characters to circumscribe subgeneric groups or hypothesize relationship among species within the genus.

Based on SEM studies of orchid seeds, Barthlott (1976) concluded that the morphological characters can be used to deduce phylogenetic relationships. Arditti (1979) also opined that seed volume in orchids reflects their size of seeds. Arditti et al. (1980) revealed that L/W (length/width) ratio of seed provides some very important information on the relative degree of truncation of orchid seeds. Arditti et al. (1980) and Augustine et al. (2001) found that seed testa cells show reticulation; if reticulation is present, the pattern may be varied from genus to genus.

Barthlott and Ziegler (1981) worked on the shape of the orchid seed. They had recognized 20 different seed types based on shape, testa cells length, sculpturing pattern of testa cells, presence of intercellular gaps and beading. An extensive review on orchid seed and their taxonomic significance was given by Barthlott (1976), Tohda (1986), Chase and Pippen (1988, 1990), Kurzweil and Weber (1991), Petersson (1991) and Arditti and Ghani (2000).

Vij et al. (1992) had done substantial work on orchid seeds and opined that the seed protected with the thickening and sculpturing pattern had varied from habitat to habitat. They also observed that the thickenings are thin in terrestrial orchids whereas thick in epiphytic orchids.

Kurzweil (1993) had done pioneering work on seed micromorphology using SEM in South African Orchidaceae and classified them into two seed types: (1) *Satyrium* type in which orchid seed testa cells have straight or slightly undulate and thickened anticlinal cell walls and (2) *Disa uniflora* type in which seed coat consists of convex cells with undulate anticlinal walls.

Molvray and Kores (1995) observed that seeds are covered by hard coat made up of testa cells and embryo loosely arranged and papery in texture. Augustine et al. (2001) also made SEM studies on *Bulbophyllum* seed micromorphology.

Swamy et al. (2004) studied the seed micromorphometry of orchids of Karnataka using SEM and found that presence of twisted ropelike testa cells in both *Aerides maculosa* and *Xenikophyton smeeanum* shows close affinity in these species. Sharma et al. (2004) studied the seed morphometry of *Paphiopedilum* spp. using SEM and reported spindle-shaped seeds in this genus. Swamy et al. (2004) also studied based on SEM of orchid seeds of Western Ghats of Karnataka and concluded that maximum relative degree of truncation is found in *Coelogyne breviscapa* and minimum in *Eria dalzelli.* They also studied SEM studies of orchid seed of Western Ghats and found that embryo volume can change during its development from zygote to seedlings; the seeds with higher percent of airspace get dispersed over wide geographical areas.

Gamarra et al. (2007) have been done pioneering work on seed micromorphology of genus *Neottinae* (Orchidaceae) and observed that all seeds are fusiform in shape with transverse ridges on the inner periclinal walls, which is the characteristic feature of genus *Neottinae.*

Gamarra et al. (2007, 2008, 2012) extensively studied the seed micromorphology in subtribe Orchidinae and found that sculpturing pattern of testa cells plays a significant role in identification of orchid species. Aytasakcin et al. (2009) studied seed morphometry of Turkish orchids. They were of opinion that some of the seed characters such as seed length, testa cell reticulation, seed volume/embryo volume and airspace are useful for taxonomy of Orchidaceae.

Verma et al. (2014) studied the seed micromorphometry of threatened orchids of Western Himalaya based on SEM and observed different seed shapes such as fusiform, spathulate, ovoid and filiform shaped seeds. They were of opinion that the embryo was tiny and most of the seed occupied with 79% airspace is found in Himalayan epiphytic orchids. The highest seed volume/embryo volume ratio is found in terrestrial orchids.

Chaudhary et al. (2014) studied the comparative seed micromorphology in *Dendrobium* (Orchidaceae) and concluded that species from temperate region have larger seed volumes and seed volume/embryo volume ratio than species from subtropical or tropical regions. They also reported maximum airspace in tropical and subtropical dendrobiums compared to temperate species. Brzosko et al. (2017) studied the seed dispersal in some terrestrial orchids in Biebrza National Park, North East Poland.

14.2 Seed Micromorphology

14.2.1 General Seed Micromorphological Features and Functional Adaptations in the Orchidaceae

Orchid seeds are unique in their tiny nature and produced in large numbers, ranging from 1300 to 10,00,000 per capsule (Garg et al. 1992) and even in a single pod of *Cycnoches ventricosum* that contains four million seeds (Arditti and Ghani 2000). Due to lack of endosperm, all seeds will not germinate, out of which very few seeds germinate whenever the fungal infection and other specific requirement is available (desired habitat and substrate); even in seedling stage itself, most of individuals will die. There is a heavy mortality at any stage of seedling development; very few seedlings successfully grow and give rise to mature plant. In order to cope with this problem, orchids have been developed some adaptive features such as occurrence of lakhs of seeds in a pod, minute seeds, extremely light in weight, etc. The seeds differ from those of the most other angiosperms and resemble the so-called dust seeds of other plants (Molvaray and Chase 1999; Arditti and Ghani 2000). The dustlike seeds (Figs. 14.1 and 14.2), a significant character exhibited by orchids, are well suited for long-distance dispersal by wind.

Seed Shape The SEM studies revealed that the seeds of all studied taxa are very minute and these varied in their shape and size (Figs. 14.1 and 14.2). Seed shape varies from quadrilateral in *Malaxis densiflora* and fusiform in *Cymbidium aloifolium* to elongated in *Coelogyne nervosa* and shorter with bulged central part in *C. nitida* (Fig. 14.1a–d). In the case of *Calanthe triplicata*, they are filamentous, whereas in *Vanda tessellata*, ropelike appearance is seen (Fig. 14.2a, b). Spindle shape has been reported in *Oberonia* and *Pholidota* (Fig. 14.2c, d). The seed shape of studied taxa varies from fusiform or short to elongated and narrowly ellipsoid, spathulate and spindle shaped. Similar observations have been reported in several other orchid taxa by many workers (Kurzweil 1995; Swamy et al. 2004; Chaudhary et al. 2014; Verma et al. 2014). Barthlott and Ziegler (1981) reported 20 different types of seed based on shape, testa cells, length and sculpturing of cells and presence of intercellular gaps and beading. Arditti et al. (1979, 1980) and Verma et al. (2012) had advocated the evolutionary significance of seed shape. Fusiform seeds that are observed in all the subfamilies appear to be basic form in orchids from which all other seed shapes evolved. Vij et al. (1992) reported that fusiform seed is found in primitive orchids and ovoid, elliptical, filamentous, cylindrical seeds are found in advanced epidendroid orchids. Molvray and Chase

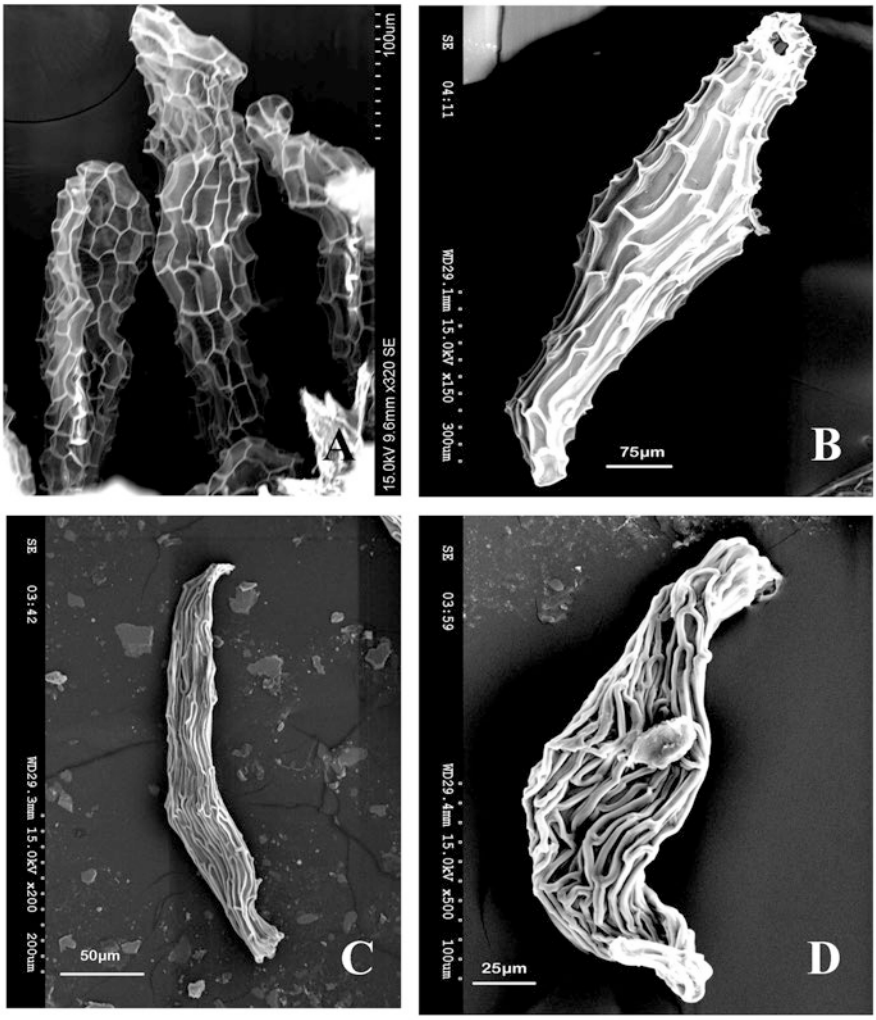

Fig. 14.1 SEM photographs of orchid seed. (**a**) *Malaxis densiflora*, transparent seed, quadrilateral-shaped seed with blunt ends; (**b**). *Cymbidium aloifolium*, fusiform-shaped seed; (**c**) *Coelogyne nervosa*, elongated seed; (**d**) *C. nitida*, shorter seed with a bulged central part having ellipsoid embryo

(1999) reported that seeds of fusiform, oblong or filiform shape are very common in Epidendroideae, whereas fusiform and ovoid seeds are typical of Orchidoideae.

Seed Size (Length of Seed) and Colour Orchid seed ranges from 100 µm (*Oberonia similis*) to 6000 µm (*Epidendrum secundum*). According to Barthlott et al. (2014), seed size has been classified into five categories.

Very small	100–200 μm
Small	200–500 μm
Medium	500–900 μm
Large	900–2000 μm
Very large	2000–6000 μm

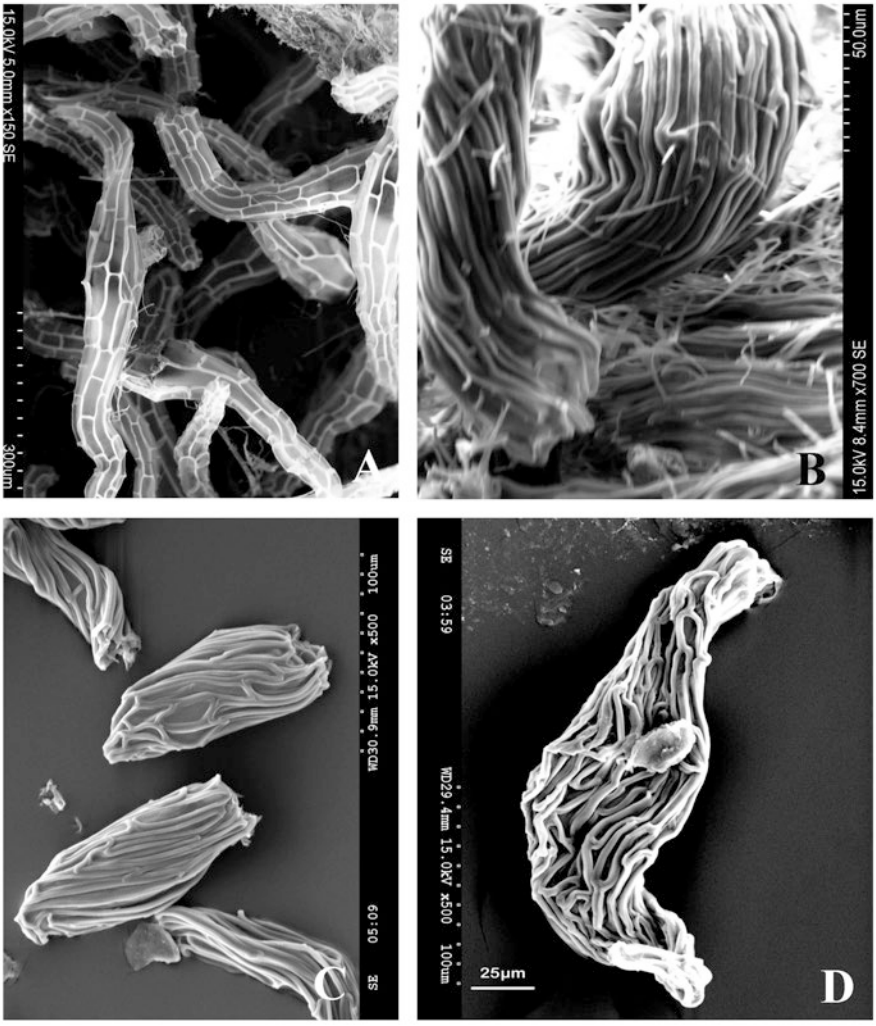

Fig. 14.2 SEM photographs of orchid seed. (**a**) *Calanthe triplicata*, filamentous shaped seed; (**b**) *Vanda tessellata*, ropelike appearance of seed; (**c**) *Oberonia arnottiana*, spindle-shaped seeds; (**d**) *Pholidota pallida*, spindle-shaped seeds with slight curvature

In most of the cases, orchid seed is whitish, brownish or dark brown and also beige, yellow, reddish, orange, greenish, yellowish brown or black. The colour is

determined by the testa especially embryo which may be intense yellow, orange or red orange colour or greenish due to presence of chloroplast pigment.

L/W Ratio According to Augustine et al. (2001), length/width ratio has some significance in expressing the relative degree of truncation of seeds in orchids. In present study, L/W ratio has been observed in 18 species belonging to 6 genera (Table 14.1). Maximum L/W ratio was observed in *Calanthe triplicata* (9.55), witnessed with highly truncated seeds, when compared to other investigated taxa. Verma et al. (2012) observed the highest L/W ratio in *Arundina graminifolia* but the lowest in *Aerides multiflora*. In the present study, maximum L/W ratio was recorded in *Calanthe triplicata*; medium L/W ratio in *C. breviscapa* (5.80) followed by *C. ovalis* (5.80*)*, *V. testacea* (4.87), *P. pallida* (4.64) and *C. nervosa* (4.53); and lowest L/W ratio recorded in *A. praemorsa* (2.67). Therefore, the genus possessing highly truncated seeds is *Calanthe* and genera possessing the medium truncated seeds are *Coelogyne, Vanda testacea* and *Pholidota* and the lowest truncated seeds are *Cymbidium*, *Oberonia* and *Acampe*.

Seed Volume The seed volume in orchids is a reflection of the size of the seeds (Arditti et al. 1979). In the present work, maximum seed volume was recorded in *Cymbidium giganteum* (16.674×10^{-3}) followed by *C. bicolor* ($11.880\,\text{mm} \times 10^{-3}$). Lowest seed volume was recorded in *Acampe praemorsa.* Almost all vandoid species studied here had lowest seed volume when compared to other taxa

Testa Cells The seed coat consists of testa cells which are transparent at maturity. Testa cells are varied in their shape and size (Fig. 14.3a, b). The thickenings in the testa cells are variously developed along the transverse and longitudinal walls. In *Malaxis densiflora* and *Calanthe triplicata*, testa cells showed transverse cell wall thickenings (Fig. 14.4a, b), whereas epiphytic species of *Cymbidium* showed very prominent longitudinally oriented cell wall thickenings (Fig. 14.4c, d). Similarly, an epiphytic orchid, viz. *Pholidota pallida*, also recorded prominent cell wall thickenings. Similar observations were recorded by Vij et al. (1992) that testa cell wall thickenings are more prominent in epiphytes but less prominent in terrestrial taxa

According to Vij et al. (1992), prominent development of cell wall thickenings in testa has got adaptive significance, provides rigidity to the seed coat and protects the embryo, and also hygroscopic nature of seed provides metabolic activities during germination.

Presence of chalazal pore in seeds of *Luisia* (Fig. 14.3c) and *Vanda* spp. possibly serves as an entry point for the fungal endophytes required for germination (Garg et al. 1992). Such chalazal openings were also reported in South African Orchidoideae (Kurzweil 1993). Each seed comprises an undifferentiated embryo enclosed within a transparent integument or seed coat. It is difficult to study its

Table 14.1 Seed characters and quantitative data

Sl. no.	Taxa	Time of fruiting	Colour	Length (mm)	Width (mm)	L/W	Seed volume $mm^3 \times 10^{-3}$	Average length of testa cells (μm)	Average width of testa cells (μm)	Average no. of testa cells
1	*Malaxis densiflora* (A.Rich) O.Kutze	Mar–Jun	White	0.3289 ± 0.0497	0.0985 ± 000983	3.33	0.0008355 0.355 $mm^3 \times 10^{-3}$	37.81	13.69	11.62
2	*Oberonia arnottiana*	Sept–Oct	Yellow	0.27398 ± 0.004986	0.09012 ± 0.004733	3.03	0.0005805 0.5605 $mm^3 \times 10^{-3}$	105.03	17.88	3.62
3	*O. ensiformis*	Sep–Oct	Light yellow	0.2657 ± 0.00546	0.08009 ± 0.00434	3.31	0.000443 0.443 $mm^3 \times 10^{-3}$	107.5	20.91	3.79
4	*Cymbidium aloifolium*	Jan–Feb	Yellow	0.8838 ± 0.1174	0.2216 ± 0.0209	3.98	0.011360 (11.36×10^{-3})	142.90	50.19	7.85
5	*C. bicolor*	Nov–Dec	Yellow	0.9568 ± 0.05001	0.2406 ± 0.0233	3.97	0.011449 (14.49×10^{-3})	148.92	53.28	8.10
6	*C. eburneum*	Nov–Dec	Yellow	0.83769 ± 0.2406	0.2462 ± 0.0770	3.40	0.01328 (13.28×10^{-3})	149.15	53.20	8.5
7	*C. giganteum*	Nov–Dec	Light yellow	0.9967 ± 0.08896	0.2529 ± 0.0377	3.94	0016674 (16.674×10^{-3})	158.21	54.15	9.5
8	*Calanthe bimuricata*	Apr–May	White	0.9474 ± 0.1701	0.0992 ± 0.0227	9.55	0.002440 2.440 $mm^3 \times 10^{-3}$	140.54	31.18	9.87
9	*Coelogyne breviscapa*	Sept–Oct	Light yellow	0.54718 ± 0.05372	0.09426 ± 0.015517	5.80	0.001271 (1.271×10)	161.29	35.38	11.21
10	*C. nervosa*	Sept–Oct	Yellow	0.54435 ± 0.5057	0.1199 ± 0.1553	4.53	0.002045 ($2.045\ mm^3 \times 10^{-3}$)	152.32	62.1	9.3

11	*C. nitida*	Sept–Oct	Pale yellow	0.2930 ± 0.0575	0.0727 ± 0.0164	4.03	0.00040534 (0.4053 $mm^3 \times 10^{-3}$)	72.71	14.16	2.20
12	*C. ovalis*	Oct–Dec	Light yellow	0.3261 ± 0.05885	0.0553 ± 0.014599	5.89	0.00026102 (0.2610 $mm^3 \times 10^{-3}$)	71.42	15.28	4.10
13	*Pholidota pallida*	Nov–Dec	Pale yellow	0.45017 ± 0.1116	0.096843 ± 0.02635	4.64	0.0011043 (1.1043 $mm^3 \times 10^{-3}$)	142.29	27.42	7.20
14	*Acampe praemorsa*	Mar–Jun	Light brown	0.1847 ± 0.06906	0.06906 ± 0.00345	2.67	0.0002306 (0.2306 $mm^3 \times 10^{-3}$)	68.56	11.19	3.66
15	*A. rigida*	Mar–Jun	Light brown	0.2402 ± 0.003910	0.0633± 0.00452	3.79	0.0002520 (0.2520 $mm^3 \times 10^{-3}$)	79.22	13.24	5.42
16	*Luisia zeylanica*	Jun–Jul	Yellow	0.2545 ± 001553	0.07445 ± 0.003838	3.39	0.00037045 (0.37045 $mm^3 \times 10^{-3}$)	84.52	12.29	3.1
17	*Vanda testacea*	Mar–Apr	Light yellow	0.2185 ± 0.0344	007232 ± 0.0004432	4.87	0.00029855 (0.2985 $mm^3 \times 10^{-3}$)	47.82	13.91	4.42
18	*V. tessellata*	Apr–May	Yellow	0.1892 ± 0.021051	0.06829 ± 0.000453	2.77	0.0002308 (0.2308 $mm^3 \times 10^{-3}$)	69.50	11.06	4.81

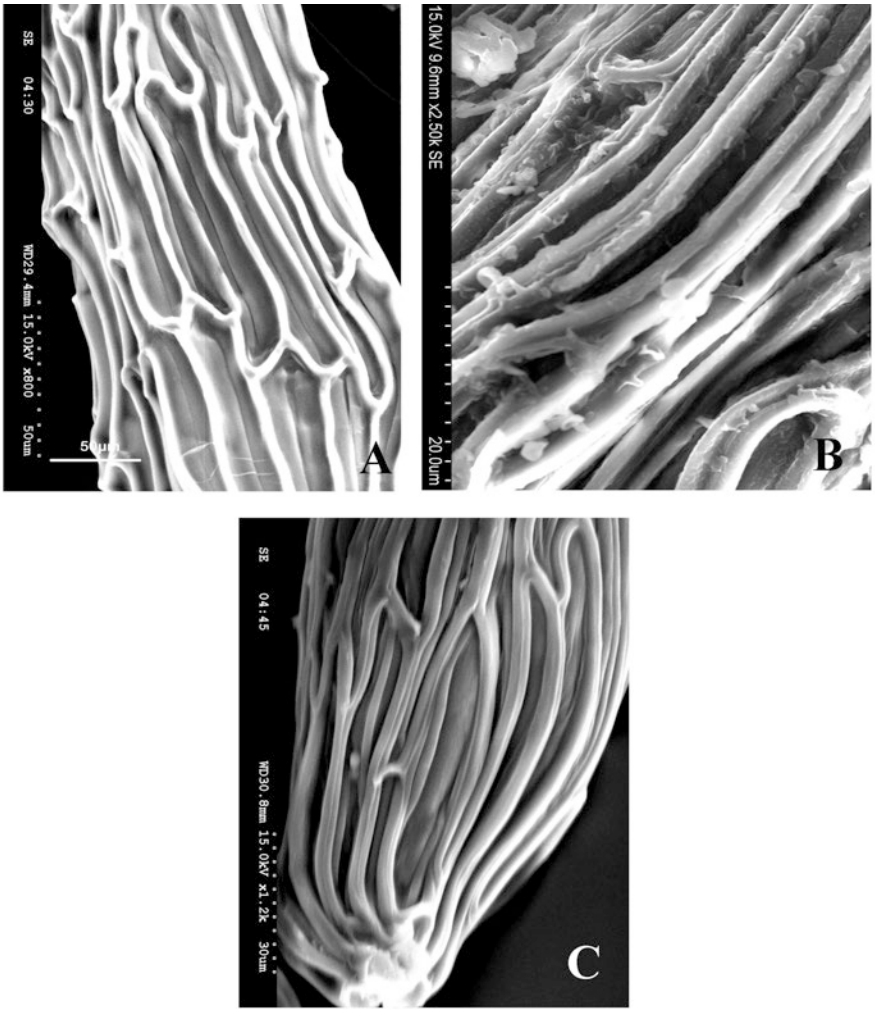

Fig. 14.3 SEM photographs of orchid seed. (**a**) *Pholidota pallida*, seed testa cells, rectangular and longitudinally oriented; (**b**). *Acampe rigida*, testa cells elongated with longitudinally oriented cell wall thickenings and blister like dots; (**c**). *Luisia zeylanica*, seed possesses opening at chalazal end

structural details with the help of optical microscope; such details can be obtained only with the help of scanning electron microscope (Arditti et al. 1979, 1980).

Embryo Characters Just like seeds, embryos are still more tiny in the Orchidaceae (Arditti and Ghani 2000). In all investigated taxa embryo, colour varies from yellow to pale yellow and light brown to white. According to Patrick et al. (1980), orchid embryos tend to be uniform in size within a genus, whereas the dimensions of testa are more variable. Orchid embryos are generally spherical or oval in shape

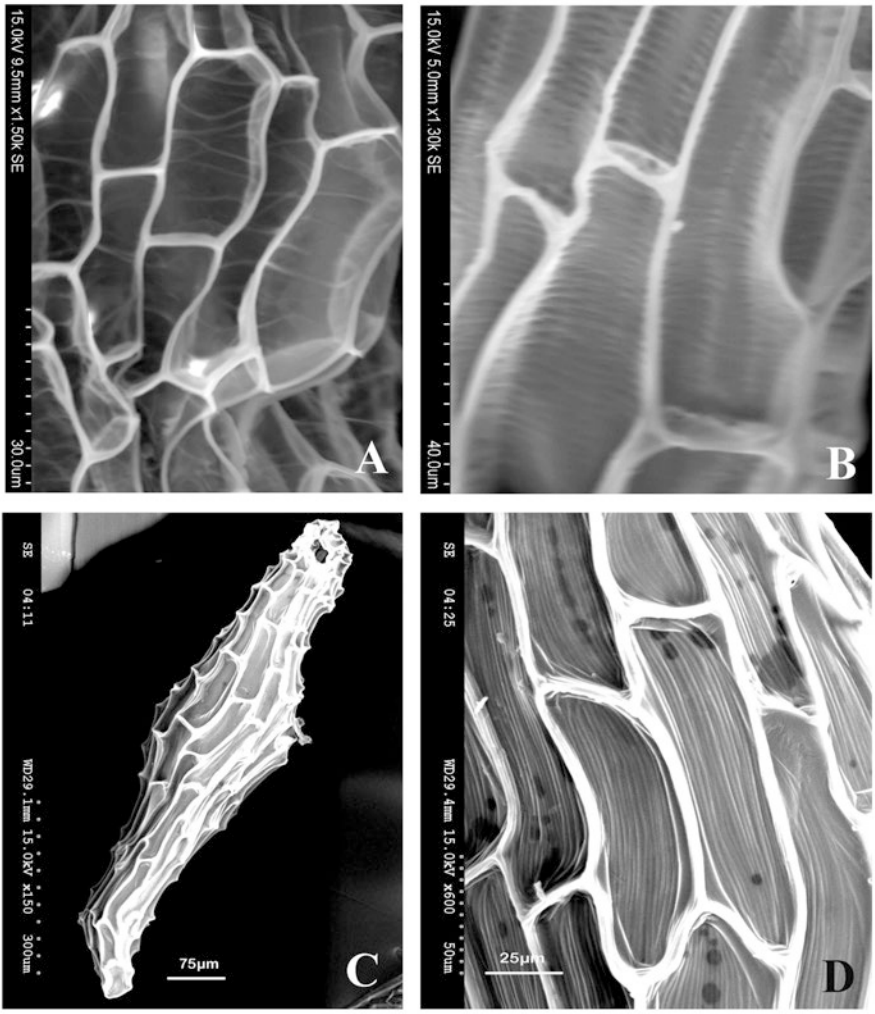

Fig. 14.4 SEM photographs of orchid seed. (**a**) *Malaxis densiflora*, seed testa cells showing fine transverse cell wall thickenings; (**b**) *Calanthe triplicata*, seed testa cells longitudinally oriented with cell wall thickenings; (**c**) *Cymbidium aloifolium*, seed testa cells with longitudinally oriented cell wall thickenings; (**d**) *Cymbidium giganteum*, seed testa cells with longitudinally oriented cell wall thickenings

In the presently studied taxa, the volume of embryo changes during the development of the seed. Young seeds have small undifferentiated embryos, whereas the mature seeds from the dehisced capsules have large volume of embryos. Embryo volume directly reflects the percentage of airspace inside the seed (Verma et al. 2014), and therefore, it has an important role in seed dispersal and species distribution. Arditti (1992) and Yan et al. (2002) reported that the tiny nature of embryos makes them exceedingly air filled, therefore helping them to float across longer distances in air for a wider dispersal (Table 14.2).

Table 14.2 Embryo characters and quantitative data

S. no.	Taxa	Colour	Length (mm)	Width (mm)	L/W	Embryo volume $mm^3 \times 10^{-3}$	Seed volume to embryo volume	Airspace (%)
1	*Malaxis densiflora*	White	0.1621 ± 0.002952	0.0628 ± 0.02501	2.58	0.0003339 (0.3339 $mm^3 \times 10^{-3}$)	2.50	60.50
2	*Oberonia arnottiana*	Yellow	0.09975 ± 0.00769	0.00937	1.20	0.00003505 (0.03505 $mm^3 \times 10^{-3}$)	1.65	39.62
3	*O. ensiformis*	Light yellow	0.09689 ± 0.01744	0.07394 ± 0.007629	1.31	0.0002756 (0.2756 $mm^3 \times 10^{-3}$)	1.60	38.19
4	*Cymbidium aloifolium*	Light yellow	0.3010 ± 0.0125	0.1108 ± 0.008485	2.716	0.001930 (1.930 $mm^3 \times 10^{-3}$)	5.886	83.00
5	*C. bicolor*	Pale yellow	0.1919± 0.0542	0.1317 ± 0.0350	1.45	0.001738 (1.738 $mm^3 \times 10^{-3}$)	8.34	88.04
6	*C. eburneum*	Yellow	0.3251 ± 0.011507	0.1119 ± 0.006677	2.905	0.002125 (2.125 $mm^3 \times 10^{-3}$)	6.249	83.99
7	*C. giganteum*	Light yellow	0.1981 ± 0.09015	0.1304 ± 0.003645	1.5191	0.001759 (1.759 $mm^3 \times 10^{-3}$)	6.479	89.47
8	*Calanthe bimuricata*	White	0.1413 ± 0.0591	0.07513 ± 0.0251	1.88	0.00041661 (0.4166 $mm^3 \times 10^{-3}$)	5.85	82.92
9	*Coelogyne brevisapa*	Yellow	0.2495 ± 0.04370	0.06437 ± 0.0755	3.816	0.000538 (0.538 $mm^3 \times 10^{-3}$)	2.362	57.67
10	*C. nervosa*	Pale yellow	0.2019 ± 0.03780	0.1032 ± 0.02336	1.95	0.0011232 (0.1232 $mm^3 \times 10^{-3}$)	1.82	47.07
11	*C. nitida*	Light yellow	0.1524 ± 0.01125	0.03912 ± 0.00168	3.89	0.00012183 0.1218 $mm^3 \times 10^{-3}$	3.327	69.94
12	*C. ovalis*	Pale yellow	0.2015 ± 0.000628	0.03125 ± 0.001663	6.44	0.00010286 0.1028 $mm^3 \times 10^{-3}$	2.53	60.59
13	*Pholidota pallida*	Pale yellow	0.2912 ± 0.01217	0.06293 ± 0.004003	4.62	0.0006017 (0.617 $mm^3 \times 10^{-3}$)	1.835	45.51

S. no.	Taxa	Colour	Length (mm)	Width (mm)	L/W	Embryo volume $mm^3 \times 10^{-3}$	Seed volume to embryo volume	Airspace (%)
14	*Acampe praemorsa*	Light brown	0.1073 ± 0.00295	0.0515 ± 0.00654	2.08	0.0001486 (0.1486 $mm^3 \times 10^{-3}$)	1.55	35.53
15	*A. rigida*	Light brown	0.1703 ± 0.02150	0.04215 ± 0.002150	4.09	0.0001579 (0.1579 $mm^3 \times 10^{-3}$)	1.59	37.34
16	*Luisia zeylanica*	Yellow	0.1212 ± 0.01217	0.05333 ± 0.00321	2.27	0.0001791 (0.179 $mm^3 \times 10^{-3}$)	2.06	51.63
17	*Vanda testaceae*	Light yellow	0.1250 ± 0.0150	0.0452 ± 0.00264	2.76	0.0001334 (0.1334 $mm^3 \times 10^{-3}$)	2.23	55.31
18	*V. tessellata*	Yellow	0.1452 ± 0.001829	0.0340 ± 0.01252	4.26	0.000087734 (008773 $mm^3 \times 10^{-3}$)	2.63	62.00

Seed Volume/Embryo Volume with Reference to Species Distribution Fahn and Werker (1972) classified the wind dispersed seeds (anemochores) into flyers (meteoranemochores) and rollers (chamaechores). The orchid seeds belong to flyers because of their tiny and light weight in nature adapted for wind dispersal. The orchid seed contains a more tiny embryo with air-filled space causing buoyancy

Seed morphological characters such as shape, size, weight, airspace play a key role in distribution of orchid species (Arditti and Ghani 2000, Murren and Ellison 1998). Kiyohara et al. (2012) and Shimizu et al. (2012) found different seed velocities based on their weight and proportion of airspace. Seeds with large airspace drop more slowly and therefore will have lower settling velocity. Further seed shape (relationship between seed length and width) is also connected with dispersal distances of seeds (Brzosko et al. 2017). Arditti and Ghani (2000) and Eriksson and Kainulainen (2011) stated that elongated seeds can disperse faraway distances. However Eriksson and Kainulainen (2011) reported that low-weight seeds could travel long distances irrespective of their shape. The other morphological character is size of the fruiting plants that reflects the distance that seed dispersed (Alexandersson and Agren 2000). Seed dispersal distance is strongly dependent on plant height (Thomson et al. 2011). Brzosko (2017) observed that the plants having shortest shoot had recorded the seed dispersal of short distance. Plants with tallest shoots had seed dispersal of farthest distances. Generally the wind dispersal was taller than the other plants in the wild habitat (Willson and Traveset 2000).

From Table 14.1, it is evident that *Cymbidium* spp. showed higher values of Sv/Ev ratio when compared to vandoid genera such as *Acampe*, *Luisia* and *Vanda*; as a result percentage of airspace is high in *Cymbidium*. Hence, *Cymbidium* seeds are more buoyant and widely distributed throughout Indo-Malayan region. Within the *Cymbidium* genus, *C. giganteum* shows the highest airspace percentage whereby it is distributed in Western Ghats and also Kumaon to Khasi Hills including Sikkim Himalaya.

Similarly among coelogynes *C. nitida* showed higher percentage of airspace. Hence, it is widely distributed in Sikkim and Khasi Hills of North East Himalaya (Bose and Bhattacharjee 1980). However, in the present study, *C. nitida* and also *Cymbidium giganteum* collected from botanical gardens, not from wild, need further study to confirm their distribution pattern in India.

In the case of tribe Vandeae, *Vanda tessellata* shows higher percent of airspace; hence it is widely distributed in Western and Eastern Ghats and also in Western Himalayan region of India. Among *Acampe* genus, *A. rigida* with 37.34% of airspace is an old world orchid restricted to peninsular region, whereas *A. praemorsa* with comparatively lower percentage (35.53%) of airspace is distributed in Southern India and nearby Sri Lanka.

As already mentioned that there is no generalised pattern of orchid distribution in India and elsewhere, Vij et al. (1998) also opined that some orchids exhibit high habitat specificity (narrow preferences towards exposure and shade, moisture, soil pH, mineral nutrients, etc.) and it is more pronounced in the mycoheterotrophs. Those species which have more buoyant seeds can successfully disperse long

distances but unable to establish themselves in the absence of biotic (mycorrhizal) and abiotic factors. As Zotz and Heitz (2001) pointed out, a more integrative approach to study the epiphytic biology is needed including physiological investigations, substrate instability, dispersal limitation and competition (intra- and interspecific level).

14.3 Taxonomic and Phylogenetic Implications

In all presently investigated taxa, testa cells are transparent and variously thickened. Their shape, size and wall thickenings have got taxonomic significance in species identification (Healey et al. 1980). Vij et al. (1992) classified the three categories of seeds based on length of testa cells; these are long (>200 μm), intermediate (>100–200 μm) and short (up to 100 μm). Based on their classification, the presently investigated taxa are grouped into following categories:

(i) ***Intermediate seeds:*** *Oberonia arnottiana*, *O. ensiformis*, *Cymbidium aloifolium*, *C. bicolor*, *C. eburneum*, *C. giganteum*, *Calanthe triplicata*, *Coelogyne breviscapa*, *C. nervosa* and *Pholidota pallida*.
(ii) ***Shorter seeds:*** Rest of all studied taxa have shorter seeds, such as *Malaxis densiflora*, *Coelogyne nitida*, *C. ovalis*, *Acampe praemorsa*, *A. regida*, *Luisia zeylanica*, *Vanda testaceae* and *V. tessellata.* Category of species having long seeds was not reported from this study.

Among intermediate category, *Coelogyne breviscapa* possesses the longest testa cells (161.29 μm) whereas *Malaxis densiflora* with the shortest testa cells (37.81; Table. 14.1). In general, the shorter testa cells were observed in the tribe Vandeae when compared to other tribes studied here. Similar observations were recorded by Swamy et al. (2004) in *Aerides maculosa* and *Vanda parviflora* of the tribe Vandeae (Table 14.3).

The micromorphological features of seed and embryo (quantitative data, Tables 14.1 and 14.2) from various species are taken and subject to hierarchical cluster analysis using Euclidean distance to determine the distance among various species (Table 14.4). The dendrogram (Fig. 14.5) based on quantitative seed micromorphological characters of various species belonging to Epidendroideae revealed the following clusters:

Cluster I:	*Coelogyne breviscapa*, *C. nervosa*, *Pholidota pallida*
II:	*C. giganteum*, *C. bicolor*, *C. eburneum*, *C. aloifolium*, *Calanthe triplicata*
III:	*Luisia zeylanica*, *Acampe praemorsa*, *A. rigida*
IV:	*Coelogyne nitida*, *C. ovalis*, *Vanda tessellata*
V:	*Oberonia arnottiana*, *O. ensiformis*, *Malaxis densiflora*, *Vanda testaceae*

It was already mentioned in the previous section that *Pholidota* is closely allied to *Coelogyne*. The dendrogram (Fig. 14.5) also showed that *Pholidota pallida* is

Table 14.3 Diagnostic quantitative characters of seed and embryo taken for dendrogram construction of subfamily Epidendroideae

	Seed/embryo characters					
Species	L/W of seed	Average length of testa cells	Average no. of testa cells	Embryo volume	Vs/Ve	Airspace percentage
Malaxis densiflora	3.33	37.81	11.62	0.0003339	2.50	60.50
Oberonia arnottiana	3.03	105.03	3.62	0.0003505	1.65	39.62
O. ensiformis	3.31	107.5	3.79	0.0002756	1.60	38.29
Cymbidium aloifolium	3.98	142.9	7.85	0.001930	5.88	83.00
C. bicolor	3.97	148.92	8.10	0.001738	8.34	88.04
C. eburneum	3.40	149.15	8.5	0.002125	6.24	83.99
C. giganteum	3.94	158.21	9.5	0.001759	6.42	89.47
Calanthe triplicata	9.55	140.54	9.87	0.00041661	5.85	82.92
Coelogyne breviscapa	5.80	161.29	11.21	0.000538	2.34	57.67
C. nervosa	4.53	152.32	9.3	0.0011232	1.82	47.07
C. nitida	4.03	72.71	2.20	0.00012183	3.32	69.94
C. ovalis	5.89	71.42	4.10	0.00010286	2.53	60.59
Pholidota pallida	4.64	142.29	7.20	0.0006017	1.83	45.51
Acampe praemorsa	2.67	68.56	3.66	0.0001486	1.55	35.53
A. rigida	3.79	79.22	5.42	0.0001579	1.59	37.34
Luisia zeylanica	3.39	84.52	3.66	0.0001791	2.06	51.63
Vanda testaceae	4.87	47.82	4.87	0.0001334	2.23	55.31
V. tessellata	2.77	69.50	4.81	0.000087734	2.63	62.00

Table 14.4 Distance matrix (Euclidean distance) based on quantitative seed features in the subfamily Epidendroideae

Name of the taxa	*Malaxis densiflora*	*Oberonia arnottiana*	*O. ensiformis*	*Cymbidium aloifolium*	*C. bicolor*	*C. eburneum*	*C. giganteum*	*Calanthe triplicata*	*Coelogyne breviscapa*
Malaxis densiflora	–								
Oberonia arnottiana	70.847	–							
O. ensiformis	73.567	2.825	–						
Cymbidium aloifolium	107.593	57.902	57.336	–					
C. bicolor	114.677	65.852	65.231	8.231	–				
C. eburneum	113.895	62.931	62.185	6.398	4.621	–			
C. giganteum	123.918	73.289	72.437	16.711	9.695	10.651	–		
Calanthe triplicata	105.400	56.878	56.369	8.378	11.701	10.730	19.674	–	
Coelogyne breviscapa	123.538	59.638	57.713	31.732	33.532	29.489	32.307	33.110	–
C. nervosa	14.084	48.233	46.020	37.398	41.646	37.344	43.058	38.285	14.084
C. nitida	89.901	44.381	47.09	71.664	78.712	78.033	88.060	69.750	89.901
C. ovalis	90.198	39.731	42.505	75.103	82.542	81.418	91.731	73.034	90.198
Pholidota pallida	22.947	37.927	35.720	37.724	43.548	39.376	47.040	38.078	22.947
Acampe praemorsa	95.689	36.701	39.04	88.419	96.346	94,282	104.910	86.783	95.689
A. rigida	47.861	25.984	28.347	78.513	86.495	84.248	94.852	76.869	84.776
Luisia zeylanica	48.209	23.774	26.576	66.519	74.381	72.561	83.159	64.870	77.414
Vanda testaceae	13.234	59.367	62.092	99.146	106.49	105.46	115.72	97.053	113.67
V. tessellata	32.453	42.020	44.817	76.484	83.848	82.793	93.069	74.607	92.165

(continued)

Table 14.4 (continued)

Name of the taxa	*C. nervosa*	*C. nitida*	*C. ovalis*	*Pholidota pallida*	*Acampe Praemorsa*	*A. rigida*	*Luisia zeylanica*	*Vanda testaceae*	*V. tessellata*
Malaxis densiflora									
Oberonia arnottiana									
O. ensiformis									
Cymbidium aloifolium									
C. bicolor									
C. eburneum									
C. giganteum									
Calanthe triplicata									
Coelogyne breviscapa									
C. nervosa	–								
C. nitida	83.149	–							
C. ovalis	82.201	9.838	–						
Pholidota pallida	10.366	73.931	72.537	–					
Acampe praemorsa	84.760	34.762	25.450	74.513	–				
A. rigida	73.851	33.445	24.667	63.628	11.012	–			
Luisia zeylanica	68.197	21.883	16.080	58.215	22.687	15.355	–		
Vanda testaceae	104.91	29.027	24.219	95.007	28.778	36.204	36.934	–	
V. tessellata	84.927	9.068	3.990	74.700	26.534	26.553	18.308	22.789	–

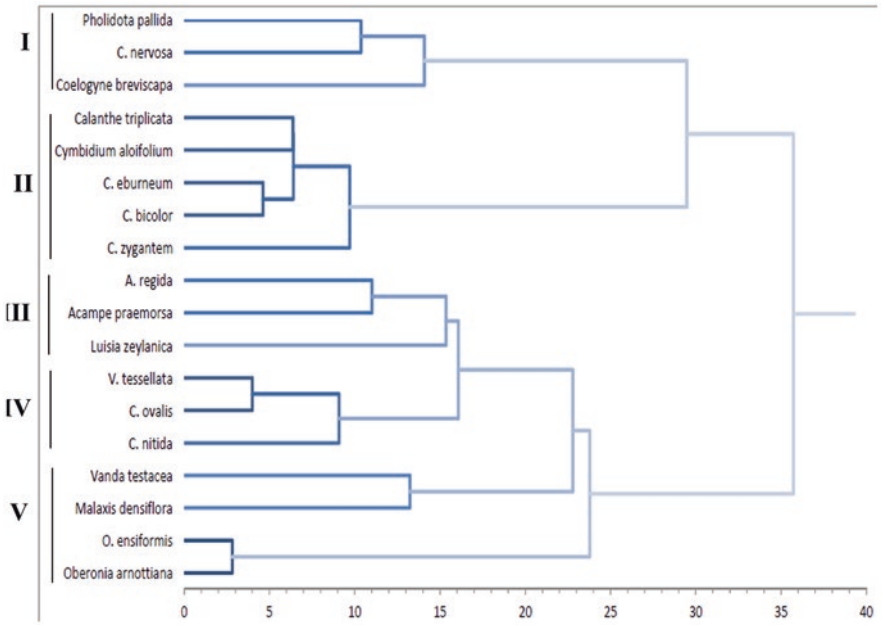

Fig. 14.5 Dendrogram of all 18 studied taxa belonging to subfamily Epidendroideae based on quantitative characters of seed and embryo

clustered with *Coelogyne breviscapa* and *C. nervosa* (cluster 5); this supports the inclusion of *Pholidota* in the subtribe Coelogyninae of the tribe Coelogyneae by Dressler (1993).

From the dendrogram (Fig. 14.5), it is also evident that there is clear species differentiation among *Cymbidium* which form a clear cluster, i.e. cluster 4; it does not share any character with other genera. Dendrogram also showed that the *Oberonia* and *Malaxis* had close affinity of *Oberonia* and *Malaxis* with that of *Vanda testaceae*. Similarly *Vanda testaceae* and *V. tessellata* are spread into cluster 1 and cluster 2 along with *Oberonia* and *Coelogyne*, respectively.

The average number of testa cells in the longest axis of seeds in *Malaxis densiflora* (11.62) is higher followed by *Coelogyne breviscapa* and lowest in *C. nitida* (2.20). From Table 14.3, it is evident that the tribe Vandeae shows lesser number of testa cells when compared to other tribes of Epidendroideae such as Malaxideae, Cymbidieae, Arethuseae and Coelogyneae.

Regarding L/W value of embryo, it is highest in *Coelogyne ovalis* (6.44) followed by *Pholidota pallida* whereas lowest in *Oberonia arnottiana* (1.20). Among all tribes studied here, Coelogyneae and Vandeae show higher L/W values than the other tribes.

Calevo et al. (2017) also opined that the morphological traits such as seed size, embryo size, cell wall thickenings, etc. are potential markers to resolve the taxonomic disputes and assess the phylogenetic relationship of various orchid taxa.

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15 Structural Adaptations of *Bulbophyllum* and *Dendrobium* (Orchidaceae) to the Epiphytic Habitat and Their Phylogenetic Implications

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Abstract

The morpho-anatomical studies in *Bulbophyllum* and *Dendrobium* (Orchidaceae) with special reference to ecological adaptation and phylogenetic implications have been carried out. The vegetative parts were collected from different parts of North-East Himalaya and Western and Eastern Ghats of India. All were epiphytes belonging to tribe Dendrobieae. These plant parts were fixed in FAA (Formaline-Acetic acid-Alcohol) and usual methods of microtomy had followed. Stomata were confined to abaxial surface in all the investigated taxa. The presence of stomatal ledges and substomatal chambers is helpful in reducing leaf transpiration and evaporation of water. Absorbing trichomes were recorded only in Sikkim collections of *D. anceps* whereas they were absent in Darjeeling collections. In case of *D. herbaceum* and *D. moschatum*, these were present only in Kerala collections and absent in Karnataka collections. Single- or multi-layered velamen has been reported in both genera. It was observed that tilosomes were always associated with single layered velamen roots whereas completely absent in multilayered velamen taxa. Based on anatomical data, sectional delineation and phylogenetic interrelationships have been discussed.

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Bulbophyllum · *Dendrobium* · Anatomical adaptations · Habitat tolerance · Phylogenetic implications

15.1 Introduction

The Orchidaceae constitutes one of the largest families of flowering plants comprising about 28,484 species (Govaerts et al. 2017). It contributes about 40% of the monocotyledons (Rasmussen 1985). In India, it represents the second largest flowering plant family with 1350 species (Jalal and Jayathi 2012) and contributes about 10% of Indian flora (Jain 1980; Kumar and Manilal 1994). A majority of orchid habitats in India are dwindling in state due to many anthropogenic activities. The present paper deals with some of the insights in anatomy related to ecological adaptability and phylogenetic interrelationship of genera *Bulbophyllum* and *Dendrobium*.

Both *Bulbophyllum* and *Dendrobium* belong to the tribe Dendrobieae Endl. and sub tribes Bulbophyllinae Schltr. and Dendrobiinae Lindl. respectively (Dressler 1993). In India, the tribe Dendrobieae is represented by about 189 species, distributed in Western and Eastern Ghats and, Eastern and Western Himalayas. Most of the taxa are primarily epiphytic, although some are lithophytic or terrestrial. In general, the great diversity of orchids and their different habitats have been made possible by structural, ecological and physiological adaptations (Mehra and Vij 1974; Khasim and Mohana Rao 1986; Mohana Rao and Khasim 1987a, b; Pridgeon 1986; Arditti 1992; Stern and Morris 1992). Vegetative structures such as roots, stems and leaves are specialized in water and nutrient absorption (Benzing et al. 1983; Moreira and Isaias 2008). Physiologically, the Crassulacean Acid Metabolism (CAM) helps in water economy by closure of stomata during the day (Luittge 2004) and, photosynthesis in roots is equally important in the maintenance of oxygen supply (Dycus and Knudson 1957; Moreira et al. 2009).

However, the vegetative anatomy of this highly evolutionary important family is completely neglected or has received little attention. From the ecological point of view Sanford (1974) did some work on African orchids, Kaushik (1983) on some Himalayan orchids and Metusala et al. (2017) on *Dendrobium* of Indonesia. During the last two decades few important monographs on orchid biology and systematics have appeared (Dressler 1993; Vermeulen 1993; Pridgeon et al. 1999, 2001, 2003, 2005; Ramesh et al. 2017). By critical reading of the available literature, it is evident that the authors had studied the anatomy with respect to systematics; but they did not explain the ecological adaptation of orchids. From the ecological point of view Sanford (1974) did some work on African orchids and Kaushik (1983) on some Himalayan orchids. As such, there has been no single paper on anatomy of orchids in relation to ecological adaptability for the last 20 years. In view of this the present anatomical investigation has been undertaken in the *Bulbophyllum* and *Dendrobium* species, the largest genera in the family Orchidaceae, so as to throw light on their

ecological adaptability and also ascertain the tribal, subtribal and sectional delineation, and phylogenetic relationships.

Plant materials were collected from Arunachal Pradesh, Darjeeling, Sikkim, Himalayas, Karnataka and Kerala at various altitudes over a period of 3 years (Table 15.1, Fig. 15.1). Plants were identified with the help of standard floras (Hooker 1894, 1895; King and Pantling 1898; Brühl 1926; Bose and Bhattacharjee 1980; Abraham and Vatsala 1981; Hegde 1984; Dressler 1993; Manilal and Kumar 2004; Mabberley 2008); these were confirmed by comparing them with the authentic herbarium specimens stocked at the Botanical Survey of India, Coimbatore,

Table 15.1 [a]Details of collections of orchid plant materials

S.No.	Species	Place, altitude and date of collection	Host tree	Accession No.
	Family: **ORCHIDACEAE**			
1	Subfamily: **EPIDENDROIDEAE**			
	Tribe: **DENDROBIEAE LINDL.**			
	Subtribe: **BULBOPHYLLINAE**			
	BULBOPHYLLUM Thouars			
	Bulbophyllum affine Lindl.	(i) Tipi (Arunachal Pradesh), 1500 m; May, 2011	*(i) Castonopsis indica*	(i) **RO1** (Arunachal Pradesh)
		(ii) Araria (Darjeeling), 1650 m; April, 2011	*(ii) Azadirachta indica*	(ii) **RO2** (Darjeeling)
2	*B. bisetum* Lindl.	(i) Jalalgarh (Darjeeling), 2250 m; February, 2011	(i) *Azadirachta indica*	***(i)*** *RO3*
3	*B. careyanum* W.J. (Hook.) Spreng.	(i) Packyong (Sikkim), 2500 m; February , 2011	(i) *Saurauia nepalensis*	(i) **RO4**
4	*B. cauliflorum* Hk. f.	(i) Jalalgarh (Darjeeling), 2250 m; February, 2011	(i) *Mangifera indica*	(i) **RO5**
5	*B. cornutum* (Lindl.) Rchb.f.	(i) Araria (Darjeeling), 1650m; February, 2011	(i) *Alnus nepalensis*	(i) **RO6**
6	*B. crassipes* J.D. Hook. f.	(i) Qasba (Darjeeling), 1250 m; February, 2011	(i) *Schima wallichii*	(i) **R07**
7	*B. fischerii* Seidenf.	(i) Jalalgarh (Darjeeling), 2250 m;	(i) *Mangifera indica*	(i) **R08**
		(ii) Lingtam (Sikkim), 2680 m; February, 2011	(ii) *Meliosma dillenifolia*	(ii) **R09**
8	*B. khasyanum* Griff.	(i) Taplejorg (Darjeeling), 1650 m; February, 2011	(i) *Schima wallichii*	(i) **R10**

(continued)

Table 15.1 (continued)

S.No.	Species	Place, altitude and date of collection	Host tree	Accession No.
9	*B. protractum* Hook. f.	(i) Ramda (Arunachal Pradesh), 1650 m, May 2011.	(i) *Elaeocarpus floribundus*	(i) **R11**
10	*B. scabratum* Rchb. f.	(i) Saddlepoint (Arunachal Pradesh, 2000 m; May 2011	(i) *Bischofia jaramica*	(i) **R12**
11	*B. stenobulbon* Par et Rchb. f.	(i) Qasba (Darjeeling), 1250 m; February, 2011	(i) *Alnus nepalensis*	(i) **R13**
12	*B. tremulum* Wight.	(i) Lingtam (Sikkim), 2680 m; February, 2011	(i) *Castanopsis indica*	(i) **R14**
13	*B. umbellatum* Lindl.	(i) Packyong (Sikkim), 2500 m; February, 2011	(i) *Alnus nepalensis*	(i) **R15** (Sikkim)
		(ii) Araria (Darjeeling), 1650 m; February, 2011	(ii) *Alnus nepalensis*	(ii) **R16** (Darjeeling)
	SUBTRIBE*: DENDROBIINAE*			
1	***DENDROBIUM* Swartz**	(i) Araria (Darjeeling), 1850 m; February, 2011	(i) *Alnus nepalensis*	(i) **R17 (**Darjeeling)
	Dendrobium anceps Sw.	(ii) Packyong (Sikkim), 2500 m; February, 2011	(ii) *Persea oderatissima*	(ii) **R18** (Sikkim)
2	*D. bicameratum* Lindl.	(i) Phidim (Darjeeling), 2000 m; February, 2011	(i) *Mangifera indica*	(i) **R19**
3	*D. densiflorum* Lindl.	(i) Phidim (Darjeeli ng), 2000 m; February, 2011	(i) *Schima wallichii*	(i) **R20**
4	*D. haemoglossum* Thw.	(i) Qasba (Darjeeling), 1785 m; February, 2011	(i) *Schma wallichii*	(i) **R21**
5	*D. herbaceum* Lindl.	(i) Karuman code (Kerala), 985 m; January, 2011	(i) *Mangifera indica*	(i) **R22** (Kerala)
		(ii) Khanapur (Karnataka), 850 m; June, 2011	(ii) *Terminalia elliptica*	(ii) **R23** (Karnataka)
6	*D. heyneanum* Lindl.	(i) Karuman code (Kerala), 985 m; June, 2011	(i) *Phoenix sylvestris*	(i) **R24**
7	*D. jenkinsii* Wall. ex. Lindl.	(i) Jalalgarh (Darjeeling), 1750 m; February, 2011	(i) *Azadirachta indica*	(i) **R25**

(continued)

Table 15.1 (continued)

S.No.	Species	Place, altitude and date of collection	Host tree	Accession No.
8	*D. microbulbon* A. Rich.	(i) Palavara (Kerala), 950 m; January, 2011	(i) *Terminalia bellirica*	(i) **R26** (Kerala)
		(ii) Halsi (Karnataka), 850 m; June, 2011	(ii) *Syzygium cumini*	(ii) **R27** (Karnataka)
9	*D. moschatum* (Buch.-Ham.) Sw.	(i) Karuman code (Kerala), 925 m; January, 2011	(i) *Mangifera india*	(i) **R28** (Kerala)
		(ii) Hanbur (Karnataka), 875 m; June, 2011	(ii) *Phoenix sylvestris*	(ii) **R29** (Karnataka)
10	*D. nobile* Lindl.	(i) Araria (Darjeeling), 2210 m; February, 2011	(i) *Alnus nepalensis*	(i) **R30**
11	*D. nutantiflorum* Hawkes & Heller	(i) Peringammala (Kerala), 950 m; June, 2011	(i) *Madhuca latifolia*	(i) **R31**
12	*D. pendulum* Roxb.	(i) Rongli (Sikkim), 1950 m; February, 2011	(i) *Albizia gamblei*	(i) **R32**

[a]Arranged according to Dressler (1993)

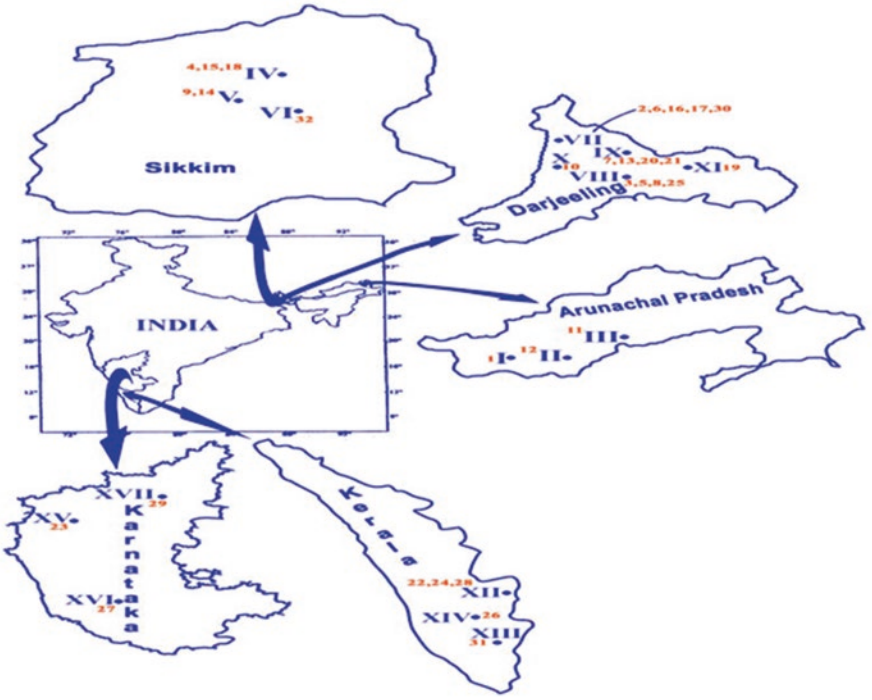

Fig. 15.1 India map and places of collection of orchid plant material

India. Voucher specimens were deposited in the Department of Botany and Microbiology, Acharya Nagarjuna University, India.

Vegetative organs such as leaves, stems, pseudobulbs and roots were fixed in FAA (5 cc formalin + 5 cc acetic acid + 90 cc 70% ethanol) for 24 h and then they were transferred to 70% alcohol and stored in it for laboratory studies. Free-hand cross sections of all vegetative organs were made at standardized levels (Metcalfe 1963; Cutter 1978). Cross section of mature leaves was done in a region equidistant from the base and apex of lamina. Stems were sectioned at median internodes, and mature roots at half way between the apex and junction with the rhizome. Sections were stained with safranin and fast green. For leaf epidermal peelings, small bits of leaves were put in 10% potassium hydroxide solution and then boiled until the epidermis was loosened from the mesophyll and veins. These peelings were mounted in 50% glycerine.

15.2 General Anatomy of *Bulbophyllum* and *Dendrobium*

The genera *Bulbophyllum* and *Dendrobium* are sympodial orchids, in which growth of the stem is arrested at certain stage and shoots are produced laterally from the base.

15.2.1 Leaf

Leaf anatomical features of *Bulbophyllum* and *Dendrobium* were given in the Tables 15.2 and 15.3. In a majority of the taxa studied here, the leaf is thick and fleshy. A fully developed leaf consists of a tubular leaf sheath and a lamina, often separated by an abscission layer, which involves in shedding and consequently helps in reducing the transpiring surfaces under stress conditions (Goh and Kluge 1989). In cross section, the leaf is generally V-shaped at the midrib and flattened at the laminar region.

Epidermal cells possess smooth and thin walls in almost all investigated taxa belonging to tribe Dendrobieae. According to Solereder and Meyer (1930) smooth cell walls are present in advanced epiphytic orchids whereas sinuous walls in primitive terrestrial ones.

In most of the presently studied taxa, the size of the adaxial epidermal cells is comparatively larger than abaxial ones. In some cases, these cells are two or three times larger in their size than the abaxial epidermal cells (Fig. 15.2a–d). Khasim (1996) reported adaxial epidermal cells that are three times larger than abaxial ones in *Paphiopedilum fairrieanum*. Mohana Rao and Khasim (1987b) reported bulliform cells on adaxial surface in *Anthogonium gracile* of Thuniinae. Bulliform cells are also reported in presently investigated taxon viz., *D. moschatum*.

Stomata The stomata are hypostomatic in distribution, restricted to abaxial surface of leaf. Similarly hypostomatic distribution is found in other groups of Orchidaceae (Möbius 1887; Singh 1981; Williams 1979; Avadhani et al. 1982). Interestingly Vij et al. (1991) observed the hypostomatic leaves in mesophytic orchids. Rasmussen

Table 15.2 **Leaf:** anatomical features in *Bulbophyllum* (in μm)

Anat. feat.	Access. no.															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. Absorbing trichome	–	–	+	–	+	+	+	–	–	–	–	+	+	–	–	+
2. Cuticle thickness	0.008	0.015	0.011	0.009	0.007	0.004	0.012	0.009	0.008	0.005	0.005	0.019	0.008	0.008	0.007	0.005
3. Stomatal width (two gua rd cells including pore)	0.023	0.018	0.021	0.019	0.016	0.024	0.028	0.024	0.020	0.011	0.021	0.022	0.015	0.018	0.017	0.014
4. Stomatal length (only guard cell)	0.017	0.012	0.019	0.015	0.019	0.011	0.021	0.011	0.015	0.008	0.012	0.011	0.018	0.019	0.015	0.012
5. Midrib vb. Size	0.089	0.075	0.062	0.084	0.058	0078	0071	0.075	0.069	0.08	0.086	0.083	0.077	0.054	0.095	0.038
6. Laminar vb. size	0.041	0.047	0.052	0.051	0.044	0.049	0.057	0.064	0.058	0.061	0.047	0.058	0.062	0.042	0.050	0.032
7. Water storage cell	0.064	0.051	0.059	0.062	0.054	0.064	0.057	0.060	0.069	0.068	0.066	0.054	0.056	0.058	0.061	0.051
8. Substomatal chamber size	0.023	0.021	0.021	0.021	0.020	0.019	0.025	0.018	0.021	0.026	0.020	0.029	0.027	0.022	0.024	0.024
9. No.of ph. cap layers	3	4	3	3	2	3	2	3	2	3	5	3	5	4	2	2
10. No.of xy. cap layers	2	4	1	2	A	A	1	2	1	2	2	1	4	2	2	1

[1. *Bulbophyllum affine* (Arunachal Pradesh), 2. *B. affine* (Darjeeling), 3. *B. bisetum,* 4. *B. careyanum,* 5. *B. cauliflorum,* 6. *B. cornutum,* 7. *B. crassipes,* 8. *B. fischerii* (Darjeeling), 9. *B. fischerii* (Sikkim), 10. *B. khasyanum,* 11. *B. protractum,* 12. B. scabratum, 13. B. stenobulbon, 14. B. tremulum, 15. B. umbellatum (Sikkim).16. B. umbellatum (Darjeeling)]

ph. = phloem, xy. = xylem, vb. = vascular bundles; + = present, – = absent

Table 15.3 **Leaf:** anatomical features in *Dendrobium* (in μm)

Anat. feat.	Access. No.															
	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1. Absorbing trichome	–	+	–	–	–	+	–	+	+	–	+	+	–	–	–	+
2. Cuticle thickness	0.010	0.007	0.009	0.006	0.004	0.008	0.006	0.011	0.006	0.009	0.004	0.004	0.008	0.007	0.005	0.012
3. Stomatal width (two guard cells including pore)	0.020	0.025	0.028	0.019	0.029	0.015	0.014	0.022	0.019	0.021	0.015	0.022	0.017	0.025	0.028	0.011
4. Stomatal length (only guard cell)	0.015	0.018	0.010	0.012	0.012	0.014	0.017	0.012	0.019	0.022	0.015	0.012	0.018	0.011	0.015	0.025
5. Mid vb. Size	0.082	0.070	0.078	0.071	0.057	0.068	0.083	0.079	0.088	0.081	0.078	0.076	0.081	0.085	0.073	0.084
6. Laminar vb. size	0.051	0.055	0.047	0.060	0.041	0.048	0.055	0.063	0.059	0.044	0.057	0.048	0.041	0.062	0.054	0.059
7. Water storage cell	0.065	0.062	0.067	0.064	0.068	0.060	0.067	0.057	0.066	0.026	0.059	0.068	0.061	0.069	0.053	0.057
8. Substomatal chamber size	0.026	0.027	0.021	0.029	0.025	0.023	0.029	0.028	0.026	0.021	0.025	0.020	0028	0.022	0.022	0.024
9. No. of ph. Cap layers	2	2	3	3	3	3	2	3	2	2	2	2	3	2	3	2
10. No. of xy. Cap layers	1	–	2	2	1	2	1	2	2	–	1	1	-	+	1	1

[17. *Dendrobium anceps* (Darjeeling), 18. *D. anceps* (Skkim), 19. *D. bicameratum*, 20. *D. densiflorum*, 21. *D. haemoglossum*, 22. *D. herbaceum* (Kerala), 23. *D. herbaceum* (Karnataka), 24. *D. heyneanum*, 25. *D. jenkinsii*, 26. *D. microbulbon* (Kerala), 27. *D. microbulbon* (Karnataka), 28. *D. moschatum* (Kerala), 29. *D. moschatum* (Karnataka), 30. *D. nobile*, 31. *D. nutantiflorum*, 32. D. pendulum

ph. = phloem, xy. = xylem, vb. = vascular bundles; + = present, – = absent

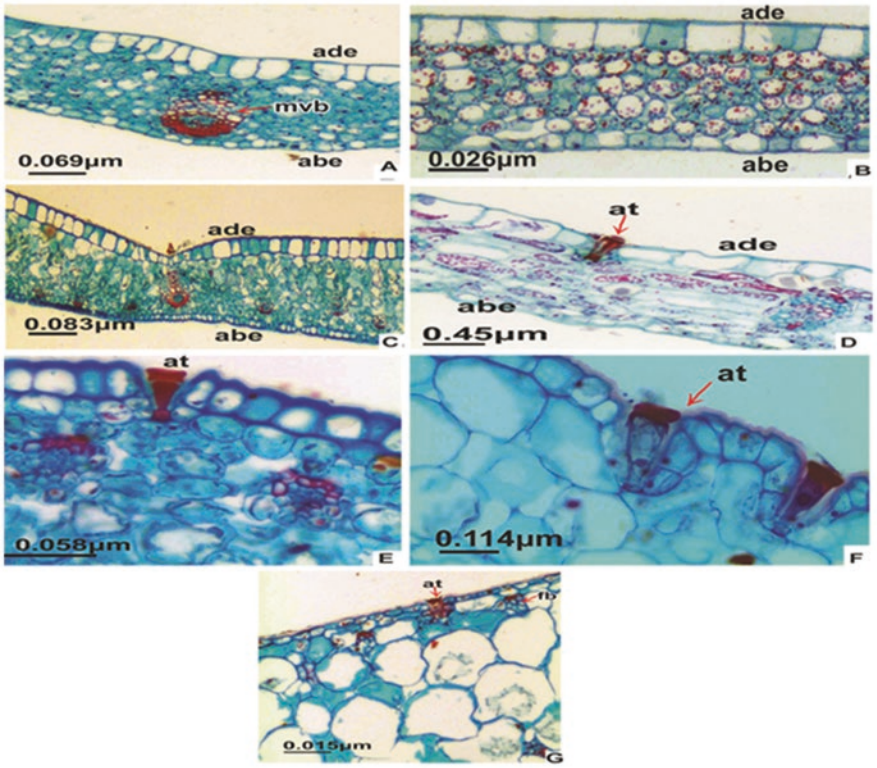

Fig. 15.2 **a**–**g**. Leaf
(**a**) *Bulbophyllum fischerii.* Leaf cross section showing larger adaxial epidermal cells and midrib vascular bundle
(**b**) *B. khasyanum.* Leaf cross section showing adaxial and abaxial epidermis
(**c**) *B. herbaceum.* Leaf cross section showing larger adaxial epidermal cells and midrib vascular bundle
(**d**) *B. umbullatum* (Sikkim collection). Leaf cross section showing absorbing trichome towards adaxial epidermis
(**e**) *B. herbacetum*. Leaf cross section indicating elongated 3-celled absorbing trichome on adaxial epidermis
(**f**) *B. umbellatum* (Darjeeling collection). Leaf cross section showing absorbing trichomes towards adaxial epidermis
(**g**) *Dendrobium anceps.* Leaf cross section indicating the absorbing trichome and fibre bundles towards adaxial epidermis

(1987) opined that hypostomaty is more frequent in mesophytic orchids whereas amphistomaty dominates in those of dry and humid habitats. Parkhurst (1978) observed that thick leaves tend to be amphistomatous, thus producing a secondary dependence of stomatal distribution on the environment. The thick leaves, generally associated with crassulacean acid metabolism have been considered an additional feature promoting amphistomaty in orchids (Rasmussen 1987). During the unfavourable period leathery leaves get folded and, in *R. retusa* two sides of lamina come so close to each other that there is no chance of transpiration from the adaxial

side of leaf (Kaushik 1983). With few exceptions, cyclocytic stomata with 5–6 subsidiary cells have been observed in presently investigated taxa. Epiphytes generally have smaller stomata than terrestrials. In the presently investigated taxa, the width of guard cells (including pore) varies among *Bulbophyllum* species (minimum of 0.011 μm to maximum 0.028 μm) and also *Dendrobium* (from 0.011 to 0.029 μm). Guard cells with prominent cuticular ledges (stomatal ledges) were observed on the leaf surface view of presently investigated taxon *B. affine* and *B. careyanum*. In *D. nobile* also, cuticular projections were observed around the stomatal apparatus; this type of projections has not been reported so far in any other orchid.

Absorbing trichomes The trichomes known to be absorbing in function, are 2 or 3-celled structures with dome-shaped apical cell and basal stalk cell (Fig. 15.2e–g). Kaushik (1983) preferred to call them as 'Handle cells'. The presence of absorbing trichomes is a regular feature in the members of Epidendroideae except tribe Vandeae (Khasim 1986). However, in the present investigation, these were observed in some species such as *Bulbophyllum bisetum, B. scabratum, B. stenobulbon*, *B. umbellatum* and also in *Dendrobium anceps, D. densiflorum, D. herbaceum, D. heyneanum* and *D. jenkinsii*. Pridgeon (1981) also studied the absorbing trichomes in Pleurothallidinae. He stated that the movement of water-soluble stain in these trichomes indicates an absorbing function similar to that of absorbing process of some bromeliad trichomes (Schimper 1888, quoted in Tomlinson 1969; Benzing et al. 1976).

Hypodermis In the presently investigated taxa, hypodermis is almost absent. However, fibre bundles at hypodermal position have appeared in *D. anceps* (Fig. 15.2g). Isaiah (1993) also reported fibre bundles in *Agrostophyllum khasianum, B. bhotanense* and *Epidendrum xanthum*. Mohana Rao and Khasim (1987b) observed these fibre bundles in *Agrostophyllum callosum, Cymbidium grandiflorum, C. lowianum, C. marstersii, C. traceyanum* and *Epidendrum radicans*. They also stated that fibre bundles provide mechanical strength to the plant body.

Mohana Rao and Khasim (1987b, c) reported multispiral thickenings in hypodermal cells in *B. dyerianum, Phaius maculatus, Pholidota imbricata* and *Otochilus alba.* Isaiah (1993) also observed hypodermal cells with multispiral thickenings in *B. bhotanense, B. gymnopus* and *D. jenkinsii*.

Mesophyll In all the investigated taxa, mesophyll is homogeneous, not differentiated into palisade and spongy parenchyma. Mesophyll tissue is tightly packed in some cases, which favours the fixation of carbon through C_4 pathway. Various tracheoidal elements including water storage cells with cellulosic thickenings and without thickenings were observed in the presently studied taxa. Olatunji and Nengim (1980), who coined the term 'tracheoidal elements', opined that certain specialized elements which possess annular, spiral or pitted secondary wall thicken-

ings, resemble the tracheids of vascular system. Pridgeon (1986) referred to these tracheoidal elements as 'spirally thickened idioblasts'.

In general, vascular bundles are arranged in a single series in all the presently investigated taxa. In all vascular bundles of leaf, phloem is situated towards abaxial side, and xylem towards adaxial side. The phloem and xylem ends possess some amount of sclerenchyma (sclerotic sheath). Tracheids with helical thickenings and vessel-like tracheids are abundant in leaves and also other parts of the plant body. Vessel-like tracheids were also reported by Ayensu and Williams (1972) in *Palumbina* and *Odontoglossum,* and also by Kaushik (1983) in several Himalayan orchids.

15.2.2 Pseudobulb/Stem

Anatomical features of Pseudobulb/stem were given in Tables 15.4 and 15.5. The stem shows morphological variation. In some species of *Dendrobium* and other orchids, the upper portion of the stem is fleshy whereas lower portion is thick and hard. Pseudobulbs are present in epiphytic orchids. Both fleshy stem and pseudobulb are concerned with storage of water. Pseudobulbs are consistent with sympodial growth, that leads to the shortening of shoots and thus to a compact habit reducing the transpiring surface; at the same time, sympodial habit promotes water storage and accumulation of starch materials (Benzing 1989a, b, c; Goh and Kluge 1989).

Fleshy stem and pseudobulb show anatomical similarities such as cuticle on the epidermis, and barrel-shaped or squarish and turgid epidermal cells; cortex and ground tissue with large polygonal to oval-shaped cells, function in storage of water (Fig. 15.3a, b). However, pseudobulb differs from stem in certain features. In pseudobulb, distinct cortex is absent; directly ground tissue in which numerous vascular bundles are scattered, appeared immediately below the epidermis (Fig. 15.3a, b). In case of stem, in the presently investigated taxa viz., *B. bisetum, B. cauliflorum* and *D. nobile*, a distinct cortex is present; this cortex is demarcated from the ground tissue by a ring of 3–4 layered sclerenchyma. Such type of demarcation was also reported by Morris et al. (1996) in some members of the subtribe Dendrobiinae.

Some of the cortical cells in the ground tissue region are showing pitted wall thickenings in most of the dendrobiums, such as *D. anceos, D. microbulbon, d. densiflorum* and *D. haemoglossum* (Fig. 15.3c–f). In some cases, cortical cells with multispiral cellulosic thickenings are involved in water storage (Fig. 15.3g). Vascular bundles showed well developed phloem cap made up of sclerenchymatous tissue. Large and small, numerous, collateral vascular bundles are scattered in the ground tissue region. In general, small vascular bundles are scattered at the peripheral region and large vascular bundles located in the centre.

Table 15.4 Stem/pseudobulb: anatomical features in *Bulbophyllum* (in μm)

	Access. no.															
Anat. feat.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. Cuticle thickness	0.006	0.004	0.005	0.006	0002	0.003	0.004	0.005	0.004	0.003	0.003	0.003	0.007	0.004	0.002	0.003
2. Water storage cells	0.021	0.024	0.020	0.021	0.026	0.021	0.024	0.018	0.021	0.019	0.017	0.025	0.038	0.022	0.024	0.025
3. Size of vb.	0.041	0.046	0.049	0.095	0.051	0.048	0.032	0.038	0.065	0.041	0.051	0.040	0.049	0.041	0.065	0.059
4. No. of ph. cap layers	3	3	3	2	1	3	2	2	3	3	3	3	2	2	2	2
5. No. of xy cap layers	2	2	1	–	–	–	–	1	2	–	1	1	–	–	1	–
6. Length of tracheid/vessel member	0.029	0.031	0.041	0.021	0.019	0.025	0.019	0.029	0.028	0.022	0.029	0.041	0.019	0.021	0.018	0.029
7. Length of xy. Fibre	0.035	0.045	0.019	0.025	0.042	0.037	0.029	0.039	0.027	0.035	0.039	0.019	0.032	0.022	0.041	0.035
8. Length of ph. Fibre	0.044	0.031	0.038	0.041	0.032	0.038	0.015	0.037	0.028	0.039	0.037	0.038	0.041	0.045	0.033	0.040

[1. *Bulbophyllum affine* (Arunachal Pradesh), 2. *B. affine* (Darjeeling), 3. *B. bisetum,* 4. *B. careyanum,* 5. *B. cauliflorum,* 6. *B. cornutum,* 7. *B. crassipes,* 8. *B. fischerii* (Darjeeling), 9. *B. fischerii* (Sikkim), 10. *B. khasyanum,* 11. *B. protractum,* 12. *B. scabratum,* 13. *B. stenobulbon,* 14. *B. tremulum,* 15. *B. umbellatum* (Sikkim), 16. *B. umbellatum* (Darjeeling)]

ph. = phloem, xy = xylem, vb. = vascular bundles; + = present, – = absent

Table 15.5 Stem/pseudobulb: anatomical features in *Dendrobium* (in μm)

	Access. No.															
Anat. feat.	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1. Cuticle thickness	0.006	0.005	0.005	0.003	0.006	0.003	0.004	0.003	0.005	0.006	0.003	0.004	0.006	0.004	0.005	0.006
2. Water storage cells	0.021	0.026	0.030	0.021	0.025	0.019	0.020	0.024	0.027	0.016	0.014	0.021	0.024	–	0.021	0.028
3. Size of vb.	0.041	0.047	0.040	0.031	0.044	0.051	0.031	0.042	0.044	0.049	0.040	0.045	0.039	0.047	0.047	0.042
4. No. of ph. cap layers	2	3	4	3	4	3	3	3	3	3	4	4	3	1	2	2
5. No. of xy. cap layers	–	1	2	–	1	2	3	–	1	1	2	2	1	–	1	–
6. Length of tracheid/vessel member	0.021	0.025	0.031	0.025	0.018	0.021	0.018	0.021	0.018	0.022	0.027	0.019	0.024	0.026	0.025	0.020
7. Length of xylem fibre	0.031	0.042	0.030	0.034	0.031	0.039	0.031	0.041	0.036	0.029	0.038	0.01	0.034	0.038	0.032	0.029
8. Length of phloem fibre	0.034	0.031	0.040	0.031	0.044	0.032	0.039	0.024	0.041	0.039	0.031	0.042	0.041	0.037	0.033	0.039

[17. *Dendrobium anceps* (Darjeeling), 18. *D. anceps* (Skkim), 19. *D. bicameratum*, 20. *D. densiflorum*, 21. *D. haemoglossum*, 22. *D. herbaceum* (Kerala), 23. *D. herbaceum* (Karnataka), 24. *D. heyneanum*, 25. *D. jenkinsii*, 26. *D. microbulbon* (Kerala), 27. *D. microbulbon* (Karnataka), 28. *D. moschatum* (Kerala), 29. *D. moschatum* (Karnataka), 30. *D. nobile*, 31. *D. nutantiflorum*, 32. *D. pendulum*]
ph. = phloem, xy. = xylem, vb. = vascular bundles; + = present, – = absent]

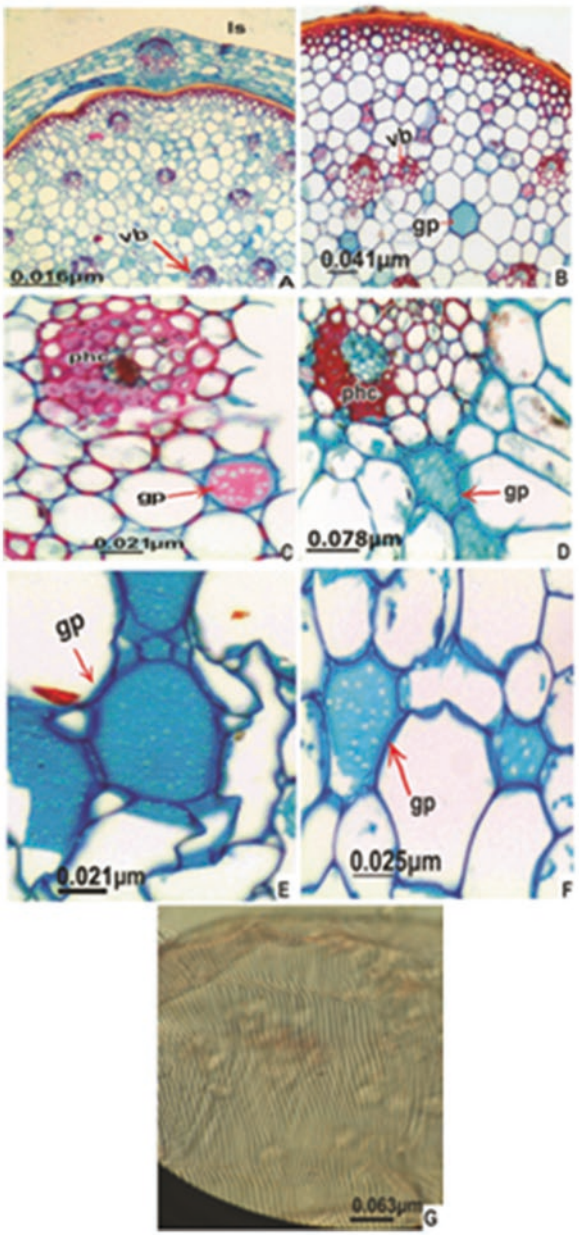

Fig. 15.3 (**a**–**g**). Pseudobulb/stem (**a**) *B. khasyanum*. Pseudobulb cross section indicating leaf sheath and scattered vascular bundles in the ground tissue (**b**) *B. tremulum.* Pseudobulb cross section showing ground tissue and vascular bundles (**c**) *D. anceps*. Fleshy stem cross section indicating the phloem cap and pitted wall thickenings in the ground tissue cell (**d**) *D. microbulbon* Pseudobulb cross section indicating vascular bundle with well-developed phloem cap and cells with pitted thickenings in ground tissue region (**e**) *D. densiflorum* Part of cross section of fleshy stem indicating pitted cell wall thickenings in ground tissue region (**f**) *D. haemoglossum* Stem cross section indicating pitted thickenings in cells of ground tissue (**g**) *D. bicameratum* Water storage cell with multispiral thickenings from stem maceration

15.2.3 Root

In general velamen roots are present in all epiphytic taxa of *Bulbophyllum* and *Dendrobium* (Tables 15.6 and 15.7) and occasionally in terrestrials. The epidermis

Table 15.6 Root: anatomical features in *Bulbophyllum* (in μm)

	Access. No.															
Anat. feat.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. No. of velamen layers	1	2–3	1	1	1	1	6	7	–	5	8	5–7	5–7	1	6–8	4–6
2. Fibrous mats/tilosomes	+	–	+	–	+	+	–	–	–	–	–	+	–	+	–	–
3.Exodermis cell lignification	0.021	0.028	0.031	0.019	0.015	0.021	0025	0.024	0.023	0.020	0.022	0.031	0.026	0.021	0.025	0.030
4.Passage cell size	0.004	0.007	0.003	0.004	0.006	0.003	0.002	0.003	0.005	0.004	0.006	0.003	0.008	0.007	0.004	0.005
5.Endoermis cell lignifiction	0.008	0006	0.010	0.009	0.004	0.007	0.011	0.008	0.005	0.015	0009	0.010	0.006	0.008	0.011	0.013
6.Vascular cylinder diameter	0.051	0.051	0.047	0.053	0.072	0.061	0.052	0.054	0.050	0049	0.044	0.047	0.051	0.049	0.057	0.041
7. No. of protoxylem poles	8	10	13	9	9	8	16	10	12	12	8	13	8	6	10	9

[1. *Bulbophyllum affine* (Arunachal Pradesh), 2. *B. affine* (Darjeeling), 3. *B. bisetum,* 4. *B. careyanum,* 5. *B. cauliflorum,* 6. *B. cornutum,* 7. *B. crassipes,* 8. *B. fischerii* (Darjeeling), 9. *B. fischerii* (Sikkim), 10. *B. khasyanum,* 11. *B. protractum,* 12. *B. scabratum,* 13. *B. stenobulbon,* 14. *B. tremulum,* 15. *B. umbellatum* (Sikkim), 16. *B. umbellatum* (Darjeeling)]

Table 15.7 Root: anatomical features in *Dendrobium* (in μm)

Anat. feat.	Access. No.															
	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
1. No. of velamen layers	9	3–4	6	5–7	1	1	1	1	1	1	2–5	3–6	5–8	1	6	3–5
2. Fibrous mats/tilosomes	–	–	–	9	+	+	+	+	+	+	–	–	–	+	–	–
3. Exodermis cell lignification	0.029	0.028	0.027	0.019	0.030	0.026	0.027	0.021	0.027	0.021	0.028	0023	0.029	0.026	0.029	0.015
4. Passage cell size	0.004	0.006	0.004	0.004	0.005	0.003	0.005	0.004	0.006	0.007	0.004	0.003	0.007	0.005	0.002	0.003
5. Endoermis cell lignifiction	0.012	0.005	0.007	0.010	0.011	0008	0.012	0.011	0.007	0.013	0.009	0.014	0.016	0.010	0.009	0.009
6. Vasclar cylinder diameter	0.0 39	0.047	0.054	0.050	0.055	0.048	0.047	0.045	0.037	0.051	0.058	0.053	0.043	0.046	0.060	0.049
7. No. of protoxylem poles	8	10	12	10	8	10	11	10	11	8	10	10	8	9	10	9

[17. *Dendrobium anceps* (Darjeeling), 18. *D. anceps* (Skkim), 19. *D. bicameratum*, 20. *D. densiflorum*, 21. *D. haemoglossum*, 22. *D. herbaceum* (Kerala), 23. *D. herbaceum* (Karnataka), 24. *D. heyneanum*, 25. *D. jenkinsii*, 26. *D. microbulbon* (Kerala), 27. *D. microbulbon* (Karnataka), 28. *D. moschatum* (Kerala), 29. *D. moschatum* (Karnataka), 30. *D. nobile*, 31. *D. nutantiflorum*, 32. *D. pendulum* + = present, – = absent

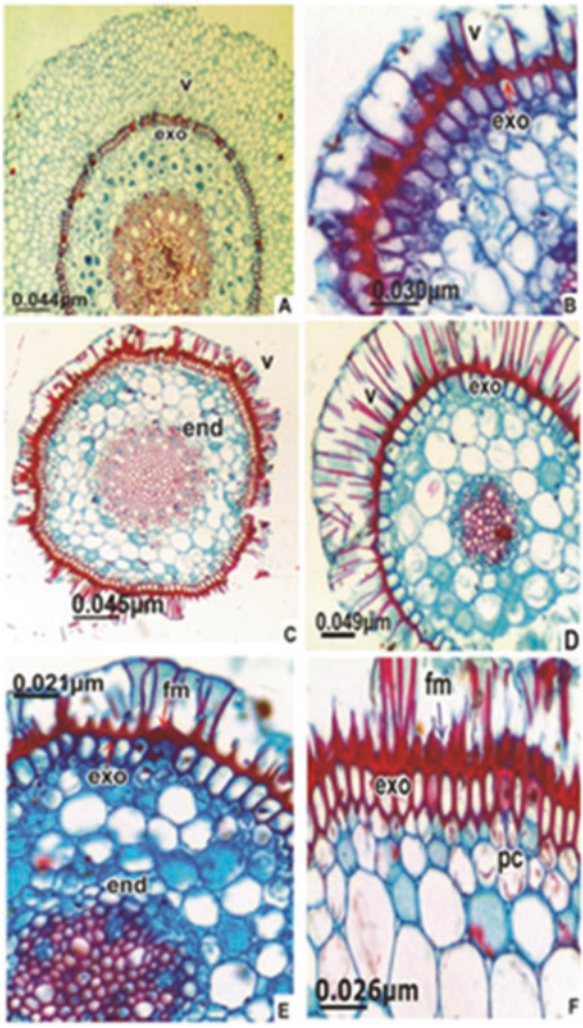

Fig. 15.4 (**a**–**f**) Root (**a**) *B. protractum*. Gross structure of root in cross-section showing multilayered velamen, exodermis and vascular cylinder (**b**) *D. haemoglossum* Root cross section showing single-layered velamen and exodermis with highly thickened inner tangential walls (**c**) *D. heyneanum* Gross structure of root in cross section showing single-layered velamen and vascular cylinder (**d**) *B. tremulum.* Water storage cell from root maceration (**e**) *B. cornutum.* Root transection showing velamen-exodermis complex with fibrous mats (tilosome) and endodermis (**f**) *D. nobile.* Root cross section indicating, fibrous mats, exodermis and also cortical cells possessing pitted thickenings

of mature root is multiseriate with velamen tissue (Fig. 15.4a). In epiphytic taxa, an extensive root system is developed to collect humus from the surrounding area. These roots are classified into two types: (1) substrata roots which penetrate the soil and absorb water and nutrients, and (2) aerial roots that are totally exposed to air and invariably they have multilayered velamen for water absorption, conservation and to provide mechanical strength to the plant body (Dycus and Knudson 1957; Morriset 1964; Benzing 1986, 1989a, b). Roots with single layered velamen (Fig. 15.4b–e) were recorded in almost all the presently studied taxa of *Bulbophyllum* and *Dendrobium*. In young roots, the cells in the outermost velamen layer are smaller than the inner ones; this layer is known as ‘epivelamen’ which is ruptured in mature

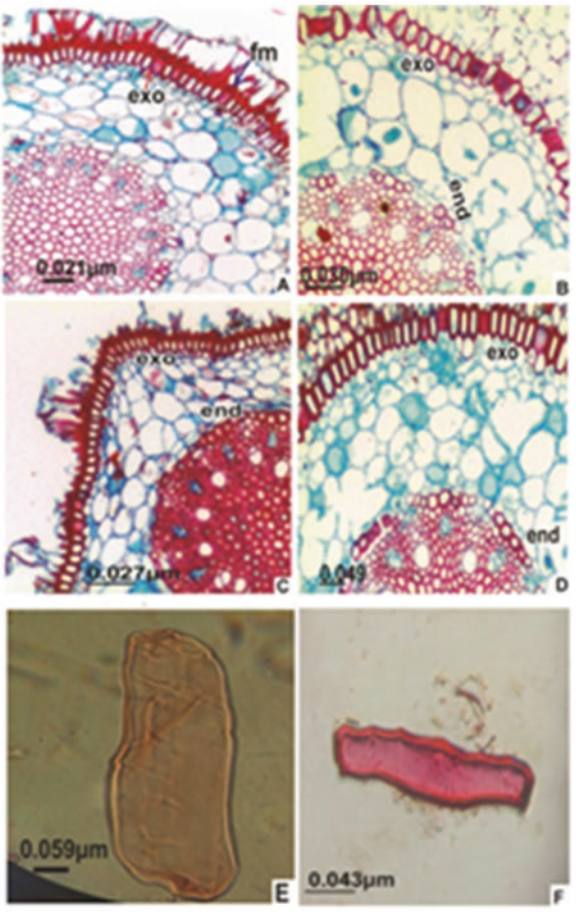

Fig. 15.5 (**a**–**f**) Root (**a**) *D. heyneanum.* Part of cross section of root showing velamen with thickened inner tangential walls, cortex and interrupted endodermis (**b**) *B. umbellatum.* Root cross section indicating exodermis and endodermis (**c**) *D. jenkinsii.* Root cross section showing peeled-off velamen with thickened inner tangential walls, exodermis, cortex, endodermis and vascular cylinder (**d**) *D. pendulum* Part of root cross section showing slightly elongated thick-walled exodermis, cortex and interrupted endodermis (**e**) *B. tremulum*. Water storage cell from root maceration (**f**) *D. moschatum.* Vessel-like tracheid from root maceration

roots. Wide bands of thickenings are found in velamen roots of some presently studied taxa.

Fibrous mats also known as tilosomes (Pridgeon et al. 1983) or fibrous bodies/fibrous mats (Benzing et al. 1983) in more specialized form are observed in some taxa of present investigation, such as *B. affine, B. bisetum, B. careyanum, B. cauliflorum, B. cornutum, B. tremulum, D. haemoglossum, D. herbaceum, D. heyneanum, D. jenkinsii, D. microbulbon and D. nobile* (Fig. 15.4f). Tilosomes also appeared in other Indian species such as *B. leopardianum, D. rotundatum* and *Otochilus alba* (Khasim 1986; Mohana Rao and Khasim 1987a). Tilosomes were observed only in epiphytes whereas absent in terrestrial taxa (Pridgeon et al. 1983; Khasim 1986; present work).

The exodermis, a single layer of cells, is situated in-between the velamen and cortex (Fig. 15.5a–d); in fact, it is the outermost layer of the cortex (Janczewski 1885; Leitgeb 1864; Engard 1944; Shushan 1959). It differs from the velamen by its greater degree of vacuolation and its elongation parallel to the long axis of the root.

At maturity, most exodermal cells do not contain protoplast and they are thick-walled, although a few remain living, thin-walled and these cells are known as "passage cells" (Fig. 15.5b). It is believed that water and solutes pass into the cortex through these cells (Dycus and Knudson 1957).

Cortex is situated in between exodermis and endodermis. It comprises thin-walled cells with cellulosic nature (Fig. 15.5e) and some may be chlorenchymatous. Some cortical cells may give an illusory appearance of vessel-like elements but the thickenings are cellulosic in nature (Fig. 15.5f). Occurrence of endotrophic mycorrhiza in the velamen and cortex is a regular feature in the family Orchidaceae.

Endodermis is uniseriate in all the investigated taxa. It is made up of thick-walled protective cells and interrupted at protoxylem poles by thin-walled passage cells. However, multiseriate endodermis was also reported in *Paphiopedilum venustum, Phragmipedium caudatum* and *P. achroederos* (Rosso 1966). Endodermal cells possess 'O' shaped thickenings (uniform lignification) in all the presently studied taxa.

Vascular cylinder comprises pericycle, phloem, xylem and pith. Phloem strands alternate with xylem strands. Vessel elements are not found in all the presently investigated taxa. However, these were reported in the roots of *Dendrobium peirardii* (Singh 1986). Vessel types and their occurrence constitute an important aspect for estimating evolutionary sequence and degree of advancement in monocotyledons (Dahlgren and Rasmussen 1983). Dahlgren and Clifford (1982) reported vessels in some orchid roots. The presence of vessels in roots is considered to be more advanced than the rhizome, stem and leaf (Cheadle and Kosakai 1980). Since all the investigated taxa are epiphytes, vessels are absent but recorded very long tracheids and vessel-like tracheids in their vegetative parts.

15.3 Anatomy in Relation to Ecological Adaptability

Some of the anatomical features of ecological interest are given below in the Table 15.8. In *Bulbophyllum* leaves are fleshy and differ in their size and form; in some cases leaf is deciduous at flowering. Leaves in *Dendrobium* are commonly distichous, conduplicate and articulate, but they range from terete and coriaceous, to laterally flattened and fleshy (Morris et al. 1996). Leaves may be persistent or deciduous. Persistent leaves are succulent ones and they commonly store water (Holttum 1960), whereas deciduous leaves remained present during wetter season of the year.

Stomatal ledges are prominent on the guard cells in *B. affine, B. careyanum, D. jenkinsii, D. moschatum* and *D. nobile*. These stomatal ledges are helpful in reducing the rate of transpiration from leaf surface and increases resistance to water loss (Yukawa et al. 1991, 1992; Ramesh et al. 2017). The presence of substomatal chambers in all taxa is an added advantage for epiphytic orchids in reducing leaf transpiration and evaporation of water.

In general, adaxial epidermal cells are larger in their size than abaxial epidermal cells. In some cases, e.g. *B. fischerii, B. khasyanum, B. pendulum, B. protractum, B. scabratum, B. stenobulbon, B. umbellatum, D. haemoglossum, D. herbaceum, D.*

Table 15.8 Morphological and anatomical features of ecological interest

Taxa	Habitat	External features	Ade cells size; stomata, distribution; ssc	Absorbing trichomes	Water storage cells and other trachaeoidal elements; fb, vlt	No. of velamen layers	Fibrous mats/ tilosomes
Bulbophyllum affine	E	Thick leaves, fleshy pseudobulbs	**ade** cells comparatively larger; with 2 or 4 subsidiary cells, prominent stomatal ledges, **h**; well-developed **ssc**	–	Simple water storage cells with abundant mucilage; **fb** absent; **vlt** abundant	Single layered	+
B. bisetum	E	Thick leaves, fleshy pseudobulb	**ade** cells comparatively larger; with 4 subsidiary cells, **h**; **ssc** present	+	Simple water storage cells with mucilages; **fb** absent	Single-layered	+
B. careyanum	E	Fleshy pseudobulb, leathery leaves	**ade** cells comp aratively larger; with 2 subsidiary cells (paracytic), stomatal ledges present **h**; well-developed **ssc**	–	Special type of water storage cells with cellulosic thickenings, other cells rich with mucilage; **fb** absent. **vlt** numerous	Single-layered	+
B. cauliflorium	E	Long-sheathed rhizome, fleshy pseudobulbs	**ade** cells comparatively larger; with 2 or 4 subsidiary cells, **h**; small **ssc**	+	Simple water storage cells rich with mucilage; **fb** absent; **vlt** numerous	Single-layered	+
B. cornutum	E	Thick leaves, fleshy pseudobulb	**ade** cells are comparatively larger; with 4–6 subsidiary cells (mostly cyclocytic, **h**; **ssc** present	–	Simple water storage cells rich with mucilage; **fb** absent; **vlt** numerous	Single-layered	+
B. crassipes	E	Leathery leaves, fleshy pseudobulb	**ade** cells are comparatively larger; with 4 subsidiary cells (tetracytic), **h**; **ssc** present	–	Simple water storage cells rich with mucilage; *fb* absent; **vlt** numerous.	6-layered	–

B. fischerii	E	Fleshy pseudobulb	**ade** *c*ells two times larger; with 4–5 subsidiary cells, **h; ssc** very small	–	Special type of water storage cells with multi- spiral cellulosic thickenings, simple water storage cells rich with mucilage; **fb** absent; **vlt** present	7–8 layered	–
B. khasyanum	E	Leaves coriaceous, fleshy pseudobulbs	**ade** cells two times larger; with 5 subsidiary cells, **h**; **ssc** present	–	Simple water storage cells rich with mucilage; **fb** absent	5- layered	–
B. protractrum	E	Fleshy pseudobulb	**ade** cells two times larger; with 5 subsidiary cells, **h**; **ssc** present	–	Simple water storage cells rich with mucilage; **fb** absent; **vlt** present	8- layered	–
B. scabratum	E	Fleshy pseudobulb	**ade** cells two times larger; with 4 subsidiary cells, **h; ssc** present.	+	Simple water storage cells with mucilage	Single-layered	+
B. stenobulbon	E	Fleshy leaves, cylindrical pseudobulbs	**ade** *c*ells 2–3 times larger; with 2–3 subsidiary cells, **h; ssc** present	+	Simple water storage cells rich with mucilage; cells with pitted thickenings **fb** absent; **vlt** present	5- layered	–
B. tremulum	E	Fleshy pseudobulb	**ade** cells comparatively larger; with 4–5 subsidiary cells, **h; ssc** absent	–	Simple water storage cells rich with mucilage, cells with pitted thickenings; **fb** absent; vessel-like tracheids	Single-layered	+

(continued)

Table 15.8 (continued)

Taxa	Habitat	External features	Ade cells size; stomata, distribution; ssc	Absorbing trichomes	Water storage cells and other trachaeoidal elements; fb, vlt	No. of velamen layers	Fibrous mats/ tilosomes
B. umbellatum	E	Ovoid pseudobulb, fleshy	**ade** cells 2–3 times larger, with 4–5 subsidiary cells or anomocytic in some cases. **h; ssc** very small	+	Special type of water storage cells with multispiral cellulosic thickenings, simple water storage cells with mucilage; **vlt** present	4–6 layered	–
Dendrobium anceps	E	Stem stout, leaves leathery	**ade** cells comparatively larger; with 4–5 subsidiary cells, **h**; no. **ssc**	+	Special water storage cells with cellulosic thickenings, cells with pitted thickenings; **fb** present, **vlt** numerous	5–9 layered	–
D. bicameratum	E	Stem fleshy	**ade** cells comparatively larger; with 4–5 subsidiary cell, **h; ssc** present	–	Special type of water storage cells with multispiral cellulosic thickenings, **fb** absent; **vlt** present.	6- layered	–
D. densiflorum	E	Thick leaves; fleshy stems	**ade** cells comparatively larger; with 45 subsidiary cells, **h**; very small, **ssc** present	–	Simple water storage cells rich with mucilage, some cells with pitted thickenings; **fb** absent; **vlt** present.	5–6 layered	–
D. haemoglossum	E	Fleshy stem	**ade** cells two times larger; with 5 subsidiary cells, **h**; smaller size, **ssc** present	–	Simple water storage cells rich with mucilage and also abundant starch **fb** absent; **vlt** numerous	Single layered	+
D. herbaceum	E	Fleshy stem	**ade** cells, 2–3 times larger; with 4–5 subsidiary cells, **h**; small **ssc**	+	Simple water storage cells with abundant mucilage; **fb** absent; **vlt** numerous	Single layered	+

D. heyneanum	E	Leathery leaves, fleshy stems	**ade** cells two times larger; with 4–6 subsidiary cells, **h**;	+	Simple water storage cells rich with mucilage, cells with pitted thickenings; **fb** absent.	Single layered	+
D. jenkinsii	E	Leathery leaves, fleshy pseudobulb	**ade** cells comparatively larger; with 4 subsidiary cells, prominent stomatal ledges, **h; ssc** present	+	Simple water storage cells with abundant mucilage; **fb** absent; **vlt** numerous.	Single layered	+
D. microbulbon	E	Thick leaves, fleshy pseudobulb	**ade** cells 2–3 times larger; with 4 subsidiary cells, **h; ssc** present	+, some times absent	Simple water storage cells with abundant mucilage, cells with pitted thickenings; **vlt** present	1–4 layered	+ (inconspicuous)
D. moschatum	E	Leaves leathery, fleshy stems	**ade** cells 2 or 3 times larger; with 5 subsidiary cells, prominent stomatal ledges, **h; ssc** not seen	+	Simple water storage cells with abundant mucilages, cells with pitted thickenings**;** **fb** absent; **vlt** numerous	6–8 layered	–
D. nobile	E	Fleshy pseudobulb like stem	**ade** cells 2 times larger; with 4 subsidiary cells, **h; ssc** present	–	Simple water storage cells with abundant mucilage, **vlt** present	Single-layered	+
D. nutantiflorum	E	Thick stems	**ade** cells 2–3 times larger; with 5 subsidiary cells, **h**; **ssc** present	–	Simple water storage cells rich with mucilage; **fb** absent, **vlt** numerous	6- layered	–
D. pendulum	E	Thick leaves, fleshy pseu- dobulbs	**ade** cells comparatively larger; with 4–5 subsidiary cells, **h; ssc** present	+	Simple water storage cells rich with mucilage, presence of bulliform ells, **fb** absent; **vlt** present	3–5 layered	–

ade = adaxial**, e** = epiphyte, **h** = hypostomatic, **fb** = fibre bundle, **ssc** = substomatal chamber, **vlt** = vessel-like tracheid; **+** = present, **–** = absent

heyniyanum, D. microbulbon, D. moschatum, D. nutantiflorum, D. nobile and *D. pendulum*, adaxial epidermal cells are 2–3 times larger in their size than abaxial ones. Such type of larger adaxial epidermal cells were also reported in *D. cumulatum, D. falconeri, D. gibsonii and D. parishii* (Isaiah 1993). All these large epidermal cells are thin-walled, mucilagenous, hyaline and function in storage of water.

Absorbing trichomes are present in both *Bulbophyllum* and *Dendrobium* species. These may be 3-celled in *B. bisetum, B. umbellatum, D. anceps*, *D. herbaceum, D. jenkinsii* and *D. pendulum;* whereas 2-celled in *B. cauliflorum, B. stenobulbon, B. heyneanum, D. microbulbon* and *D. moschatum*. Systematic occurrence of absorbing trichomes were recorded in Pleurothallidinae (Pridgeon 1981). These are sunken and glandular with a basal cell. Apical cells of trichomes on adaxial leaf surface generally rupture and a brown opaque residue covers the exposed portion of the basal cell (stalk cell). Pridgeon (1981) observed that amylacetate-alcohol dissolves the residue, rendering the stalk cell's lateral walls clearly visible. Inward movement of eosin or safranin stain from the apical cell through the stalk cell and into hypodermal cells indicates an absorptive function.

As such distinct hypodermis is absent; however, fibre bundles are observed in *D. anceps* at hypodermal position. Fibre bundles provide mechanical strength to the plant body along with special type of water storage cells with multispiral cellulosic thickenings.

Pseudobulbs are uninodal or polynodal organs. They show a great range of variation in their size and shape. Pseudobulbs are conical-ovoid in *B. bisetum* and *B. fischerii*, ellipsoidal in *B. crassipes*, cylindrical in *B. protractum, B. umbellatum* and *B. stenobulbon* and sub-globose in *B. tremulum*. In the case of *D. jenkinsii*, pseudobulbs are bottle-shaped; mostly in presently studied taxa of *Dendrobium*, stems are fleshy and erect or pendulous without bulbous nature. Thick cuticle and sinuous walls of epidermal cells are helpful in reducing the transpiration and also harden the tissue (Yukawa and Uehara 1996).

In some cases, these larger cells are modified into special type of water storage cells with multispiral cellulosic thickenings observed in *B. careyanum, B. fischerii, B. umbellatum, D. anceps* and *D. bicameratum*. All these larger parenchymatous cells with or without thickenings serve as water storage cells and comprise the succulent tissue of the organ (Wilder 1985; Koller and Rost 1988a, b; Stern and Morris 1992).

As it was opined by Moreira et al. (2013) that the well-developed velamen roots, distinct exodermis and endodermis, and specialized thick-walled cortical cells are the characteristic features of epiphytic orchids. This tissue is supposed to act as a sponge, absorbing the moisture from the atmosphere. In fact, the velamen stores water which is utilized by plant during dry conditions. Pridgeon (1987) reviewed the functional aspects of velamen. Tangential walls of cells in the innermost velamen layer are much thickened and form fibrous mats, also known as fibrous bodies or tilosomes (Benzing et al. 1982; Pridgeon et al. 1983). It was observed in the presently investigated taxa that the well-developed fibrous mats (tilosomes) are always associated with single-layered velamen (Table 15.8). This type of situation was also reported from *D. rotundatum* (Khasim and Mohana Rao 1984). Dycus and Knudson

(1957) called these fibrous bodies as "layers of even mats" situated on velamen cell walls immediately above the passage cells in several epiphytic taxa. The fibrous bodies in *Sobralia macrantha* (Benzing et al. 1982) and *D. rotundatum* (Khasim and Mohana Rao 1984) have been described as hygroscopic device designated to facilitate the condensation of atmospheric moisture prior to its absorption through underlying passage cells, and as a possible aid to the acquisition of atmospheric ammonia (Haberlandt 1914). The fibrous mats/tilosomes promote water economy in orchids. The labyrinthine structure of tilosome lengthens the pathway, the water vapour must traverse to breach the exodermis-velamen barrier during transpiration (Khasim and Mohana Rao 1984). This notion parallels to the plug hypothesis of (Leitgeb 1856). If the fibrillar components of tilosome alternately shrink and swell upon desiccation and hydration, its mass would function as a one way valve and not as a plug (Benzing et al. 1982).

Velamen may be single-layered or multi-layered. In single-layered velamen roots, exodermis is well developed with long thick-walled and short thin-walled passage cells. This single-layered velamen is peeled-off in mature roots. As a result, the entire interior part of the root is exposed out and there is a possibility of losing water from the root very easily. So as to prevent the water loss, tilosomes were conspicuously seen just above the exodermis in these single-layered velamen roots. Tilosomes are completely absent from the multi-layered velamen roots of *Bulbophyllum* and *Dendrobium* species. From this discussion, it can be presumed that the single-layered velamen roots have undergone anatomical adaptations so as to protect the root from desiccation and transpiration of water from interior parts of root.

The diversification of velamen characters is also exemplified by the type of habitat and host tree on which *Bulbophyllum* and *Dendrobium* species are growing continuously (Ramesh et al. 2017). When compared to Darjeeling collections, plants collected from Arunachal Pradesh habitat show some xeric characters; with respect to velamen, it is well-developed in Arunachal collections of *B. umbellatum* at altitude 1500 m when compared to Darjeeling at 1650 m elevation. However, velamen is well developed in *D. anceps* plants collected from Darjeeling (9-layered) than that of Sikkim collections at 2500 m elevation (3–4 layered velamen). Besides, tilosomes are observed in *B. affine* of Arunachal collections whereas absent in Darjeeling accessions. Isaiah (1993) reported 1–2 layered velamen with tilosomes in *B. protractum*, whereas in the presently studied collection at 1650 m elevation from Arunachal Pradesh velamen is multilayered without tilosomes. This report further indicates that Arunachal Pradesh habitat shows more xeric elements than that of Darjeeling where luxuriant growth of orchids is found. Similarly Karnataka region at 850 m altitude shows some xeric condition when compared to Kerala at 950 m elevation, both come under Western Ghats of India. This is evident from the well developed velamen roots of *D. microbulbon* and *D. moschatum* collected from Karnataka region (Tables 15.6 and 15.7). Moreover, more number of water storage cells with abundant mucilage is observed in *D. microbulbon* collected from Karnataka than that of Kerala collections. From the above discussion, it is evident

that those plants growing in lower elevation are showing more xeric conditions than those plants of higher elevation.

Exodermis in root possesses long, thick-walled and broad thin-walled passage cells. The thick-walled cells prevent water escaping from the conducting tissues in the interior of roots; thin-walled passage cells allow the water-soluble nutrients to pass through from outside into the conducting tissues. Water-soluble nutrients are checked by tilosomes and these pass through the passage cells into the interior of roots. Just like velamen, the exodermal thickenings aid in the reduction of water loss by root transpiration (Benzing et al. 1983), turning into an important apoplastic barrier (Hose et al. 2001; Ma and Peterson 2003).

Endodermis is interrupted by thin-walled passage cells at protoxylem poles. In all the investigated taxa, endodermal cells are uniformly lignified ('O' shaped thickenings). In both samples of *D. anceps* collected from Darjeeling and Sikkim have showed poor lignification of endodermal cells. But Isaiah (1993) reported high lignification in endodermis of some species, also collected from Sikkim. This can be attributed that not only the habitat conditions but also the supply of nutrients by host plant plays a vital role for the survival of epiphytic orchids.

Vascular cylinder in the root consists of pericycle, phloem, xylem and pith. In *Bulbophyllum* the number of protoxylem poles is 6–16 whereas in *Dendrobium*, it is 8–12 (Tables 15.6 and 15.7). On the basis of the number of protoxylem poles Rosso (1966) classified orchids belonging to Cypripedioideae into two groups: (I) protoxylem points 8 or less and (ii) protoxylem points 9 or more.

In orchid root, vascular cylinder is polyarch in nature. In most of the investigated taxa, fibre sheath was observed around xylem and phloem. Vessels were not found in majority of taxa, instead vessel-like tracheids were abundant in almost all the taxa. However, vessels were reported from the roots of *Dendrobium pierardii* (Singh 1986), *D. amplum* and *D. thyrsiflorum* (Isaiah 1993). Carlquist and Schneider (2006) reported vessels in other members of Epidendroideae. Cheadle (1942) found vessels with scalariform perforation plates and also with simple perforation plates in some orchid taxa; he opined that the vessels do not occur in the shoot system of typically bulbose or cormose plants, but occur most commonly in roots, less commonly in leaves, and an intermediate way in aerial roots. This can be interpreted as an adaptation for rapid uptake of water during brief periods of water availability (Carlquist and Schneider 2006). Kaushik (1983) also opined that vessels must have been eliminated due to development of other water storage mechanisms in the plant body; in fact, epiphytes, which are cut-off from the ground perhaps, have no need of possessing vessels.

Mycorrhizal association is found in the roots of presently investigated taxa. It was also observed in rhizomes of *Zeuxine gracilis* (Muthu Kumar et al. 2011). In fact orchid seed germinates only after being infected by fungal mycelium. No other members of angiospermous family, except Orchidaceae, have maximum exploitation of endotrophic fungus for their nutritional requirements. Withner (1974) postulated that orchid-fungus association in various types of soils as indicative of deficient soil nutrient supply rather than that of a particular host-fungus specificity. Rayer (1927) opined that the possession of mycorrhiza is infrequently beneficial to

vascular plants. Phytoalexins undoubtedly play an important role in this respect (Arditti 1979).

Having no direct root contact to the soil, epiphytes lack access to the most important nutrient source of ground-rooted plants. Sources for epiphytic orchids are atmospheric inputs (rain, dust and intercepted mist), nutrients released from ground-rooted host plants through leaching or decomposition and to a lesser extent, remains of animals as well as mineral and organic matter (Benzing 1990). Awasti et al. (1995) reported that stemflow leachates are the main source of ammonium-N and nitrate-N for uptake by orchids of Sikkim Himalaya. Nutrient scavenging in epiphytes is assisted by unusual morphological structures such as velamen roots with tilosomes, extensive development of roots, absorbing trichomes etc. However, some ecologists pointed out that though nutrients are scarce, this may not be of much importance, but the prime limiting factor is water (Zotz and Heitz 2001). So as to store and conserve the water, orchid has undergone various morphological adaptations such as presence of pseudobulbs, succulent/leathery leaves, presence of water storage cells with multispiral cellulosic thickenings.

From the entire discussion of this chapter, it is evident that there is no generalized pattern of growing of epiphytic orchids; not only the geographical conditions and type of habitat, but also the host-tree on which orchid grows, is playing vital role in survivability of epiphytic orchids (Ramesh et al. 2017) However, this needs further study to confirm. Those orchids that are getting poor supply of nutrients from host plant, undergo anatomical adaptations so as to survive under extreme environmental conditions (Khasim and Ramesh 2010; Ramudu et al. 2012). Sikkim-Himalaya is known to be congenial for orchid growth as it has sufficient rainfall and, warm and humid conditions prevailing throughout the year. However, leaves and roots of *B. fischerii* collected from Sikkim itself showed much larger size of leaf adaxial epidermal cells and well-developed, 12-layered velamen roots. This could be attributed to the host-tree, *Meliosma dillenifolia*, on which it is growing (Table 15.1); leaves and roots have undergone structural adaptations so as to conserve the nutrient supply appropriately (Khasim and Ramesh 2010). As Zotz and Heitz (2001) pointed out, a more integrative approach to study the epiphytic biology is needed including physiological investigations, substrate instability, dispersal limitation and competition (intra and inter specific level).

15.3.1 Tribal and Subtribal Delineation in Dendrobieae of Subfamily Epidendroideae

Lindley (1830–1840), Bentham and Hooker (1883), Rolfe (1909), Mansfeld (1937a, b), Hatch (1954), Dressler and Dodson (1960), and Melchior (1964) treated the Epidendroideae as one of the major tribes in family Orchidaceae; whereas Vermeulen (1966), Garay (1972), Thorne (1976), Dressler (1981), Rasmussen (1985) and Dressler (1986, 1993) regarded the Epidendroideae as a subfamily of Orchidaceae.

The subfamily Epidendroideae resembles the other members of Orchidaceae in possessing both terrestrial and epiphytic habits, larger adaxial epidermal cells and

homogeneous mesophyll. However, Epidendroideae deviate markedly in having hypodermis in leaf; stomata of cyclocytic, diacytic and in some cases paracytic type, heterogeneous mesophyll; well-developed sclerenchymatous sheath around vascular bundles; and velamen roots with uniformly thickened or U-shaped thickenings in exodermal and endodermal cells. With respect to embryology, they show similarities with members of Orchidaceae in possessing zygomorphic flowers, column, rostellum, unilocular ovary, parietal placentation, capsular fruit and numerous tiny transparent non-endospermic seeds. But Epidendroideae deviate from other members of Orchidaceae in possessing well-developed suspensor and thick cell-walled seed coat.

With respect to chemistry, Epidendroideae show affinity with members of Orchidoideae in having flavone C-glycosides, anthocyanins, phenanthrenes and coumarins, but they deviate significantly in other chemical constituents like alkaloids, 9, 10-dihydrophenanthropyrans and pyrones, steroides, triterpenoids and bibenzyls (Veerraju 1990). Due to these significant differences in anatomy, embryology and chemical constituents, Epidendroideae deserves the status of subfamily.

The members of tribe Dendrobieae are characterized by the presence of naked pollinia without caudicles or any other appendages, a prominent column foot and *Dendrobium* seed type. *Bulbophyllum* belonging to subtribe Bulbophyllinae is quite distinct from subtribe Dendrobiinae in its habit (pseudobulbs of a single internode and basal inflorescence), absence of silica bodies, presence of leaf hypodermis and more number of protoxylem poles upto 16 in roots (Table 15.9). However, both genera *Bulbophyllum* and *Dendrobium* share several common anatomical features such as presence of prominent stomatal ledges, substomatal chambers, well-developed phloem cap, water storage cells with multispiral cellulosic thickenings and single-layered velamen roots (Table 15.10). These resemblances strongly

Table 15.9 Dissimilar anatomical features (quantitatively) of *Bulbophyllum* and *Dendrobium*

Anatomical features	*Bulbophyllum*	*Dendrobium*	References
Leaf Cuticle thickness in leaf	0.005–0.019	0.004–0.012 µm	Present study
Length of guard cells	0.008–0.021	0.010–0.025 µm	Present study
Hypodermis	Present; however, absent in most of the taxa of present study	Absent	Isaiah (1993); present study
Laminar vascular bundles	0.032–0.062 µm	0.041–0.063 µm	Present study
Root Exodermis lignification	0.015–0.31 µm	0.015–0.029 µm	Present study
Endodermis lignification	0.003–0.013 µm	0.005–0.016 µm	Present study
Vascular cylinder diameter	0.041–0.061 µm	0.037–0.060 µm	Present study
No. of protoxylem poles	6–16	8–12	Kaushik 1983; present study

Table 15.10 Common anatomical features shared by *Bulbophyllum* and *Dendrobium*

Anatomical features	*Bulbophyllum*	*Dendrobium*	References
Stomata	With 2–6 subsidiary cells (cyclocytic)	With 4–6 subsidiary cells (cyclocytic)	Mohana Rao and Khasim (1987c), present study
Stomatal ledges	Prominent	Prominent	Mohana Rao and Khasim (1987c), present study
Substomatal chambers	Present	Present	Mohana Rao and Khasim (1987c), Isaiah 1993 present study
Absorbing trichomes	Present	Present	Mohana Rao and Khasim (1987c), present study
Fibre bundles in leaf	Present in few cases	Present in few cases	Mohana Rao and Khasim (1987c), present study
Mesophyll	Homogeneous, in few cases differentiated	Homogeneous	Mohana Rao and Khasim (1987c), present study
Phloem cap in leaf and stem	Well developed	Well developed	Mohana Rao and Khasim (1987c), present study
Special water storage cells	Present, columnar or oval shaped	Present, club or oval shaped	Mohana Rao and Khasim (1987c), present study
Single-layered velamen	Present in some cases with fibrous mats (tilosomes)	Present, in some cases with fibrous mats (tilosomes)	Isaiah (1993), present study

support the view of Dressler (1993) that both subtribes Bulbophyllinae and Dendrobiinae are sister groups of tribe Dendrobieae.

15.4 Interrelationships and Phylogenetic Implications

Morris et al. (1996) stated that the comparative anatomy and systematics are common strategies to understand the relationships among *Dendrobium* and also *Bulbophyllum* spp. Nor Hazlina et al. (2013) opined that morphological characters are very important in distinguishing various species and also interspecific hybrid progenies. They also stated that the data on relationships among the species and hybrids are useful to select the parents for hybrid production.

In *Bulbophyllum* sections (Table 15.11), viz., *Desmosanthus* is characterized by larger adaxial epidermal cells (two to three times larger in their size); similarly in the section *Cirrhopetalum* species such as *B. scabratum* and *B. umbellatum* larger adaxial epidermal cells are found; further phloem cap is well developed in the section *Cirrhopetalum*. Both single- and multi-layered velamen is observed in the

Table 15.11 [a]Sectional delineation in *Bulbophyllum* Thou. and *Dendrobium* Sw

Section	Species
BULBOPHYLLUM Thouars	
Sestochilos (Breda) Benth. Hk.f.	(i) *Bulbophyllum affine* Lindl.
Desmosanthes (Bl.) J.J.Sm.	(i) *B. cauliflorum* Hk. f.
	(ii) *B. protractum* Hk.f.
	(iii) *B. stenobulbon* Par. et Rchb. f.
Racemosae Benth. ex Hk. f.	(i) *B. bisetum* Lindl.
	(ii) *B. careyanum* (Hook.) Sprngl.
	(iii) *B. crassipes* Hk.f.
Cirrhopetalum Lindl.	(i) *B. cornutum* (Lindl.) Rchb.f. (*Cirrhopetalum cornutum* Lindl.)
	(ii) *B. fischerii* Seidenf. (*Cirrhopetalum gamblei* Hk.f. *C. thomsonii* Hk.f.)
	(iii) *B. scarbratum* Rchb. f. (*C. caespitosum* Lindl.)
	(iv) *B. umbellatum* Lindl. [=*B.maculosum* (Lindl.) Rchb. f.]
Globiceps Schltr.	(i) *B. khasyanum* Griff.
Pleiophyllus J.J. Sm.	(i) *B. tremulum* Wt.
DENDROBIUM Swartz	
Aporum Bl.	(i) *D. anceps* Sw.
Breviflores Hk.f.	(i) *D. bicameratum* Lindl.
Dendrobium Sw.	(i) *D. nobile* Lindl.
	(ii) *D. pendulum* Roxb. (*D. crassinode* Benson & Rchb.f.)
Densiflora Finet	(i) *D. densiflorum* Lindl.
	(ii) *D. jenkinsii* Wall. ex Lindl.
Grastidium Bl.	(i) *D. haemoglossum* Thw.
Formosae (Benth. et. Hk.f.) Hk.f.	(i) *D. nutantiflorum* Hawk. et Helr. (D. *jerdonianum* Wt.)
Herbacea Krzl.	(i) *D. herbaceum* Lindl.
Holochrysa Lindl.	(i) *D. moschatum* (Buch.-Ham.) Sw.
Stachyobium Lindl.	(i) *D. heyneanum* Lindl.
	(ii) *D. microbulbon* A.Rich.

[a]According to Garay et al. (1994), Wood (2006)

section *Cirrhopetalum.* Single-layered velamen is also found in other studied taxa belonging to *Sestochilus* and *Pleiophyllus*. Mohana Rao and Khasim (1987c) reported the same type of velamen roots in *B. andersonii*. Further well-developed multi-layered velamen is also recorded in sections *Desmosanthes, Racemosae* and *Globiceps*. This anatomical data clearly indicates that the section *Cirrhopetalum* is a unique one, from which other groups have originated. This strengthens the Schlechter's (1912) opinion that *Cirrhopetalum* species are true bulbophyllums. The assemblage of some vegetative characters in the section *Cirrhopetalum* and appearance of these characters in other sections of *Bulbophyllum*, justify that *Cirrhopetalum* must have existed prior to the origin of other *bulbophyllums*. Molecular data also support this assumption (Ramesh et al. 2017). However, some more studies are needed to ascertain this statement.

In case of genus *Dendrobium*, both single and multi-layered velamen was recorded in the sections *Dendrobium* and *Densiflora*. The section *Stachyobium* in the present study had sheerly showed single-layered with well-developed tilosomes and also with larger adaxial epidermal cells in leaf; whereas in the sections of *Aporum, Breviflores, Formosae* and *Holochrysa*, well-developed multi-layered velamen was recorded.

The presently investigated taxon *D. anceps* of *Dendrobium* section *Aporum*, is characterized by the presence of 3-celled absorbing trichomes, suberized epidermal cells and fibre bundles at subepidermal region in leaf. Similar anatomical features were recorded in *D. aloifolium* also belongs to the section *Aporum* by Solereder and Meyer (1930) and Morris et al. (1996).

Leaf anatomy of *D. anceps*, only species representing the section *Aporum* in the present investigation, is similar to that of species of the section *Rhizobium* in possessing three-celled absorbing trichomes, suberized epidermal cells and fibre bundles (Carlsward et al. 1997). This anatomical data supports the view of Stern et al. (1994) that the section *Aporum* is a sister group of the section *Rhizobium*. Based on cladistic analysis with leaf anatomical features, Carlsward et al. (1997) demonstrated that both these groups are monophyletic.

The morphological characters (quantitative data; Tables 15.12 and 15.13) from various species of *Bulbophyllum* and *Dendrobium* are taken and subjected to Hierarchical cluster analysis using Euclidean distance to determine the distance among various species (Tables 15.14 and 15.15).

Bulbophyllum In *Bulbophyllum*, a range of 1.00–11.87 Euclidean distance values are observed (Table 15.14). *B. crassipes* has highest (11.87) and *B. careyanum* lowest (1.00) values. The dendrogram based on anatomical features of *Bulbophyllum* (Fig. 15.6) revealed 3 clusters as follows:

Cluster I –	*B. umbellatum, B. scabratum, B. fischerii, B. khasyanum, B. stenobulbon, B. protractum.*
Cluster-II –	*B. cauliflorum, B. careyanum, B. cornutum, B. affine, B. tremulum, B. bisetum.*
Cluster-III –	*B. crassipes*

From the dendrogram (Fig. 15.6), it is evident that *B. umbellatum* is closely related to *B. scabratum*; in the same way *B. stenobulbon* has close affinity with *B. protractum*; similarly *B. cauliflorum* with *B. careyanum*.

It is also noted from the dendrogram (Fig. 15.6) that the section *Cirrhopetalum* species, such as *B. cornutum, B. fischerii, B. scabratum* and *B. umbellatum*, are scattered among two clusters. This indicates that all species of the section *Cirrhopetalum* are in one way or other related to other sections of *Bulbophyllum*. In other words, other *Bulbophyllum* species show some affinity with this section. This supports the view that all other *Bulbophyllum* species might have derived from the section

Table 15.12 Diagnostic anatomical features (quantitatively) in *Bulbophyllum* used for dendrogram construction

Taxa	Size of adaxial epidermal cells in leaf (μm)	No. of subsidi- ary cells in stoma	No. of phloem cap layers in leaf	No. of velamen layers in root	No. of proto- xylem poles in root
Bulbophyllum affine	0.025	2–4	2–4	1	8–10
B. bisetum	0.023	4	3	1	13
B. careyanum	0.026	4–5	3	1	9
B. cauliforum	0.028	4–5	2	1	9
B. cornutum	0.025	4–6	3	1	8
B. crassipes	0.029	4	2	5–7	16
B. fischerii	0.029	4–5	2–3	7–8	10–12
B. khasyanum	0.005	5	2	5	12
B. protractum	0.024	5	5	8	8
B. scabratum	0.037	4	3	5–7	10
B. stenobulbon	0.025	4–5	6	5–7	8
B. tremulum	0.024	4–5	4	1	6
B. umbellatum	0.031	4	2	6–8	10

Table 15.13 Diagnostic anatomical features (quantitatively) in *Dendrobium* used for dendrogram construction

Taxa	Size of adaxial epidermal cells in leaf (μm)	No. of subsidi- ary cells in stoma	No. of phloem cap layers in leaf	No. of velamen layers in root	No. of proto- xylem arches in root
Dendrobium anceps	0.019	4–5	2	7–9	8–10
D. bicameratum	0.021	2	3	6	12
D. densiflorum	0.025	4	3	5–7	8
D. haemoglosum	0.031	5	3	1	8
D. herbaceum	0.027	4–5	2–3	1	10–11
D. heyneanum	0.032	4–6	3	1	10
D. jenkinsii	0.002	4–5	2	1	11
D. microbulbon	0.024	5	2	2–5	8–10
D. moschatum	0.026	4	2–3	5–8	8–10
D. nobile	0.029	4	2	1	9
D. nutantiflorum	0.003	4	3	6	7
D. pendulum	0.028	4–5	2	3–5	9

Table 15.14 Distance Matrix (Euclidean Distance) based on anatomical features in *Bulbophyllum*

Taxa	*B. affine*	*B. bisetum*	*B. careyanum*	*B. cauliflorum*	*B. cornutum*	*B. cressipes*	*B. fischeri*	*B. Khasyanum*	*B. protractum*	*B. scabratum*	*B. stenobulbon*	*B. tremulum*	*B. umbellatum*
Bulbophyllum affine	–												
B. bisetum	3.162	–											
B. careyanum	1.732	4.123	–										
B. cauliflorum	2.449	4.243	1.000	–									
B. cornutum	3.000	5.385	1.414	1.732	–								
B. crassipes	8.718	6.782	9.327	9.274	10.247	–							
B. fischerii	7.416	7.141	7.616	7.681	8.124	4.359	–						
B. khasyanum	5.000	4.359	5.099	5.000	5.831	4.583	3.162	–					
B. protractum	7.416	8.888	7.348	7.681	7.348	8.660	4.472	5.831	–				
B. scabratum	6.083	6.708	6.164	6.245	6.633	6.083	2.450	3.162	3.162	–			
B. stenobulbon	6.708	8.426	6.782	7.280	6.782	9.000	5.099	6.000	1.414	3.742	–		
B. tremulum	4.123	7.141	3.162	3.606	2.449	11.874	9.274	7.483	7.348	7.348	6.633	–	
B. umbellatum	7.280	7.681	7.211	7.141	7.616	6.083	2.449	3.742	3.742	1.414	4.690	8.367	–

Table 15.15 Distance Matrix (Euclidean Distance) based on anatomical features in *Dendrobium*

Taxa	*D. anceps*	*D. bicameratum*	*D. densiflorum*	*D. haemoglosum*	*D. herbaceum*	*D. heyneanum*	*D. jenkinsii*	*D. microbulbon*	*D. moschatum*	*D. nobile*	*D. nutantifium*	*D. pendulum*
Dendrobium anceps	–											
D. bicameratum	4.796	–										
D. densiflorum	3.162	4.583	–									
D. haemoglosum	8.307	7.071	6.083	–								
D. herbaceum	8.124	5.916	6.782	3.000	–							
D. heyneanum	8.124	6.708	6.633	2.236	1.414	–						
D. jenkinsii	8.063	6.000	6.856	3.162	1.000	1.732	–					
D. microbulbon	4.000	3.873	3.162	4.583	4.243	4.243	4.123	–				
D. moschatum	1.732	3.464	2.236	7.348	7.141	7.280	7.211	3.317	–			
D. nobile	8.124	6.245	6.164	1.732	2.449	2.449	2.236	4.243	7.141	–		
D. nutantiflorum	4.472	5.385	1.414	5.196	6.481	6.164	6.557	3.464	3.606	5.477	–	
D. pendulum	4.123	4.472	2.646	4.243	4.583	4.359	4.472	1.000	3.464	4.123	2.646	–

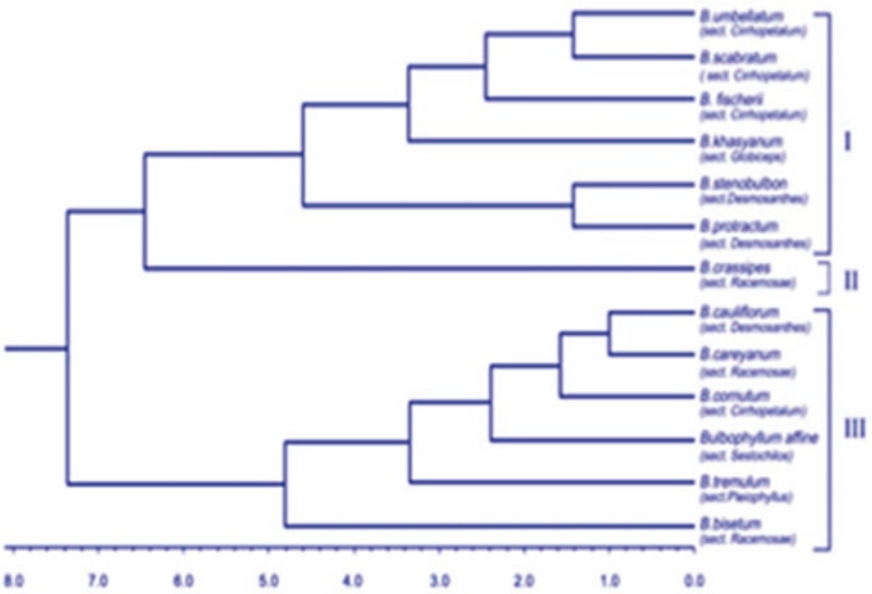

Fig. 15.6 Dendrogram showing dissimilarity among *Bulbophyllum* based on anatomical features

Cirrhopetalum which is considered to be the ancestral one to others. However, this needs further study. *B. crassipes* showing 6.5 dissimilarity value, does not form cluster with any other group of species.

Dendrobium

In *Dendrobium*, a range of 1.00–8.30 Euclidean Distance values are observed (Table 15.15); *D. haemoglossum* has the highest (8.30) whereas *D. jenkinsii* lowest (1.00) Euclidean distance values. The dendrogram (Fig. 15.7) reveals the following clusters.

Cluster-I – *D. nutantiflorum, D. densiflorum, D. pendulum, D. microbulbon, D. moschatum, D. anceps, D. bicameratum.*

Cluster-II – *D. nobile, D. haemoglossum, D. jenkinsii, D. herbaceum, D. heyneanum.*

The *Dendrobium* section *Formosae*, to which *D. nutantiflorum* (=*D. jerdonianum* Wt.) belongs (Table 15.11), was thoroughly analysed by Sathapattayanom (2008); according to him, the two morphologically aberrant species, such as *D. nutantiflorum* and *D. trigonopus*, remain unplaced. But from this study, preliminarily dendrogram shows that *D. nutantiflorum* has close affinity with *D. densiflorum* (section *Densiflora*) based on quantitative anatomical features.

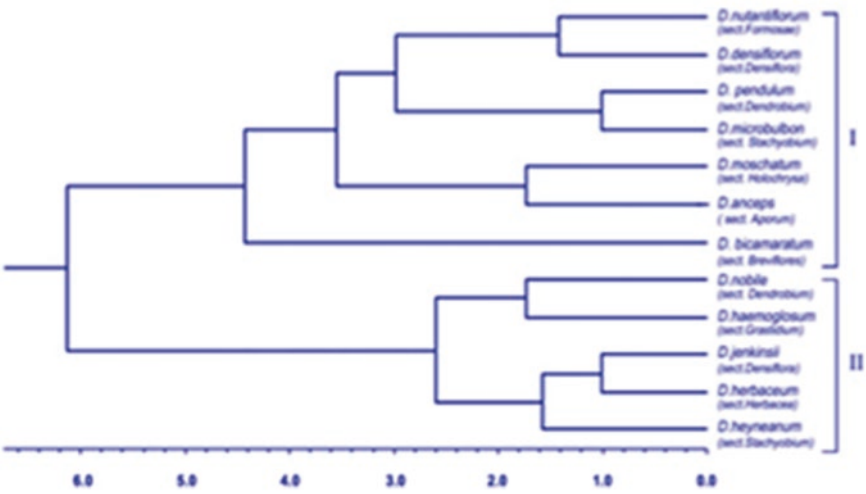

Fig. 15.7 Dendrogram showing dissimilarity among *Dendrobium* based on anatomical features

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Physiological Response of *Dendrobium* cv. Earsakul to Plant Growth Promoters and Growing Systems

16

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Abstract

This chapter discusses the investigation on 'Physiological Response of *Dendrobium* cv. Earsakul to Plant Growth Promoters and Growing Systems' that was conducted at the College of Horticulture, Vellanikkara, Thrissur, Kerala, from April 2011 to March 2013. This experiment was carried out with an objective to study the response of *Dendrobium* cv. Earsakul to nutrients, plant-growth-promoting root endophyte, *Piriformospora indica* (PGPRE) and plant growth regulators under three microclimatic conditions. The experiment was laid in CRD with six treatments and four replications. The results revealed that maximum leaf area (29.99 cm^2), relative growth rate (0.013 g g^{-1} day^{-1}) and number of stomata (41.14) were observed in 6-month-old plants, whereas maximum dry matter production (20.92 g $plant^{-1}$) and crop growth rate (0.148 g m^{-2} day^{-1}) were observed in 3-year-old plants treated with T_4. Higher levels of dry matter production (14.27 g $plant^{-1}$), crop growth rate (0.131 g m^{-2} day^{-1}), rate of photosynthesis (6.36 µmol CO_2 m^{-2} s^{-1}) and transpiration rate during day time (6.56 µmol m^{-2} s^{-1}) were observed in 6-month-old plants treated with T_3. Maximum rate of transpiration during nighttime was recorded in plants treated with T_2 (0.26 µmol m^{-2} s^{-1}). Among the systems of growing, rate of photosynthesis

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(6.86 µmol CO_2 m^{-2} s^{-1}), transpiration rate during night (0.32 µmol m^{-2} s^{-1}) and transpiration rate during day (6.00 µmol m^{-2} s^{-1}) were observed to be higher in 6-month-old plants grown under top-ventilated polyhouse system (S_2). Maximum values were also observed for the remaining physiological parameters of plants grown in top-ventilated polyhouse. The association between plant growth promoters and systems of growing significantly influenced the physiological attributes of plants.

Keywords
Dendrobium cv. Earsakul · Nutrients · *Piriformospora indica* (PGPRE) · Growing systems · Physiological traits

16.1 Introduction

Among the orchid genera, *Dendrobium* is a very complex and extremely large genus widely used in the commercial production of cut flowers. It is the second largest genus in the family with nearly 1600 species and is one of the commercially important species. Most *Dendrobium* species are epiphytic and are from tropical and subtropical regions. It is a popular genus for cut-flower production. Many growers in the states of Kerala, Tamil Nadu and Coastal Karnataka are cultivating *Dendrobium* on a commercial scale. *Dendrobiums* occupy nearly 90% of the area under orchid cultivation in Kerala due to the easy management practices and plant material availability (Rajeevan and Sobhana 1993). These hybrids are in the foremost position in floriculture trade especially in ornamental cut-flower sprays and its capability in blooming continuously and a prolonged post-harvest life relative to other orchid hybrids (Puchooa 2004). The type of nutrients, their quality and frequency of application play an important role on the growth and quality of flower. In orchids, growth and floral initiation is determined by the genotype and its interaction with the environmental conditions. Temperature, humidity, light and photoperiod are some of the important environmental conditions that influence growth and reproductive biology of orchids. Regulation of light intensity is essential for successful orchid culture. During plant development, the transition from vegetative to reproductive growth is triggered by a number of environmental and endogenous signals. Under controlled conditions of greenhouse, the flowers exhibit the best quality attributes required for the market. For better growth, yield and quality of the flowers, the system of growing is very important. Microclimate inside the growing system may drastically influence the growth, flowering and quality of flowers (Femina et al. 2006). In most *Dendrobium* orchids, rapid vegetative growth occurs at temperatures between 24 °C and 30 °C (Leonhardt 2000). In their natural habitat, epiphytes usually meet with a greater degree of environmental stress. Fernandez (2001) reported that in *Dendrobium*, remarkable increase in plant height was noticed in treatments with 35% and 50% shading (both at double level) and 50% single-level shading. The plant height was considerably less in intense light conditions. The major constraints

encountered in *Dendrobium* orchid cultivation are growing conditions, long pre-blooming period and susceptibility to pest and diseases. It is envisaged that growing tropical orchids for cut-flower production and potted plants will benefit from the recent advances in plant physiology and biotechnology. For the orchid industry, producing an improved hybrid, through conventional breeding or genetic engineering, is only the beginning. Optimization of the production processes and ensuring a quality product for the market are equally important. To achieve this goal, a good basic understanding of orchid physiology is essential to solve key physiological issues. However, we lack information on some physiological aspects on tropical orchids under greenhouse cultivation, particularly at a commercial level. This information is crucial in the optimization of the growth and yield of orchids in commercial farms. Keeping in view all these, the present investigation was taken up with the objective to study the physiological traits of *Dendrobium* cv. Earsakul as influenced by nutrients under three microclimatic conditions.

The experiments were carried out at the orchidarium of the All India Coordinated Floriculture Improvement Project (AICFIP) in the Department of Pomology and Floriculture, College of Horticulture, Vellanikkara, Thrissur, Kerala. Studies were conducted over a period from April 2011 to March 2013 in three types of growing systems, viz., two-level shade house (S_1), top-ventilated polyhouse (S_2) and fan and pad system (S_3). Two groups of commercially cultivated orchid hybrid variety *Dendrobium* cv. Earsakul was used for the study: 6-month-old plants and 3-year-old plants at the time of planting (starting time of investigation). Plants were grown under 50% shade in two-level shade house (size, 21.00 m × 6.00 m × 3.50 m × 2.0 0 m; top one-layer shade net; lower one-layer poly film with a size of 200 micron with misting system), top-ventilated polyhouse (size, 21.00 m × 6.00 m × 3.50 m × 2.00 m; poly film with a size of 200 micron covering with shade net and misting system) and in 75% shade in fan and pad system (size, 12.50 m × 8.00 m × 6.00 m × 4.00 m; poly film 200 micron covering; UV-stabilized shade net with fan and pad for cooling system). The major nutrients $N:P_2O_5:K_2O$ at two different ratios, viz., 3:1:1 and 1:2:2, at 0.2% were applied as foliar sprays during vegetative and flowering stages, respectively. The frequency of application was twice weekly. Nutrient combinations were made using ammonium nitrate, orthophosphoric acid and potassium nitrate.

Six types of treatments were followed:

T_1 – POP recommendations of KAU (foliar feeding with fertilizer mixture of $N:P_2O_5:K_2O$ at a ratio of 3:1:1 during vegetative period and 1:2:2 during flowering period at 0.2%, spraying of ammonium nitrate, orthophosphoric acid and potassium nitrate twice weekly)

T_2 – POP + PGPRE (1 g of fungal culture *Piriformospora indica* was mixed with 100 g of vermiculite and applied near the root zone at the time of planting) + bone meal (15 g per plant applied near root zone at the time of planting)

T_3 – POP + OM (bone meal, neem cake and groundnut cake 100 g each, soaked in water for 3–4 days and diluted 10–15 times with water, filtered and sprayed over

plants at 15 days interval) + vermiwash (diluted to 3% and sprayed at 15 days interval) + PGPRE + bone meal

T_4 – POP + OM + VW + PGPRE + bone meal + GR (BA 50 mg/l and GA_3 10 mg/l sprayed at monthly intervals)

T_5 – 10:20:10 NPK + GR

T_6 – NPK + GR + OM + VW + PGPRE + bone meal.

The experiment was laid out in completely randomized design comprising six treatments, four replications and five plants per treatment for recording observations. The observations on physiological attributes were recorded. The experimental data were analyzed by the ANOVA (Analysis of Variance technique (Panse and Sukhatme 1985). The data on physiological observations were recorded by the following methods suggested in RGR by (Blackman 1919) in NAR (Williams 1946) and in CGR (Yaduraju and Ahuja 1996).

16.2 Plant Growth Regulators and their Physiological Response in *Dendrobium* cv. Earsakul

16.2.1 Leaf Area

Leaf area was significantly influenced by treatments (Tables 16.1 and 16.2). In 6-month-old plants, T_4 recorded significantly higher leaf area (29.99 cm^2) which was on par with T_3 (29.33 cm^2) and T_2 (27.43 cm^2). In 3-year-old plants, the treatment T_4 recorded significantly higher leaf area (30.58 cm^2) which was followed by T_3 (27.17 cm^2). The treatment T_3 was on par with T_2 (26.13 cm^2). This could be well explained that the leaf area was determined by the number of leaves per plant. The number of leaves was higher in the treatment POP + OM + VW + PGPRE + bone meal + growth regulators and hence the maximum leaf area. Similar types of findings are reported by Bichsel and Starman (2008) in *Dendrobium nobile*.

Response of growing systems on leaf area was significant. Significantly higher leaf area was recorded in S_2 (28.92 cm^2) which was followed by S_3 (24.94 cm^2). The leaf area in S_3 was on par with S_1 (23.97 cm^2). The growing systems had no significant influence on production of leaf area in 3-year-old plants. The increase in leaf number results in increase in leaf area.

T × S interaction on leaf area was significant in both groups of plants. Significantly higher leaf area of 34.41 cm^2 was recorded under the combination T_3S_2, which was on par with T_4S_1 (31.25 cm^2) and T_4S_2 (30.46 cm^2). In 3-year-old plants, significant higher leaf area was recorded under T_4S_1 interaction (32.73 cm^2), which was on par with T_3S_3 (31.53 cm^2) and T_3S_2 (31.41 cm^2). In 6-month-old plants grown in top-ventilated polyhouse with high temperature, high light intensity and low relative humidity, P. indica increased the number of leaves per plant, which in turn enhanced the leaf area. Foliar feeding of organic manures may also be the reason for the highest leaf area. Whereas in 3-year-old plants, *P. indica* along with growth regulators increased the number of leaves per plant, which ultimately resulted in more leaf area.

Table 16.1 Influence of plant growth promoters (T), growing systems (S) and T × S interaction on physiological parameters in 6-month-old plants of *Dendrobium* cv. Earsakul at 6 months after treatment

	Leaf area (cm^2)				Dry matter production (g $plant^{-1}$)				Crop growth rate (g m^{-2} day^{-1})				Relative growth rate (g g^{-1} day^{-1})			
Treatments	S_1	S_2	S_3	Mean	S_1	S_2	S_3	Mean	S_1	S_2	S_3	Mean	S_1	S_2	S_3	Mean
T_1	19.26	26.88	28.73	25.03	5.38	9.27	7.93	7.53	0.076	0.104	0.089	0.090	0.009	0.010	0.007	0.009
T_2	23.71	29.66	28.94	27.43	7.10	12.43	6.92	8.82	0.067	0.107	0.080	0.085	0.012	0.012	0.009	0.011
T_3	24.85	34.41	28.95	29.33	13.93	16.07	12.80	14.27	0.169	0.130	0.093	0.131	0.011	0.011	0.008	0.010
T_4	31.25	30.46	28.27	29.99	6.72	15.47	6.12	9.43	0.084	0.116	0.075	0.091	0.018	0.013	0.019	0.013
T_5	21.49	27.42	16.51	21.81	5.10	10.25	6.93	7.43	0.071	0.085	0.079	0.078	0.008	0.009	0.007	0.008
T_6	23.25	25.72	18.23	22.06	14.80	8.05	7.98	10.28	0.179	0.147	0.011	0.125	0.007	0.008	0.006	0.007
Mean	23.97	28.92	24.94		8.84	11.92	8.11		0.107	0.115	0.078		0.010	0.010	0.008	
CD (0.05)	T: 2.71				T: 1.95				T: 0.040				T: 0.003			
	S: 1.91				S: 1.38				S: 0.028				S: NS			
	T × S: 4.69				T × S: 3.38				T × S: 0.069				T × S: 0.005			

	Net assimilation rate (g m^{-2} day^{-1})				Number of stomata				Rate of photosynthesis (μmol CO_2 m^{-2} s^{-1})				Rate of transpiration (Night) (μmol m^{-2} s^{-1})				Rate of transpiration (Day) (μmol m^{-2} s^{-1})			
Treatments	S_1	S_2	S_3	Mean	S_1	S_2	S_3	Mean	S_1	S_2	S_3	Mean	S_1	S_2	S_3	Mean	S_1	S_2	S_3	Mean
T_1	0.004	0.009	0.004	0.006	34.32	31.80	28.26	31.46	4.24	8.86	3.73	5.61	0.14	0.23	0.11	0.16	1.73	4.40	3.15	3.09
T_2	0.006	0.009	0.004	0.006	31.80	44.92	39.38	38.70	4.83	6.10	3.49	4.81	0.16	0.45	0.18	0.26	3.81	5.27	3.11	4.06
T_3	0.007	0.009	0.005	0.007	34.82	34.33	39.38	36.17	4.94	9.73	4.41	6.36	0.14	0.23	0.10	0.16	8.83	7.77	3.09	6.56
T_4	0.009	0.009	0.006	0.008	40.33	41.38	41.72	41.14	3.62	6.01	3.26	4.29	0.21	0.46	0.07	0.25	3.88	5.39	3.24	4.17
T_5	0.006	0.006	0.003	0.005	28.55	37.85	38.35	34.91	2.48	6.90	3.88	4.42	0.10	0.37	0.12	0.19	4.46	9.19	2.47	5.37
T_6	0.004	0.011	0.002	0.006	32.29	39.36	43.00	38.21	4.20	3.58	2.58	3.45	0.15	0.14	0.15	0.15	2.41	3.95	2.96	3.10
Mean	0.006	0.009	0.004		33.68	38.27	38.34		4.05	6.86	3.55		0.15	0.32	0.12		4.18	6.00	3.00	
CD (0.05)	T: NS				T: 3.41				T: 1.72				T: 0.032				T: 1.29			
	S: 0.002				S: 2.41				S: 1.21				S: 0.023				S: 0.91			
	T × S: 0.007				T × S: 5.91				T × S: 2.98				T × S: 0.056				T × S:2.23			

Table 16.2 Influence of plant growth promoters (T), growing systems (S) and T × S interaction on physiological parameters in 3-year-old plants of *Dendrobium* cv. Earsakul at 6 months after treatment

	Leaf area (cm^2)				Dry matter production (g $plant^{-1}$)				Crop growth rate (g m^{-2} day^{-1})				Relative growth rate (g g^{-1} day^{-1})			
Treatments	S_1	S_2	S_3	Mean	S_1	S_2	S_3	Mean	S_1	S_2	S_3	Mean	S_1	S_2	S_3	Mean
T_1	20.72	25.66	18.66	22.36	6.76	8.46	6.93	7.38	0.105	0.106	0.090	0.100	0.016	0.011	0.008	0.011
T_2	25.43	27.52	24.01	26.13	12.33	13.33	9.66	11.77	0.092	0.108	0.065	0.088	0.014	0.013	0.010	0.012
T_3	25.05	31.41	31.53	27.17	17.66	24.33	12.30	18.09	0.156	0.122	0.091	0.123	0.012	0.012	0.009	0.011
T_4	32.73	26.30	23.80	30.58	22.33	26.66	13.76	20.92	0.180	0.159	0.104	0.148	0.011	0.015	0.014	0.012
T_5	22.15	19.36	23.48	21.22	7.88	11.78	8.88	9.51	0.098	0.128	0.086	0.104	0.010	0.010	0.007	0.009
T_6	18.84	20.81	20.81	19.49	15.25	16.16	11.81	14.41	0.117	0.114	0.100	0.110	0.039	0.009	0.007	0.018
Mean	24.15	25.18	24.14		13.70	16.78	10.56		0.125	0.123	0.089		0.017	0.012	0.008	
CD (0.05)	T: 2.28				T: 1.64				T: 0.033				T: NS			
	S: NS				S: 1.16				S: 0.023				S: NS			
	T × S: 3.96				T × S: 2.84				T × S: 0.057				T × S: 0.021			

	Net assimilation rate (g m^{-2} day^{-1})				Number of stomata				Rate of photosynthesis (μmol CO_2 m^{-2} s^{-1})				Rate of transpiration (night) (μmol m^{-2} s^{-1})				Rate of transpiration (daytime) (μmol m^{-2} s^{-1})			
Treatments	S_1	S_2	S_3	Mean	S_1	S_2	S_3	Mean	S_1	S_2	S_3	Mean	S_1	S_2	S_3	Mean	S_1	S_2	S_3	Mean
T_1	0.011	0.007	0.004	0.007	32.79	34.80	37.35	34.98	3.72	5.92	4.49	4.71	0.12	0.09	0.26	0.15	2.40	2.33	3.56	2.76
T_2	0.013	0.012	0.003	0.009	40.39	37.33	38.33	38.68	3.78	3.58	3.22	3.53	0.19	0.12	0.04	0.12	3.88	6.15	3.28	4.44
T_3	0.012	0.010	0.004	0.009	38.37	38.86	42.36	39.86	3.71	3.63	4.93	4.09	0.15	0.09	0.05	0.10	3.14	6.71	2.64	4.16
T_4	0.007	0.014	0.005	0.009	30.33	44.92	39.85	38.36	2.82	3.51	8.72	5.01	0.22	0.06	0.29	0.19	7.20	1.42	1.96	3.53
T_5	0.009	0.014	0.004	0.009	34.32	37.85	36.33	36.16	2.87	2.96	6.58	4.14	0.11	0.20	0.23	0.17	4.05	0.57	3.10	2.57
T_6	0.028	0.017	0.004	0.016	35.83	38.86	39.38	38.02	2.95	3.98	3.26	3.40	0.16	0.06	0.22	0.14	8.73	3.06	3.40	5.06
Mean	0.013	0.012	0.004		35.34	38.77	38.93		3.30	3.93	5.20		0.16	0.10	0.18		4.90	3.37	2.98	
CD (0.05)	T: NS				T: 3.22				T: NS				T: NS				T: 0.81			
	S: 0.007				S: 2.28				S: 1.84				S: NS				S: 0.57			
	T × S: 0.017				T × S: 5.57				T × S: 4.51				T × S: 0.301				T × S: 1.41			

16.2.2 Dry Matter Production (DMP)

The results indicated that different treatments markedly influenced the DMP (Tables 16.1 and 16.2). In 6-month-old plants, treatment T_3 recorded significantly higher DMP (14.27 g plant^{-1}) which was followed by T_6 (10.28 g plant^{-1}), and this was on par with T_4 (9.43 g plant^{-1}) and T_2 (8.82 g plant^{-1}). Different treatments had significant influence on DMP in 3-year-old plants. The treatment T_4 recorded significantly higher DMP of 20.92 g plant^{-1} which was followed by T_3 (18.09 g plant^{-1}), T_6 (14.41 g plant^{-1}), T_2 (11.77 g plant^{-1}) and T_5 (9.51 g plant^{-1}). The plant height and number of shoots per plant were more in the treatment POP + OM + VW + PGPRE + bone meal in 6-month-old plants, whereas the number of leaves per plant and leaf area were more in the treatment POP + OM + VW + PGPRE + bone meal + GR. This might be the reason for more DMP observed in those treatments in 6-month-old and 3-year-old plants, respectively. This was in accordance with the findings of Cardoso et al. (2012) in *Phalaenopsis* orchid.

Multiple growing sites had significant influence on DMP. Significantly higher DMP was recorded in S_2 (11.92 g plant^{-1}). In 3-year-old plants, significantly higher DMP of 16.78 g plant^{-1} was recorded under S_2 which was followed by S_1 (13.70 g plant^{-1}). The plant height, number of leaves, number of shoots and leaf area were maximum in top-ventilated polyhouse which might have resulted in increased DMP in plants grown under top-ventilated polyhouse.

T × S interaction had significant influence on DMP in both stage plants. T_3S_2 interaction recorded significantly higher DMP of 16.07 g plant^{-1} which was on par with T_4S_2 (15.47 g plant^{-1}), T_6S_1 (14.80 g plant^{-1}), T_3S_1 (13.93 g plant^{-1}) and T_3S_3 (12.80 g plant^{-1}). In 3-year-old plants, T_4S_2 interaction recorded significantly highest DMP of 26.66 g plant^{-1} which was on par with T_3S_2 (24.33 g plant^{-1}). These results are in conformity with earlier results of plant growth promoters and systems of growing on DMP.

16.2.3 Crop Growth Rate

CGR was significantly influenced by various plant growth promoters (Tables 16.1 and 16.2). The plant growth promoter T_3 recorded significantly higher CGR of 0.131 g m^{-2} day^{-1} which was on par with T_6 (0.125 g m^{-2} day^{-1}), T_4 (0.091 g m^{-2} day^{-1}), T_1 (0.090 g m^{-2} day^{-1}) and T_2 (0.085 g m^{-2} day^{-1}). In 3-year-old plants, the treatment T_4 recorded significantly higher CGR of 0.148 g m^{-2} day^{-1} which was followed by T_3 (0.123 g m^{-2} day^{-1}), and this was on par with all other treatments. The CGR is the proportion of dry matter production and time period of growth. The results proved that more DMP was recorded in 6-month-old plants treated with POP + OM + VW + PGPRE + bone meal and in 3-year-old plants treated with POP + OM + VW + PGPRE + bone meal + GR. A similar trend was also observed in the case of CGR. The highest CGR was recorded in those plants which received POP + *P. indica*. The results of the present investigation find support from Dhinesh (2009) in *Dendrobium*. CGR was significantly influenced by three multiple sites in

both crop stages. Significantly higher CGR was recorded in S_2 (0.115 g m^{-2} day^{-1}). In 3-year-old plants, significantly higher CGR of 0.125 g m^{-2} day^{-1} was recorded in S_1.

Regarding T × S interaction, in 6-month-old plants, significantly higher CGR was recorded in T_6S_1 (0.179 g m^{-2} day^{-1}) which was on par with T_3S_1 (0.169 g m^{-2} day^{-1}), T_6S_2 (0.147 g m^{-2} day^{-1}), T_3S_2 (0.130 g m^{-2} day^{-1}) and T_4S_2 (0.116 g m^{-2} day^{-1}). In 3-year-old plants, the highest CGR was recorded in T_4S_1 (0.180 g m^{-2} day^{-1}) which was on par with T_4S_2 (0.159 g m^{-2} day^{-1}), T_3S_1 (0.156 g m^{-2} day^{-1}) and T_5S_2 (0.128 g m^{-2} day^{-1}). The possible reason could be due to the treatments NPK + GR + PGPRE + OM + VW + bone meal and POP + PGPRE + OM + VW + bone meal + GR under the environmental condition of two-level shade house may result in high CGR.

16.2.4 Relative Growth Rate

RGR differed significantly among various plant growth promoters applied in 6-month-old plants (Table 16.1). The treatment T_4 recorded significantly higher RGR (0.013 g g^{-1} day^{-1}) which was on par with T_2 (0.011 g g^{-1} day^{-1}) and T_3 (0.010 g g^{-1} day^{-1}). In 3-year-old plants, none of the inputs show significant influence on RGR (Table 16.2). Since the 6-month-old plants were in active growth phase, it was significantly showing the unit increasing DMP. This may lead to increase in RGR. The result in the present study was parallel with the findings of Dhinesh (2009) in *Dendrobium*. RGR did not vary significantly under three growing conditions (Tables 16.1 and 16.2).

The interaction of plant growth promoters and growing systems influenced significantly the RGR. In 6-month-old plants, the highest RGR of 0.019 g g^{-1} day^{-1} was recorded in T_4S_3 which was on par with T_4S_1 (0.018 g g^{-1} day^{-1}). In 3-year-old plants, significantly higher RGR was recorded in T_6S_1 (0.039 g g^{-1} day^{-1}). Under fan and pad system, a uniform environmental condition with high relative humidity may facilitate the maximum RGR in 6-month-old plants which are in active growth stage, whereas in 3-year-old plants, NPK + GR + OM + VW + PGPRE + bone meal combination was performing well under two-level shade house in increasing RGR.

16.2.5 Net Assimilation Rate

None of the plant growth promoters responded significantly on NAR.

Growing systems had significant influence on NAR. In 6-month-old plants, significantly higher NAR of 0.009 g m^{-2} day^{-1} was recorded in S_2. In 3-year-old plants, significantly higher NAR was recorded in S_1 (0.013 g m^{-2} day^{-1}) which was on par with S_2 (0.012 g m^{-2} day^{-1}). This finding was in consonance with results of Samasya (2000) in *Dendrobium*.

In T × S interaction, in 6-month-old plants, the highest NAR was recorded under the combination T_6S_2 (0.011 g m^{-2} day^{-1}) which was on par with all other treatments except T_5S_3 (0.003 g m^{-2} day^{-1}) and T_6S_3 (0.002 g m^{-2} day^{-1}). In 3-year-old plants, the highest NAR was recorded under combination of T_6S_1 (0.028 g m^{-2} day^{-1}) which was on par with T_6S_2 (0.017 g m^{-2} day^{-1}), T_5S_2 (0.014 g m^{-2} day^{-1}), T_4S_2 (0.014 g m^{-2} day^{-1}), T_2S_1 (0.013 g m^{-2} day^{-1}), T_2S_2 (0.012 g m^{-2} day^{-1}) and T_3S_1 (0.012 g m^{-2} day^{-1}). The interaction effect was clearly suggesting the results of plant growth promoters and systems of growing in independent cases on NAR. The results of the present study also collaborate with the findings of Jin et al. (2009) in *Dendrobium.*

16.2.6 Number of Stomata

The response of number of stomata to plant growth promoters was significant. T_4 recorded significantly higher number of stomata (41.14 per mm^2) which was on par with T_2 (38.70 per mm^2) and T_6 (38.21 per mm^2) in 6-month-old plants. In 3-year-old plants, T_3 recorded significantly higher number of stomata (39.86 per mm^2) which was on par with T_2 (38.68 per mm^2), T_4 (38.36 per mm^2) and T_6 (38.02 per mm^2). The number of leaves per plant in 6-month-old plants might be high due to the influence of growth regulators, and in 3-year-old plants, the individual leaf area was more. This could be the result of more number of stomata due to increasing number of leaves and larger area of the leaves in 6-month-old and 3-year-old plants. A similar trend was recorded in *Dendrobium* by Yukawa et al. (1992).

Among growing conditions, in 6-month-old plants, the highest number of stomata was recorded under S_3 (38.34 per mm^2) which was on par with S_2 (38.27 per mm^2). In 3-year-old plants, higher number of stomata was recorded under S_3 (38.93 per mm^2) which was on par with S_2 (38.77 per mm^2). The fan and pad system recorded highest number of stomata in both the stages of plants. Under fan and pad system, the uniform environmental conditions were maintained throughout the growth phase of the plants. This may be the adaptations for maintaining the physiological processes of the plants.

In 6-month-old plants, the T × S interaction showed that the combination of T_2S_2 recorded significantly higher number of stomata (44.92 per mm^2) which was on par with T_6S_3 (43.00 per mm^2), T_4S_3 (41.72 per mm^2), T_4S_2 (41.38 per mm^2), T_4S_1 (40.33 per mm^2), T_3S_3 (39.38 per mm^2), T_2S_3 (39.38 per mm^2), T_6S_2 (39.36 per mm^2) and T_5S_3 (38.35 per mm^2). In 3-year-old plants, the combination T_4S_2 recorded significantly higher number of stomata (44.92 per mm^2) which was on par with T_3S_3 (42.36 per mm^2), T_2S_1 (40.39 per mm^2), T_4S_3 (39.85 per mm^2) and T_6S_3 (39.38 per mm^2). This might be due to the fact that in top-ventilated polyhouse, the favourable environmental conditions would have influenced the number of stomata in the leaves of both stages of plants.

16.2.7 Rate of Photosynthesis

Photosynthetic rate was significantly influenced by various inputs applied. In 6-month-old plants, the treatment T_3 recorded significantly higher rate of photosynthesis (6.36 µmol CO_2 m^{-2} s^{-1}) which was on par with T_1 (5.61 µmol CO_2 m^{-2} s^{-1}) and T_2 (4.81 µmol CO_2 m^{-2} s^{-1}). In 3-year-old plants, different treatments had no significant influence on photosynthetic rate (Table 16.2).

Among growing systems, in 6-month-old plants, the highest photosynthetic rate was recorded under S_2 (6.86 µmol CO_2 m^{-2} s^{-1}) which was followed by S_1 (4.05 µmol CO_2 m^{-2} s^{-1}), and this was on par with S_3 (3.55 µmol CO_2 m^{-2} s^{-1}). In 3-year-old plants, the highest photosynthetic rate was recorded under S_3 (5.20 µmol CO_2 m^{-2} s^{-1}) which was on par with S_2 (3.93 µmol CO_2 m^{-2} s^{-1}). Rate of photosynthesis in S_2 was on par with S_1 (3.30 µmol CO_2 m^{-2} s^{-1}). The positive effect of POP + OM + VW + PGPRE + bone meal in increasing DMP and CGR were recorded in earlier results which indicated that higher rate of photosynthesis would increase the food reserves which subsequently increased DMP and CGR. This may be explained by the fact that the 6-month-old plants were in active growth stage. In 6-month-old plant, under top-ventilated polyhouse system, high temperature and high light intensity result in higher rate of photosynthesis, whereas in 3-year-old plants, uniform environmental conditions of fan and pad system resulted in higher rate of photosynthesis.

T × S interaction on photosynthetic rate was significant. In 6-month-old plants, the combination T_3S_2 recorded significantly higher rate of photosynthesis (9.73 µmol CO_2 m^{-2} s^{-1}) which was on par with T_1S_2 (8.86 µmol CO_2 m^{-2} s^{-1}) and T_5S_2 (6.90 µmol CO_2 m^{-2} s^{-1}). In 3-year-old plants, combination of T_4S_3 recorded significantly higher rate of photosynthesis (8.72 µmol CO_2 m^{-2} s^{-1}) which was on par with T_5S_3 (6.58 µmol CO_2 m^{-2} s^{-1}), T_1S_2 (5.92 µmol CO_2 m^{-2} s^{-1}), T_3S_3 (4.93 µmol CO_2 m^{-2} s^{-1}) and T_1S_3 (4.49 µmol CO_2 m^{-2} s^{-1}). The interaction results in the 6-month-old plants conformed the earlier results in independent observations, whereas in 3-year-old plants, the treatment POP + OM + VW + PGPRE + bone meal + GR was performed well under fan and pad system for recording the highest photosynthetic rate.

16.2.8 Transpiration Rate at Night Time

Rate of transpiration varied significantly among treatments. In 6-month-old plants, the treatment T_2 recorded significantly higher rate of transpiration (0.26 µmol m^{-2} s^{-1}) which was on par with T_4 (0.25 µmol m^{-2} s^{-1}). The plant growth promoters had no significant influence on transpiration rate in 3-year-old plants. The *Piriformospora indica* and plant growth promoters access to more growth and thereby more water and hence promoted higher rate of transpiration.

The response of rate of transpiration to the growing systems was significant. In 6-month-old plants, the highest transpiration rate was recorded under S_2 (0.32 µmol m^{-2} s^{-1}). Rate of transpiration during night-time grown under three

growing conditions was not significant in 3-year-old plants. This could be due to higher temperature; lower relative humidity would result in gradient in vapour-pressure deficit resulting in higher rate of transpiration. These results are in agreement with the findings of Nagoaka et al. (1984) and Samasya (2000) in *Dendrobium*.

Rate of transpiration during night was significantly influenced by plant growth promoters and growing systems. The combination of T_4S_2 recorded significantly higher rate of transpiration (0.46 μmol m^{-2} s^{-1}) which was on par with T_2S_2 (0.45 μmol m^{-2} s^{-1}). In 3-year-old plants, T_4S_3 recorded significantly higher rate of transpiration (0.29 μmol m^{-2} s^{-1}) which was on par with all other interactions except T_4S_2 (0.06 μmol m^{-2} s^{-1}), T_6S_2 (0.06 μmol m^{-2} s^{-1}), T_3S_3 (0.05 μmol m^{-2} s^{-1}) and T_2S_3 (0.04 μmol m^{-2} s^{-1}). This phenomenon could be due to the fact that plant growth promoters have a positive influence on the growth of the plants, i.e. number of leaves per plant, leaf area, number of stomata; similar result were obtained in earlier results. Higher temperature and lower relative humidity prevailing inside the top-ventilated polyhouse influenced higher transpiration rate in 6-month-old plants.

16.2.9 Transpiration Rate at Daytime

A perusal of the data revealed that various plant growth promoters had significant influence on rate of transpiration. In 6-month-old plants, the treatment T_3 recorded significantly higher rate of transpiration (6.56 μmol m^{-2} s^{-1}) which was on par with T_5 (5.37 μmol m^{-2} s^{-1}). In 3-year-old plants, the treatment T_6 recorded significantly higher rate of transpiration (5.06 μmol m^{-2} s^{-1}) which was on par with T_2 (4.44 μmol m^{-2} s^{-1}). This might be due to positive influence of all applied plant growth promoters' favour for luxurious growth of the plants thereby resulted in increased rate of transpiration during daytime, i.e. the indication for healthy growth of the plants.

The data presented in tables indicate that growing systems had significant influence on rate of transpiration. In 6-month-old plants, the highest transpiration rate of 6.00 μmol m^{-2} s^{-1} was recorded under S_2. In 3-year-old plants, significantly higher rate of transpiration was recorded under S_1 (4.90 μmol m^{-2} s^{-1}) which was followed by S_2 (3.37 μmol m^{-2} s^{-1}), and this was on par with S_3 (2.98 μmol m^{-2} s^{-1}). The reasons for the highest transpiration rate under top-ventilated polyhouse are higher temperature, high light intensity and low relative humidity. In high light intensity, the water presented in mesophyll cells diffuses rapidly resulting in increase in humidity of internal air, and this increases the rate of transpiration (Cho and Kwack 1996). In 3-year-old plants, the environmental conditions prevailing in two-level shade house would also have influenced higher rate of transpiration during daytime.

In T × S interaction, the combination of T_5S_2 recorded significantly higher rate of transpiration (9.19 μmol m^{-2} s^{-1}) which was on par with T_3S_1 (8.83 μmol m^{-2} s^{-1}) and T_3S_2 (7.77 μmol m^{-2} s^{-1}) in 6-month-old plants. In 3-year-old plants, combination of T_6S_1 recorded significantly higher rate of transpiration (8.73 μmol m^{-2} s^{-1}).

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Anatomical Studies in Some Indian Coelogyneae (Orchidaceae)

17

J. Ramudu and S. M. Khasim

Abstract

Root anatomical studies in some Indian Coelogyneae species, such as *Coelogyne breviscapa*, *C. corymbosa*, *C. flaccida*, *C. nervosa*, *C. nitida*, *C. ovalis*, *C. prolifera*, *C. stricta* and *Pholidota pallida*, have been taken up. Multiseriate velamen, parenchymatous cortex and defined endodermis were recorded in all studied species. The root anatomical characters could be considered as adaptation to epiphytism and their survival and sustainability in their respective habitats. The geographical conditions and type of habitat, also the host tree on which orchid grows, are playing a vital role in the survivability of epiphytic orchids. Those orchids that are getting poor supply of nutrients by host plant undergo adaptations so as to survive under extreme environmental conditions.

Keywords

Root anatomy · *Coelogyne* · *Pholidota* · Epiphytism · Ecological adaptability

17.1 Introduction

The Orchidaceae is one of the largest families of flowering plants comprising about 28,484 species worldwide (Govaerts et al. 2017). In India, with 1350 species, it represents the second largest flowering plant family and contributes about 10% of Indian flora (Kumar and Manilal 1994; Jalal and Jayathi 2012).

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In South India, there are about 250 species spreading to 70 genera reported (Abraham and Vatsala 1981). The vegetative anatomy of this highly evolutionary important family is neither completely taken up nor received attention. During the last two decades, few important monographs on orchid biology and systematics have appeared (Dressler 1993; Vermeulen 1993; Pridgeon et al. 1999, 2001, 2003, 2005). By critical reading of the available literature, it is evident that authors have studied the anatomy in relation to systematics; but they did not explain the ecological adaptation of orchids. From the ecological point of view, Sanford (1974) did some work on African orchids, Kaushik (1983) on some Himalayan orchids and Metusala (2017) on *Dendrobium* of Indonesia. As such, there has been no single paper on anatomy of orchids in relation to ecological adaptability for the last 20 years.

The orchid plant materials were collected from different parts of Southern India. Orchid species taken for anatomical studies were listed in Table 17.1. Roots of various species were collected and fixed in FAA (formalin 0.5 parts, acetic acid 0.5 parts, 70% ethanol 9 parts); later these were preserved in 70% ethanol before processing. Free-hand cross sections of roots were made at standardized levels (Cutter 1978). Roots of the plant were dehydrated in alcohol and xylene series, infiltrated and embedded in paraffin wax (melting point 60–62 °C) and sectioned with a rotary

Table 17.1 List of species taken for anatomical studies[a]

S.No.	Species	Place of collection and elevation	Habitat and host tree	Voucher No.
I.	Subfamily **Epidendroideae**			
	Tribe **Coelogyneae**			
	Subtribe **Coelogyninae**			
1	*Coelogyne breviscapa* Lindl.	Shevaroy Hills, Yercaud (TN), 2000 m.	Epi and*Alnus nepalensis*	ANUH 1001
2	*C. corymbosa* Lindl.	Doddabetta, Ooty (TN), 2200 m.	Epi and*Mangifera indica*	ANUH 1002
3	*C. flaccida* Lindl.	Doddabetta, Ooty (TN), 2200 m.	Epi and *Castanopsis indica*	ANUH 1003
4	*C. nervosa* A. rich.	Doddabetta, Ooty (TN), 1800 m.	Epi and *Schima wallichii*	ANUH 1004
5	*C. nitida* Lindl.	National Orchidarium,Yercaud (TN)	___	ANUH 1005
6	*C. ovalis* Lindl.	Doddabetta, Ooty (TN), 2200 m.	Epi and *Terminalia bellirica*	ANUH 1006
7	*C. prolifera* Lindl.	Doddabetta, Ooty (TN), 2100 m.	Epi and *Terminalia alata*	ANUH 1007
8	*C. stricta* (D.Don) Schltr.	National Orchidarium,Yercaud (TN)	___	ANUH 1008
9	*Pholidota pallida* Lindl.	Shevaroy Hills, Yercaud (TN), 1800 m.	Epi and*Mangifera indica*	ANUH 1009

[a]Arranged according to Dressler (1993)
Epi Epiphyte, *TN* Tamil Nadu

microtome at a thickness of 15–20 μm. Double staining was done by safranin-fast green combination, and sections were mounted in DPX mount (Vijayaraghavan and Shukla 1990; Khasim 2002). Optical photomicroscope (Motic 2.0, 5 megapixels) was used to take anatomy photographs.

17.2 Root Anatomy of Indian Coelogyneae

The present investigation roots of all studied taxa were circular in outline (Fig. 17.1a). The velamen was formed by dead tissue; velamen cells are polygonal to oval shaped (Fig. 17.1a). Numbers of velamen layers are highest in *C. stricta*, i.e. 7 (Table. 17.2). In *C. flaccida* epivelamen is distinct (Fig. 17.1b). However in C. corymbosa velamen was bistratified in roots of characteristic wall thickenings (Fig. 17.1c).

Outer layer of cortex consists of long, thick-walled passage cells which do not necessarily alternate each other (Fig. 17.1c). Exodermal size is equal in all studied taxa. However the presence of secondary thickenings in these cell walls plays an important role in water storage function and mechanical support. Exodermal cell lignifications are highest in *P. pallida* compared to other taxa (Fig. 17.2a, b).

The highest cortical layers are found in *C. breviscapa* (Fig. 17.2c, d), *C. flaccid* and *C. nervosa* (Fig. 17.2e). Cortices consist of small and large oval-shaped cells with intercellular spaces. Some cortical cells are with pitted thickenings (Fig. 17.1e). In *C. stricta* some layers of cortical cells are hyaline and mucilaginous and have water storage function (Fig 17.1e).

The endodermis cell walls were thickened. However in *C. stricta*, the highest endodermal cell lignifications were observed (Fig. 17.1d). The vascular cylinder, phloem and xylem, strands alternate each other. In *P. pallida* xylem comprises tracheids with helical thickenings (Fig. 17.2a, b). Vessel members and vessel-like tracheids were abundantly observed in root macerations of *C. ovalis*. Pith is parenchymatous (Figs. 17.1f and 17.2f).

17.3 Anatomy in Relation to Ecological Adaptability

All the presently investigated taxa possess velamen roots. The epidermis of mature root is multiseriate with velamen tissue. According to Dycus and Knudson (1957) and Benzing (1986, 1989a, 1989b), epiphytic roots are of two types: (1) substrata roots which penetrate the soil and absorb water and nutrients and (2) aerial roots that are totally exposed to air and provide mechanical strength to plant body. Epivelamen which differs from inner velamen layers was also reported by Khasim (1986) in *Cymbidium grandiflorum*, *C. mastersii* and *Oberonia wightiana*,but it is peeled off at maturity.

Velamen is dead tissue filled with air in the dry condition, giving the root the characteristic grey colour. This tissue is supposed to act as a sponge, absorbing the moisture from the atmosphere. In fact, the velamen stores water which is utilized by

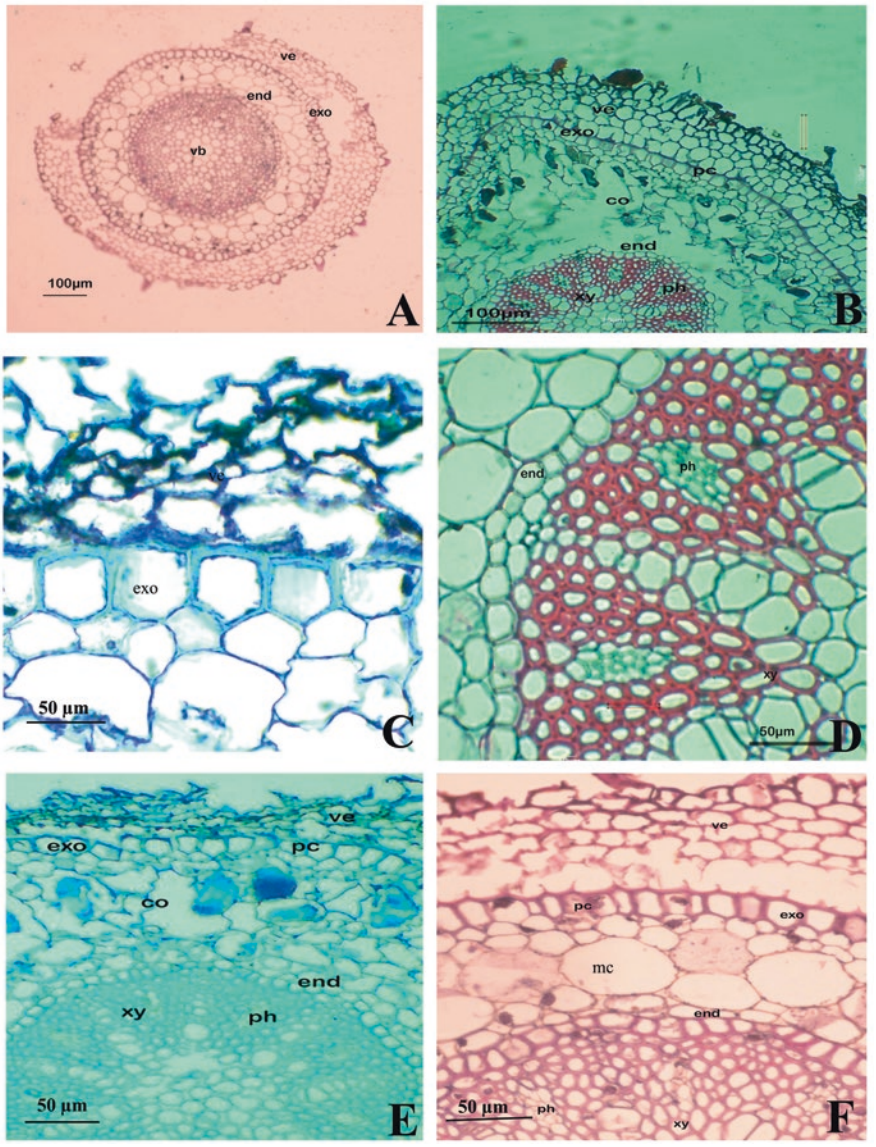

Fig. 17.1 (**a**–**f**). Anatomical studies of *Coelogyne nervosa*, *C. flaccid*, *C.corymbosa*, *C. stricta* and *C.ovalis*roots. (**a**). Root cross section showing gross structure with cortex and vascular cylinder of *C. nervosa*; (**b**). Root cross section showing velamen, exodermis, cortex and endodermis in *C.flaccida*; (**c**). Root cross section showing epivelamen and exodermis and cortex of *C. corymbosa*; (**d**). Root cross section showing endodermis of *C. stricta*; (**e**). Root cross section showing cortex of *C.* stricta; (**f**). Root cross section showing velamen, cortex and endodermis of *C. ovalis* (***co*** Cortex, ***end*** Endodermis, ***exo*** Exodermis, ***mc*** Mucilage cavity, ***pc*** Passage cell, ***ph*** Phloem, ***pt*** Pith, ***vb*** Vascular bundle, ***ve*** Velamen, ***xy*** Xylem)

Table 17.2 Root: anatomical features in Epidendroideae (in μm)

Species / Anatomical feature	*Coelogyne breviscapa*	*C. corymbosa*	*C. flaccida*	*C. nervosa*	*C. nitida*	*C. ovalis*	*C. prolifera*	*C. stricta*	*Pholidota pallida*
1. No. of velamen layers	5	4	5	6	5	4	4	7	6
2. Exodermis size	32.12	33.32	34.01	30.08	34.04	31.87	29.85	31.90	33.46
3. Exodermis cell lignification	2.1	2.0	2.1	2.2	1.9	1.8	2.1	2.0	2.3
4. Cortex no. of layers	7	6	7	7	5	6	6	6	6
5. Endodermis thickness	18.81	23.41	21.59	18.48	16.61	21.21	14.53	21.21	14.53
6. Endodermal cell lignification	12.11	13.22	12.91	11.11	13.00	15.99	9.08	16.44	9.20
7.Vascular cylinder diameter	316.02	499.9	452.05	5808	486.6	730.16	328.32	395.5	664.17
8. No. of protoxylem poles	10	9	8	9	10	19	8	9	14
9. Vessel member	+	+	+	+	+	+	+	+	−

(+), Present; (−), Absent

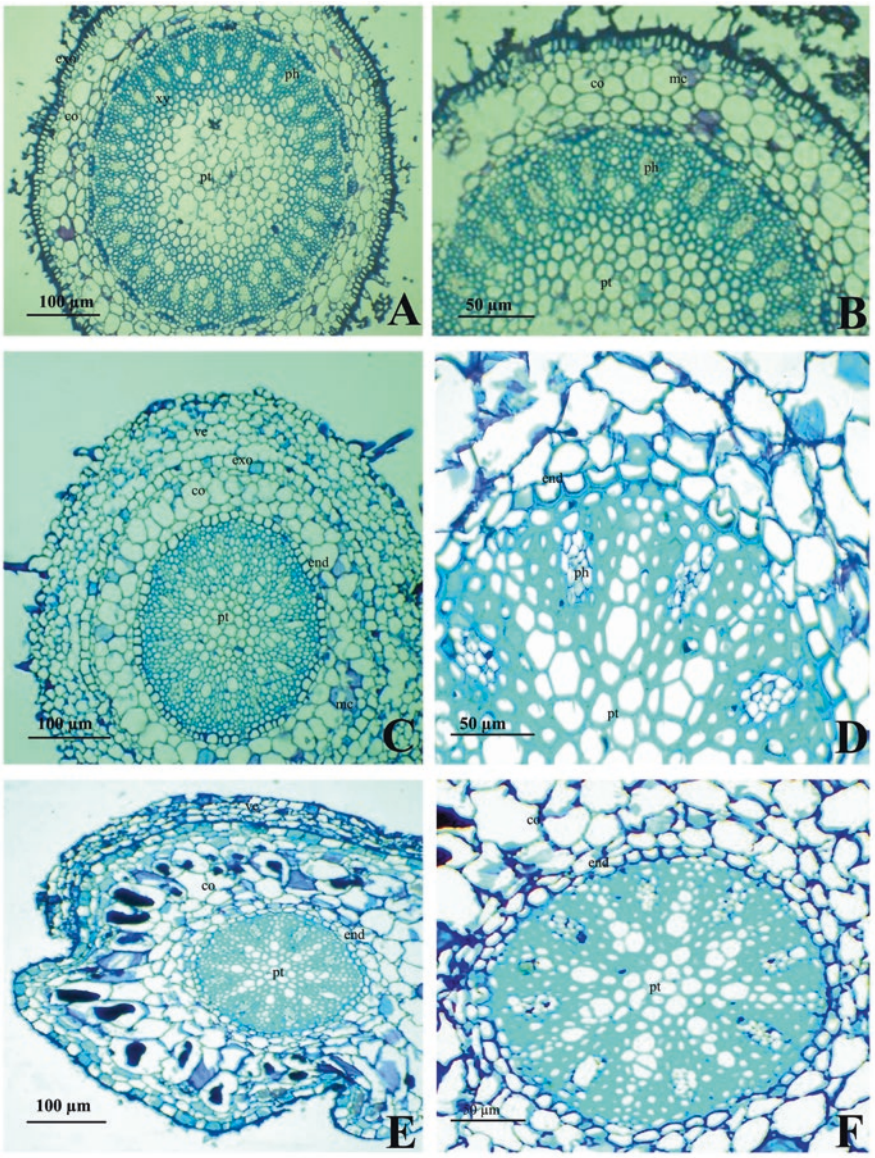

Fig. 17.2 (**a–f**). Anatomical studies of *Pholidota pallida*, *C. breviscapa*, *C. nitida* and *C. flaccida* roots. (**a**). Root cross section showing gross structure with cortex and vascular cylinder of *P. pallida*; (**b**). Root cross section showing exodermis, cortex and endodermis of *P. pallida*. (**c**). Root cross section showing epivelamen and exodermis and cortex of *C. breviscapa*. (**d**). Root cross section showing cortex and endodermis of *C. breviscapa*. (**e**). Root cross section showing cortex of *C. nitida*. (**f**). Root cross section showing velamen, cortex and endodermis of *C. flaccida* (*co* Cortex, ***end*** Endodermis, ***exo*** Exodermis, ***mc*** Mucilage cavity, ***pc*** Passage cell, ***ph*** Phloem, ***pt*** Pith, *vb* Vascular bundle, ***ve*** Velamen, ***xy*** Xylem)

plant during dry conditions. Velamen also protects the inner cortex and other tissues against UV damage (Zotz and Winkler 2013; Chomicki 2015).

The diversification of velamen character is also exemplified by type of habitat and host tree on which both *Coelogyne* and *Pholidota* species are growing continuously. However, velamen is well developed in taxa collected from Yercaud (Tamil Nadu) at an elevation of 1500 m with six to seven layers than that of Doddabetta (Ooty, 2200 m) plant collections. The present investigation further indicates that the Yercaud plant collections, viz. *C. breviscapa* and *P. pallida*, growing in Eastern Ghats show more xeric elements than that of Ooty and Doddabetta that come under Western Ghats where luxuriant growth of orchids is found.

In some orchids, flavonoids are accumulated in the aerial photosynthetic roots. These flavonoids are helpful in protecting the velamen root against the UV radiation damage and also providing long-lasting nature to the aerial roots even after their death (Chomicki et al. 2015), whereas in terrestrial orchid root, flavonoids are completely absent.

Fibrous mats are completely absent from the presently studied taxa except *Pholidota pallida*. Fibrous mats (tilosomes) are reported in the tribes Polystacheae and Dendrobieae and other subtribes of Epidendroideae (Pridgeon 1983; Khasim 1986; Mohana Rao and Khasim 1987a, c; Ramesh 2014).

According to Shushan (1959), the outermost layer of cortex, close to the velamen, is differentiated as an exodermis. Exodermis consists of two types of cells: (1) larger cells, along with root axis, with thickened walls without protoplast, and isodiametric and (2) shorter cells with thin walls, known as passage cells with a dense cytoplasm and prominent nucleus. The longer lignified cells of exodermis protect the root cortex against dehydration, while shorter cells, with thin walls, drive nutrients from velamen to root cortex (Dycus and Knudson 1957; Oliveira and Sajo (1999). In almost all presently investigated taxa, exodermis comprises 'O'-shaped thickenings.

Benzing et al. (1983) reported that just like velamen, the exodermal thickenings aid in the reduction of water loss by root transpiration. Thickenings of exodermal cell wall are also reported maximum in *P. pallida* collected from Yercaud (Eastern Ghats of Tamil Nadu). Cortical cells with pitted thickenings are found in *C. ovalis*. These were also reported in *C. cristata* (Mohana Rao and Khasim 1987b) and *Eria bicolor* (Isaiah et al. 1990). Bur and Barthlott (1991) described these cells as pseudovelamen cells. These cells provide mechanical strength to the plant body. Moreira et al. (2013) opined that the well-developed velamen, distinct exodermis and specialized thick-walled cortical cells are the characteristic features of epiphytic orchids.

Cortex is situated in between exodermis and endodermis. It consists of thin-walled oval- to circular-shaped cells of various sizes. The cortical layers close to the exodermis and endodermis are smaller than those of the central region. Cortex comprises thin-walled chlorenchymatous cells. Occurrence of endotrophic mycorrhiza in the velamen and cortex is a regular feature in the family Orchidaceae (Leitgeb 1864).

Exodermis in root possesses long, thick-walled and broad thin-walled passage cells. The thick-walled cells prevent water escaping from the conducting tissues in the interior of roots; thin-walled passage cells allow the water-soluble nutrients to pass through from outside into the conducting tissue.

Endodermis is interrupted by thin-walled passage cells at protoxylem poles. In all investigated taxa, endodermal cells are uniformly lignified ('O'-shaped thickenings). Species such as *C. nervosa*, *C. prolifera* and *Pholidota pallida* collected from Ooty, Doddabetta (both under Western Ghats) and Yercaud (Eastern Ghats), respectively, showed high lignification in exodermal cells. This must be attributed that not only habitat conditions (including altitude) but also the host tree supplying nutrients play a vital role for the survival of epiphytic taxa. Ramesh (2014) also made similar observations in *Dendrobium anceps* collected from Darjeeling and Sikkim Himalaya. However, this needs further study on interpopulation diversity and its ecology.

In vascular cylinder of roots, the maximum number of protoxylem poles (protoxylem points) was observed in *C. ovalis* followed by *Pholidota pallida*. Based on number of protoxylem poles, Rosso (1966) classified orchids belonging to Cypripedioideae into two groups: (I) protoxylem points 8 or less and (ii) protoxylem points 9 or more.

Vascular cylinder is polyarch in orchid root. Xylem strands alternate with phloem. In most of the investigated taxa, fibre sheath is present around xylem and phloem. Sclerenchymatous pith in some of taxa is merging with vascular sclerenchyma. Vessel members are present in all investigated *Coelogyne* whereas absent in *Pholidota*. Carlquist and Schneider (2006) also reported vessels in other members of Epidendroideae. Cheadle (1942) reported vessels with scalariform perforation plates and also with simple perforation plates. However, in most of the cases, vessels were not reported, but vessel-like tracheids are abundant. In this context, Kaushik (1983) opined that vessels must have eliminated due to development of other water storage mechanisms in the plant body; in fact, epiphytes, which are cutoff from the ground perhaps, have no need of possessing vessels.

According to Cheadle and Kosakai (1980), the presence of vessels in roots is considered to be more advanced than the rhizome, stem and leaf. Since most of the investigated taxa are epiphytes, vessels rarely appeared but very long tracheids and vessel-like tracheids are abundant in their vegetative parts.

From the entire discussion of this section, it is evident that there is no generalised pattern of growth of epiphytic orchids; not only the geographical conditions and type of habitat but also the host-tree on which orchid grows is playing a vital role in survivability of epiphytic orchids. Those orchids that are getting poor supply of nutrients by host plant undergo adaptations so as to survive under extreme environmental conditions (Khasim and Ramesh 2010; Ramudu et al. 2012).

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Part IV

Pollination Biology

Beauty of Orchid Flowers Are Not Adequate to Lure Indian Biologists

18

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Abstract

Orchids are always receiving a special attention among biologists globally, owing to their stunning colours, peculiar shapes, unique floral architecture and significantly their mystical deceptive pollination. Nearly, one-third of the orchids are achieving pollination through deceptive mechanisms. Throughout the world, biologists are working towards the understanding of deceptive pollination in various dimensions. Though India is holding more than 1200 reported orchid species, the deceptive pollination studies on Indian orchid species are very meagre. Our meta-analysis and field visits clearly revealed that the deceptive pollination is present in the Indian orchids. The genera level comparison on deceptive pollination between India and other parts of the world indicates that more number of Indian orchid genera holds the generalized food deception, brood-site imitation and sexual deception mechanisms. Since many orchid species are under threat, the studies related to deceptive pollination are vital to develop better conservation strategies.

Keywords

Deception · Indian orchids · Indian orchidologist · Orchid conservation · Orchid pollination

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18.1 Introduction

Angiosperms are one of the most successful groups in the plant kingdom, which have occupied almost every habitat on the earth's surface (Crepet and Niklas 2009; Hiscock 2011; Zeng et al. 2014). An estimate of about 405 families comprising 352,000 species have been recorded in it (De Storme and Mason 2014). Orchidaceae is the second largest family of flowering plants constituting nearly 10% of the total flowering species (Roberts and Dixon 2008; Tsai et al. 2013). Among the angiosperm families, Orchidaceae is considered to be highly evolved as evidenced in their unique floral architecture, condensed pack of pollinia, existence in different habitats and particularly their specialized pollination mechanism. These features have been enticing to evolutionary biologists over several decades incessantly to study their evolution. Charles Darwin also stated, "I never was more interested in any subject in my life, than in this of Orchids," in his letter to Hooker on the 13th of October 1861 (Roberts and Dixon 2008). One-third of orchids lure their pollinator through various deceptive pollination mechanisms such as generalized food deception, Batesian floral mimicry, brood-site imitation, sexual deception, shelter imitation, pseudo-antagonism and rendezvous attraction (Jersáková et al. 2006). Among them the major deceptive mechanisms hold by orchids are as follows: (i) generalized food deception, which exhibits the mimicry of general advertising signals of rewarding flowers such as colour, shape, scent, spur, nectar guides and importantly pseudopollen and false anthers (Jersáková et al. 2006); (ii) Batesian floral mimicry, which involves the mimic of particular rewarding flower (model) characteristics for specific pollinator attraction (Schlüter and Schiestl 2008); (iii) brood-site imitation occurs by mimicking the conditions of female insect (pollinator) oviposition sites by false odour and visual signals (Jersáková et al. 2006); and (iv) sexual deception, which involves the mimic of female insects in terms of their olfactory, visual and tactile cues to engage the pollinator for pseudocopulation (Jersáková et al. 2006). Over several decades, researchers around the world have been working to unravel the mystery of evolution and mechanism of deceptive pollination in orchids (Neiland and Wilcock 1998).

As one of the mega diversity countries in the world, India serves as a rich site for orchids by holding 1295 species across 179 genera (http://www.bsienvis.nic.in/Database/bsi_3949.aspx). So far, Indian biologists have been exploring the orchid diversity in various phytogeographical regions of India, establishing various conservation centres and producing hybrid orchid flowers for ornamental purpose (Hegde 1997). However, there are very limited reports on the deceptive pollination mechanism (model flower, pollinator, deception mode, etc.) of Indian orchids (Yadav 1995). This raises a question among Indian biologists whether the orchids of India possess any unique deceptive pollination mechanism or still it has been not studied in the angle of deception. Moreover, many Indian orchid species have been listed as threatened species. So, studying and understanding the pollination mechanism of Indian orchids is very crucial to conserve them. Thus, the aim of this report is to bring the attention of Indian orchid researchers to study the deceptive pollination mechanism of Indian species to develop better conservation strategies.

Tracing the deceptive pollination mechanism of orchids is almost similar to experiencing Arthur Conan Doyle's portrayal of Sherlock Holmes' mystery deduction. While reading the interesting deductions on deceptive pollination strategies, we found that most of them are from other countries, and similar reports from India are meagre. In order to assess the quantum of work carried out on orchid's deceptive pollination mechanism in India, Google search engine was used to collect data through search terms such as "orchid pollination in India", "pollination biology of Indian orchids" and "Indian orchid pollination". Only very few literatures were found to be available in the context of deceptive pollination in orchids. To prepare species-wise pollination data, Indian Orchid species list was collected from ENVIS Centre on Floral Diversity database (http://www.bsienvis.nic.in/Database/bsi_3949.aspx). Since the species-wise pollination data was not much available from Indian literature for Indian orchids, the available data on deception was gathered up to genera level from other parts of the world (Bänziger 1996, 2002; Bino et al. 1982; Borba and Semir 1999; Chen 2009; Dafni and Ivri 1981a, b; Davies and Turner 2004; Gaskett 2012; Jersáková et al. 2009; Jin et al. 2014; Kelly et al. 2013; Kjellsson et al. 1985; Li et al. 2010; Nilsson 1983; Pellegrino et al. 2008; Peter and Johnson 2008, 2013; Ren et al. 2014; Schiestl and Cozzolino 2008; Shi et al. 2007; Sugahara et al. 2013; Tang et al. 2014; van der Niet 2011; Xiaohua et al. 2012). We also conducted a field survey at the National Orchidarium, Experimental Garden and Laboratories (NOEGL), Yercaud, Tamil Nadu, on August 2015 to observe any cues for deception in Indian orchid species. The orchid flowers were photographed and qualitatively analyzed for deceptive parameters. Then the deceptive pollination mechanism for the observed orchids in NOEGL was collected up to generic level from the available literature (Bänziger 1996; Borba and Semir 1999; Chen et al. 2009; Davies and Turner 2004; Jersáková et al. 2009; Kjellsson et al. 1985; Ren et al. 2014; Shi et al. 2007; Sugahara et al. 2013; Tang et al. 2014). The habitat, flowering and fruiting period for the orchids in NOEGL were adopted from India Biodiversity Portal (http://www.indiabiodiversity.org/) and eMonocot database (http://e-monocot.org/). The conservation status of orchids in NOEGL was collected from "The IUCN Red List of Threatened Species™" (http://www.iucnredlist.Org/search) and from other existing reports (Averyanov and Averyanova 2003; Gogoi et al. 2012, 2015; Jalal 2012; Karuppusamy et al. 2009; Kumar et al. 2001; MOE 2012; Rokaya et al. 2013; http://www.efloras.org/florapage.aspx?flora_id=2; http://www.kew.org/scienceconservation/plants-fungi/epidendrum-radicans).

18.2 Deceptive Pollination Mechanisms in Orchids

From the meta-analysis, we found that out of the 179 orchid genera available in India, only 18 genera had reports on deceptive pollination. However, the salient feature in the reported orchid genera is that they lure their pollinators through various deceptive strategies such as generalized food deception, Batesian floral mimicry, brood-site imitation and sexual deception. There are different types of pollinators belonging to the orders such as Hymenoptera, Diptera and Coleoptera

involved in the pollination process of the reported 18 genera. The genera *Bulbophyllum* and *Satyrium* render reward but also deceit their pollinators, "Milichid flies" and *Sarcophaga redux*, respectively, with an olfactory cue mimicking the oviposition sites (brood-site imitation). Similarly, the genus *Corybas* exerts brood-site imitation by mimicking the scent, colour and gills appearance of mushroom to pull female fungus gnats (pollinator) for pollination without any reward. *Paphiopedilum* species exhibit brood-site mimic with no reward, attracting "hoverflies" (pollinator) to lay their eggs on the labellum by depicting the dummy aphids-like colony on its lateral petals. The genus *Epipactis* exhibits reward/non-reward and achieves brood-site deceptive pollination by luring "hoverflies" (pollinator) through the sex hormone of aphids. The genera *Eulophia*, *Dactylorhiza*, *Doritis* and *Cephalanthera* produce rewardless flowers and exhibit both Batesian floral and generalized food deception by mimicking their rewarding neighbour's floral shape and colour. Some genera of orchids such as *Holcoglossum*, *Calanthe*, *Coelogyne* and *Phaius* are producing rewardless flower and lure their pollinators through generalized food deception. The genus *Cryptostylis* mimics the sex pheromone or the morphology of the female wasp to draw the male wasps of *Lissopimpla excelsa* to achieve pollination through sexual deception. Orchids are not always exhibiting a single deceptive pollination mechanism, but in some cases they hold a combination of two or three types of deception, for instance, in the genus *Cypripedium*, which produces rewardless flowers and lures their pollinator through generalized food, Batesian floral and brood-site deceptive pollination mechanism (Table 18.1).

In order to observe the orchids and their pollinators, our team made a field survey at the National Orchidarium, Experimental Garden and Laboratories, Yercaud, Tamil Nadu, on August 2015. In the Orchidarium, several native and exotic (*Epidendrum radicans*) orchids collected from various phytogeographical regions of India are being maintained. Among them, we were able to observe only 87 orchid species which belong to 38 genera. In the observed orchid species, 60 are epiphytes, 9 are terrestrial, 7 are geophytes, 6 orchid species have both epiphytes and lithophyte habitats, 2 are lithophytes and 2 species have lithophytic/terrestrial habitats. Based on the literature, we found that 8 out of 38 orchid genera present in the orchidarium have deceptive pollination through various deceptive strategies (Table 18.1). But for the remaining 30 orchid genera, it is unknown whether these orchids maintain a deceptive pollination or not due to lack of studies in the pollination biology of those orchids. In the Orchidarium, we found that only 14 genera were in the blossom/initiation of flowering during the field survey. The observed orchid genera in flowering are *Epidendrum radicans* (Fig. 18.1a), *Calanthe sylvatica* (Fig. 18.1b), *Coelogyne fimbriata* (Fig. 18.1c), *C. ovalis*, *C. prolifera*, *C. nervosa* (Fig. 18.1e), *Habenaria rariflora* (Fig. 18.1d), *Dendrobium wightii* (Fig. 18.1f), *D. aqueum*, *D. heyneanum*, *Liparis atropurpurea*, *L. viridiflora*, *Malaxis versicolor*, *Eria pseudoclavicaulis*, *Oberonia brunoniana*, *Sirhookera latifolia*, *Xenikophyton smeeanum* and *Bulbophyllum* sp. The orchid genera in the budding stage are *Paphiopedilum spicerianum*, *Pinalia polystachya*, *Eria pauciflora*, *Dendrobium chrysanthum* and *Habenaria* roxburghii. During the field visit, we observed not even a single

Table 18.1 Reward, type of deceptions and pollinator details of the orchids in India (up to genera level) reported from other parts of the world

S. no.	Name of the genus	Reward/ non-reward	Type of deception reported at genus level	Pollinator			References
				Order	Common name	Scientific name	
1.	*Bulbophyllum*	Reward	Brood-site deception	Hymenoptera	Milichid flies	Not available	Borba and Semir (1999)
2.	*Calanthe*	Non-reward	Generalized food deception	Diptera	Drone fly	*Eristalis tenax*	Ren et al. (2014) and Tang et al. (2014)
				Hymenoptera	Bumble bee	*Bombus patagiatus*	
3.	*Cephalanthera*	Non-reward	Batesian floral/ generalized food deception	Hymenoptera	Megachilid bee	*Chelostoma* sp.	Dafni and Ivri (1981a, b) and Nilsson (1983)
					Solitary bee	*Halictus* sp.	
4.	*Coelogyne*	Non-reward	Generalized food deception	Hymenoptera	Eastern honey bee	*Apis cerana cerana*	Chen et al. (2009) and Tang et al. (2014)
					Social wasps	*Vespula* sp.	
5.	*Corybas*	Non-reward	Brood-site deception	Diptera	Female fungus gnats	Not available	Kelly et al. (2013)
6.	*Cryptostylis*	Non-reward	Sexual deception	Hymenoptera	Orchid dupe wasp	*Lissopimpla excelsa*	Gaskett (2012)
7.	*Cymbidium*	Non-reward/ reward	Batesian floral/ generalized food/ sexual deception	Hymenoptera	Honey bee	*Apis* sp.	Kjellsson (1985), Sugahara et al. (2013) and Tang et al. (2014)
				Rodentia	Bumble bee	*Bombus eximius*	
					Mouse	*Mus* sp.	
8.	*Cypripedium*	Non-reward	Batesian floral/ brood-site/ generalized food deception	Hymenoptera	Bumble bee	*Bombus* sp.	Bänziger (2009) and Jersáková et al. (2009)
					Sweat bee	*Lasioglossum* sp.	
9.	*Dactylorhiza*	Non-reward	Batesian floral/ generalized food deception	Diptera	Flies	Not available	Pellegrino et al. (2008) and Schiestl and Cozzolino (2008)
				Coleoptera	Beetles	Not available	
				Hymenoptera	Bumble bees	*Bombus* sp.	

(continued)

Table 18.1 (continued)

S. no.	Name of the genus	Reward/ non-reward	Type of deception reported at genus level	Pollinator			References
				Order	Common name	Scientific name	
10.	*Dendrobium*	Non-reward/ reward	Batesian floral/ brood-site/ generalized food deception	Hymenoptera	Bumble bee	*Bombus eximius*	Davies and Turner (2004), Kjellsson (1985) and Tang et al. (2014)
					Mining bee	*Andrena parvula*	
11.	*Doritis*	Non-reward	Generalized food deception	Hymenoptera	Bees	*Amegilla nigritar*	Xiaohua et al. (2012)
12.	*Epipactis*	Reward	Brood-site deception	Diptera	Hoverfly	*Eupeodes corollae*	Jin et al. (2014)
						Episyrphus balteatus	
13.	*Eulophia*	Non-reward	Batesian floral/ generalized food deception	Hymenoptera	Carpenter bee	*Xylocopa* sp.	Peter and Johnson (2008, 2013)
					Sweat bee	*Lipotriches* sp.	
14.	*Holcoglossum*	Non-reward/ reward	Generalized food deception	Coleoptera	Beetle	*Hybovalgus bioculatus*	Tang et al. (2014)
				Diptera	Fly	*Micraphis and reniformis*	
15.	*Orchis*	Non-reward	Batesian floral/ generalized food/ sexual deception	Diptera	Large beefly	*Bombylius* sp.	Bino et al. (1982), Dafni and Ivri (1981a) and Jersáková et al. (2009)
				Hymenoptera	Miner bee	*Anthophora* sp.	
					Long-horned bees	*Eucera clypeata*	
					Halictid bee	*Halictus marginatus*	
16.	*Paphiopedilum*	Non-reward	Brood-site/ generalized food deception	Diptera	Hoverfly	*Betasyrphus serarius*, *Episyrphus alternans*, *E. balteatus* (females), *Syrphus fulvifacies*	Bänziger (1996), Shi et al. (2007) and Tang et al. (2014)
				Hymenoptera	Bumble bee	*Pyrobombus flavescens*	
17.	*Phaius*	Non-reward	Generalized food deception	Hymenoptera	Bumble bee	*Bombus hypnorum*, *B. lepidus*	Li et al. (2010) and Tang et al. (2014)
18.	*Satyrium*	Reward	Brood-site deception	Diptera	Fly	*Sarcophaga redux*	van der Niet (2011)

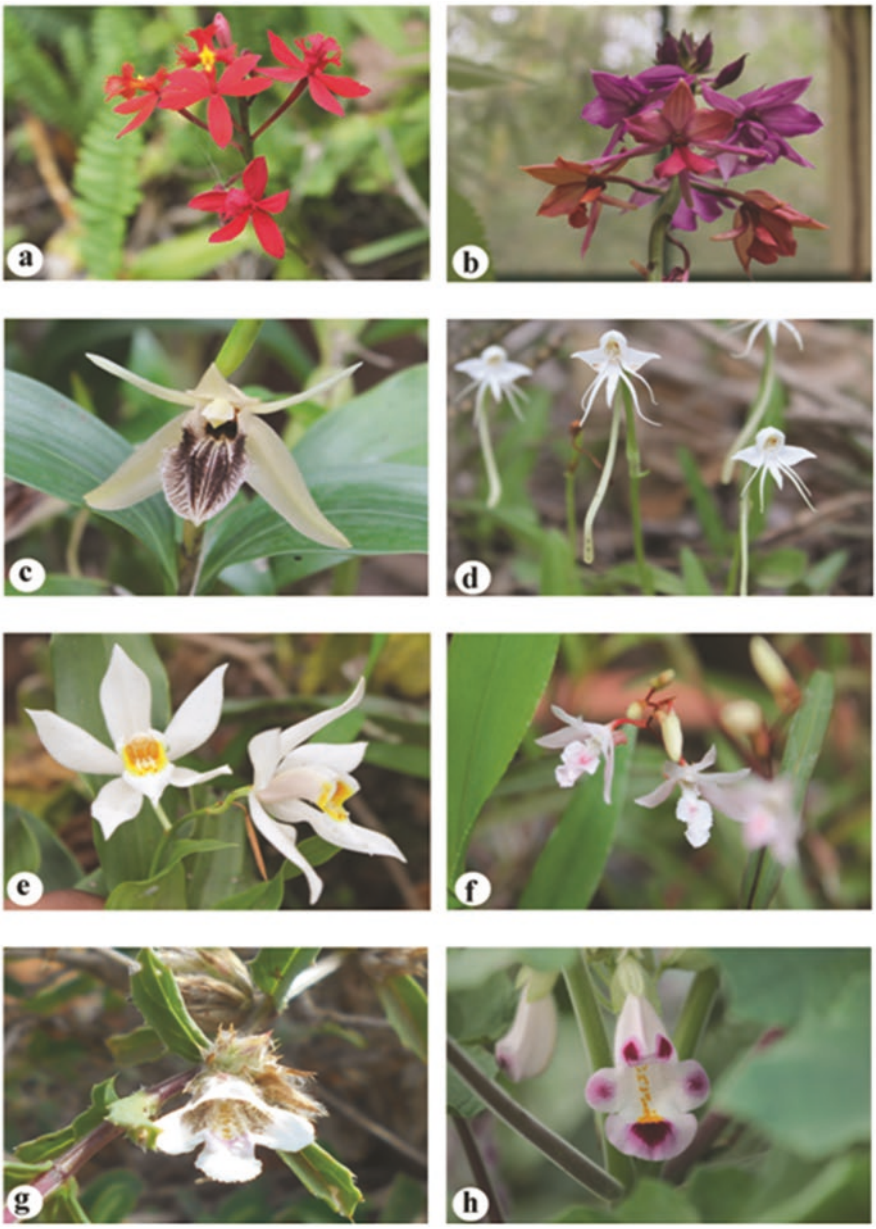

Fig. 18.1 Beautiful orchid flowers observed in NOEGL, Yercaud. (**a**) *Epidendrum radicans*, (**b**) *Calanthe sylvatica*, (**c**) *Coelogyne fimbriata*, (**d**) *Habenaria rariflora*, (**e**) *Coelogyne nervosa*, (**f**) *Dendrobium wightii* and flowers (other than orchids) with mimic syndrome; (**g**) *Lepidagathis cristata* and (**h**) *Martynia annua*

pollinator available for the orchid species. Moreover, the officials in the orchidarium are maintaining these orchid species mainly through vegetative propagation methods (Table 18.2).

We also found that the deceptive pollination mechanism is not only occurring among the Orchidaceae family but also in some other families of Angiosperms in India. Some of them we observed are *Lepidagathis cristata* of Acanthaceae family (Fig. 18.1g) and *Martynia annua* of Martyniaceae family (Fig. 18.1h). In *L. cristata*, we observed numerous distinct yellow spots present inside the corolla tube just beneath the original stamen. These yellow spots might mimic the pseudo-pollen and act as visual cue for landing platform for their pollinator (honey bee) which guides to collect the nectar. The presence of yellow spots mimicking the pseudo-pollen is also observed in *M. annua*.

18.3 Pollination and Conservation in Orchids

From the analysis, it is clear that Indian orchid species also exhibits deceptive pollination mechanism. However, the number of studies on pollination of deceptive orchids in India is very limited. This state has to be changed by studying, identifying and understanding the pollination mechanism and the pollinator involved in various phytogeographical regions. Among the observed 38 orchid genera in NOEGL, majority of them have been listed in IUCN Red List of Threatened Species and other local reports. Hence, it is clear that pollination studies are vital to develop conservation strategies. The pollination studies also help to understand any unique deceptive mechanism hold by Indian orchids. In many cases, deceptive orchids exhibit species-specific pollinator for its pollination success. This may possess a very high chance for the speciation event (Borba and Semir 1999). Studying the pollination biology of orchids will help test the pollinator-driven speciation event and also to know the reason for the declining of orchid population in several orchid reserves. Moreover, identifying the model for a particular orchid species and growing the model species in conservation centres is essential for the attraction of pollinators. This will assist the natural mode of pollination in addition to the vegetative propagation of orchids in conservation centres. Orchids often offer many puzzles to the evolutionary biologists in the evolution and maintenance of deception. Among the deceptive orchids, the evolution of sexually deceptive species such as *Ophrys* and *Chiloglottis* has long been enticing the evolutionary biologists (Peakall et al. 2010). The Australian orchid genus *Chiloglottis* has evolved to mimic the sex pheromone of the female wasp to provoke precopulatory behaviour of the male wasp (pollinator) to achieve pollination (Peakall et al. 2010; Peakall and Whitehead 2014; Schiestl et al. 2003). But still the complete mechanism of deception is not well understood. However, the evolutionary lineage of certain sexually deceptive orchid species coincides in the molecular level, species-specific pollinator level and also in the olfactory chemical level phylogenetic tree construction (Ågren et al. 1984; Mant et al. 2005; Peakall et al. 2010). Hence, it is crucial to study the Indian orchid

Table 18.2 Habitat, flowering, fruiting period and type of deception (up to genera level) for orchids maintained in National Orchidarium and Experimental Garden and Laboratories (NOEGL), Yercaud, Tamil Nadu

S. no.	Name of the orchid species	Habitat	Flowering and fruiting period	Type of deception reported at genus level	Conservation status (IUCN and Local)	References
1.	*Acampe ochracea*	Epiphyte	February–April	Not available	VU	MOE (2012) and http://www.indiabiodiversity.org/
2.	*A. praemorsa*	Epiphyte	March–April	Not available	O	Karuppusamy et al. (2009) and http://www.indiabiodiversity.org/
3.	*Acanthephippium bicolor*	Terrestrial	March–April	Not available	R	Karuppusamy et al. (2009) and http://www.indiabiodiversity.org/
4.	*Aerides maculosa*	Epiphyte	June–August	Not available	EN	Kumar et al. (2001) and http://www.indiabiodiversity.org/; http://e-monocot.org/
5.	*A. ringens*	Epiphyte	June–July	Not available	S	Karuppusamy et al. (2009) and http://www.indiabiodiversity.org/; http://e-monocot.org/
6.	*Anoectochilus elatus*	Terrestrial	November–December	Not available	R	Karuppusamy et al. (2009) and http://www.indiabiodiversity.org/
7.	*Bulbophyllum acutiflorum*	Epiphyte	March–April	Brood-site deception	EN	Borba and Semir (1999), Kumar et al. (2001) and http://www.indiabiodiversity.org/
8.	*B. fischeri*	Epiphyte	October–November	Brood-site deception	R	Borba and Semir (1999), Karuppusamy et al. (2009) and http://www.indiabiodiversity.org/
9.	*B. fuscopurpureum*	Epiphyte	April–May	Brood-site deception	EN	Borba and Semir (1999), Kumar et al. (2001) and http://www.indiabiodiversity.org/
10.	*B. pteroglossum*	Epiphyte	April–June	Brood-site deception	Not available	Borba and Semir (1999), Gogoi et al. (2015) and http://e-monocot.org/
11.	*Bulbophyllum* sp.	Not available	Not available	Brood-site deception	Not available	Borba and Semir (1999)
12.	*Calanthe sylvatica*	Terrestrial	August–December	Generalized food deception	C	Gogoi et al. (2012), Ren et al. (2014), Tang et al. (2014) and http://www.indiabiodiversity.org/; http://e-monocot.org/
13.	*Callostylis bambusifolia*	Epiphyte	December (Flowering)	Not available	Not available	http://e-monocot.org/; http://www.efloras.org/flora_page.aspx?flora_id=2
14.	*Cleisostoma tenuifolium*	Epiphyte	July–August	Not available	NT	MOE (2012) and http://www.indiabiodiversity.org/; http://e-monocot.org/
15.	*Coelogyne breviscapa*	Epiphyte	March–April	Generalized food deception	O	Karuppusamy et al. (2009), Tang et al. (2014) and http://www.indiabiodiversity.org/

(continued)

Table 18.2 (continued)

S. no.	Name of the orchid species	Habitat	Flowering and fruiting period	Type of deception reported at genus level	Conservation status (IUCN and Local)	References
16.	*C. corymbosa*	Epiphyte	April and May (Flowering)	Generalized food deception	Not available	Tang et al. (2014) and http://www.indiabiodiversity.org/
17.	*C. cristata*	Epiphyte/ lithophyte	February and March	Generalized food deception	C	Jalal (2012), Tang et al. (2014) and http://www.indiabiodiversity.org/;
18.	*C. fimbriata*	Epiphyte	August–October (flowering) and next April–August (fruiting)	Generalized food deception	EN	Karuppusamy et al. (2009), Gogoi et al. (2012), Tang et al. (2014) and http://e-monocot.org/
19.	*C. flaccida*	Epiphyte	March–April	Generalized food deception	Not available	Tang et al. (2014) and http://www.indiabiodiversity.org/
20.	*C. mossiae*	Epiphyte	August–November	Generalized food deception	EN	Kumar et al. (2001), Tang et al. (2014) and http://www.indiabiodiversity.org/
21.	*C. nervosa*	Lithophyte	June–July	Generalized food deception	EN	Kumar et al. (2001), Tang et al. (2014) and http://www.indiabiodiversity.org/; http://e-monocot.org/
22.	*C. ovalis*	Epiphyte	August–November (flowering) and next September (fruiting)	Generalized food deception	EN	Gogoi et al. (2012), Tang et al. (2014) and http://www.indiabiodiversity.org/; http://www.efloras.org/flora_page.aspx?flora_id=2
23.	*C. prolifera*	Epiphyte	June (flowering)	Generalized food deception	Not available	Tang et al. (2014) and http://www.indiabiodiversity.org/; http://www.efloras.org/flora_page.aspx?flora_id=2
24.	*C. stricta*	Epiphyte	September–December	Generalized food deception	SP	Gogoi et al. (2015), Jalal (2012), Tang et al. (2014) and http://www.indiabiodiversity.org/
25.	*Conchidium braccatum*	Epiphyte	July–October	Not available	NT	MOE (2012) and http://www.indiabiodiversity.org/; http://e-monocot.org/
26.	*C. filiforme*	Epiphyte	September–December	Not available	Not available	http://www.indiabiodiversity.org/
27.	*Cottonia peduncularis*	Epiphyte	March–May	Not available	NT	http://www.indiabiodiversity.org/ and MOE (2012)

28.	*Cymbidium aloifolium*	Epiphyte	April–May	Batesian floral/ generalized food/ sexual deception	C	Gogoi (2012), Kjellsson et al. (1985), Sugahara et al. (2013), Tang et al. (2014) and http://www.indiabiodiversity.org/; http://www.efloras.org/flora_page.aspx?flora_id=2
29.	*C. bicolor*	Epiphyte	March–April	Batesian floral/ generalized food/ sexual deception	EN	Gogoi (2012), Kjellsson et al. (1985), Sugahara et al. (2013), Tang et al. (2014) and http://www.indiabiodiversity.org/
30.	*C. haematodes*	Geophyte	September–October (flowering) and November–April (fruiting)	Batesian floral/ generalized food/ sexual deception	Not available	Kjellsson et al. (1985), Sugahara et al. (2013), Tang et al. (2014) and http://e-monocot.org/; http://www.efloras.org/flora_page.aspx?flora_id=2
31.	*C. mastersii*	Geophyte	October–December (flowering) and February–April (fruiting)	Batesian floral/ generalized food/ sexual deception	Not available	Kjellsson et al. (1985), Sugahara et al. (2013), Tang et al. (2014) and http://e-monocot.org/; http://www.efloras.org/flora_page.aspx?flora_id=2
32.	*Dendrobium aqueum*	Epiphyte	September–December	Batesian floral/ brood-site/ generalized food deception	R	Davies and Turner (2004), Karuppusamy (2009), Kjellsson (1985), Tang (2014) and http://www.indiabiodiversity.org/
33.	*D. chrysanthum*	Epiphyte/ lithophyte	May–September	Batesian floral/ brood-site/ generalized food deception	SP	Davies and Turner (2004), Gogoi (2015), Jalal (2012), Kjellsson (1985), Tang (2014) and http://e-monocot.org/
34.	*D. chrysotoxum*	Epiphyte	May–August	Batesian floral/ brood-site/ generalized food deception	EN	Averyanov and Averyanova (2003), Davies and Turner (2004), Gogoi (2015), Kjellsson (1985), Tang (2014) and http://e-monocot.org/
35.	*D. herbaceum*	Epiphyte	December–January	Batesian floral/ brood-site/ generalized food deception	O	Davies and Turner (2004), Karuppusamy (2009), Kjellsson (1985), Tang (2014) and http://www.indiabiodiversity.org/; http://e-monocot.org/

(continued)

Table 18.2 (continued)

S. no.	Name of the orchid species	Habitat	Flowering and fruiting period	Type of deception reported at genus level	Conservation status (IUCN and Local)	References
36.	*D. heyneanum*	Epiphyte	August–November	Batesian floral/ brood-site/ generalized food deception	EN	Davies and Turner (2004), Kjellsson (1985), Kumar et al. (2001), Tang (2014) and http://www.indiabiodiversity.org/; http://e-monocot.org/
37.	*D. khasianum*	Epiphyte	Not available	Batesian floral/ brood-site/ generalized food deception	Not available	Davies and Turner (2004), Kjellsson (1985), Tang et al. (2014) and http://e-monocot.org/
38.	*D. macraei*	Epiphyte	Not available	Batesian floral/ brood-site/ generalized food deception	Not available	Davies and Turner (2004), Kjellsson (1985), Tang et al. (2014) and http://e-monocot.org/
39.	*D. moschatum*	Epiphyte	June–September	Batesian floral/ brood-site/ generalized food deception	C	Davies and Turner (2004), Gogoi et al. (2012, 2015), Kjellsson (1985), Tang et al. (2014) and http://e-monocot.org/
40.	*D. nanum*	Epiphyte	September–August	Batesian floral/ brood-site/ generalized food deception	R	Davies and Turner (2004), Karuppusamy et al. (2009), Kjellsson (1985), Tang et al. (2014) and http://www.indiabiodiversity.org/; http://e-monocot.org/
41.	*D. nodosum*	Epiphyte	March–April	Batesian floral/ brood-site/ generalized food deception	Not available	Davies and Turner (2004), Kjellsson (1985), Tang et al. (2014) and http://www.indiabiodiversity.org/; http://e-monocot.org/
42.	*D. ochreatum*	Epiphyte	May–June (flowering)	Batesian floral/ brood-site/ generalized food deception	VU	Averyanov and Averyanova (2003), Kjellsson (1985), Davies and Turner (2004), Rokaya et al. (2013), Tang et al. (2014) and http://www.indiabiodiversity.org/

43.	*D. ovatum*	Epiphyte	December–January	Batesian floral/ brood-site/ generalized food deception	VU	Davies and Turner (2004), Kjellsson (1985), Kumar et al. (2001), Rokaya et al. (2013), Tang et al. (2014) and http://www.indiabiodiversity.org/
44.	*D. wightii*	Lithophyte	September–October	Batesian floral/ brood-site/ generalized food deception	R	Davies and Turner (2004), Karuppusamy et al. (2009), Kjellsson (1985), Rokaya et al. (2013), Tang et al. (2014) and http://www.indiabiodiversity.org/
45.	*Diplocentrum recurvum*	Epiphyte	May–June	Not available	C	Karuppusamy et al. (2009) and http://www.indiabiodiversity.org/
46.	*Epidendrum radicans* (exotic)	Terrestrial	Not available	Batesian floral and generalized food deception	Not available	Jersáková et al. (2009) and http://www.kew.org/science-conservation/plants-fungi/epidendrum-radicans
47.	*Epigeneium amplum*	Epiphyte/ lithophyte	November (flowering)	Not available	LR	Averyanov and Averyanova (2003) and http://e-monocot.org/; http://www.efloras.org/flora_page.aspx?flora_id=2
48.	*Eria mysorensis*	Epiphyte	August–September	Not available	EN	Kumar et al. (2001) and http://www.indiabiodiversity.org/
49.	*Eria pauciflora*	Epiphyte	August–September	Not available	C	Karuppusamy et al. (2009) and http://www.indiabiodiversity.org/
50.	*Eria pseudoclavicaulis*	Epiphyte	August–September	Not available	VU	Kumar et al. (2001) and http://www.indiabiodiversity.org/; http://e-monocot.org/
51.	*Gastrochilus acaulis*	Epiphyte	October–November	Not available	C	Karuppusamy et al. (2009) and http://www.indiabiodiversity.org/; http://e-monocot.org/
52.	*G. calceolaris*	Epiphyte	March–April (flowering)	Not available	CR[a]	http://e-monocot.org/; http://www.iucnredlist.org/search; http://www.efloras.org/flora_page.aspx?flora_id=2
53.	*Geodorum densiflorum*	Terrestrial	April–June	Not available	C	Karuppusamy et al. (2009) and http://www.indiabiodiversity.org/
54.	*Goodyera procera*	Terrestrial	February–March	Not available	R	Karuppusamy et al. (2009) and http://www.indiabiodiversity.org/
55.	*Habenaria rariflora*	Geophyte	July–September	Not available	C	Karuppusamy et al. (2009) and http://www.indiabiodiversity.org/; http://e-monocot.org/
56.	*Habenaria roxburghii*	Geophyte	August–October	Not available	R	Karuppusamy et al. (2009) and http://www.indiabiodiversity.org/; http://e-monocot.org/
57.	*Liparis atropurpurea*	Terrestrial	July–October	Not available	EN	MOE (2012) and http://www.indiabiodiversity.org/

(continued)

Table 18.2 (continued)

S. no.	Name of the orchid species	Habitat	Flowering and fruiting period	Type of deception reported at genus level	Conservation status (IUCN and Local)	References
58.	*L. viridiflora*	Epiphyte	September–October	Not available	C	Karuppusamy et al. (2009) and http://www.indiabiodiversity.org/; http://e-monocot.org/
59.	*Luisia tenuifolia*	Epiphyte	Not available	Not available	Not available	http://e-monocot.org/
60.	*Luisia tristis*	Epiphyte	March–August	Not available	C	Jalal (2012) and http://www.indiabiodiversity.org/; http://e-monocot.org/
61.	*Malaxis versicolor*	Geophyte	Not available	Not available	LC	MOE (2012) and http://e-monocot.org/
62.	*Nervilia plicata*	Geophyte	February–March	Not available	C	Karuppusamy et al. (2009) and http://www.indiabiodiversity.org/; http://e-monocot.org/
63.	*Oberonia brunoniana*	Epiphyte	December–January	Not available	R	Karuppusamy et al. (2009) and http://www.indiabiodiversity.org/; http://e-monocot.org/
64.	*O. santapaui*	Epiphyte	November–December	Not available	C	Karuppusamy et al. (2009) and http://www.indiabiodiversity.org/; http://e-monocot.org/
65.	*Paphiopedilum druryi*	Terrestrial	January–April	Brood-site/ generalized food deception	CR[a]	Bänziger (1996), Shi et al. (2007), Tang et al. (2014) and http://www.indiabiodiversity.org/; http://www.iucnredlist.org/search
66.	*P. hirsutissimum*	Lithophyte/ terrestrial	April–May (flowering)	Brood-site/ generalized food deception	VU[a]	Bänziger (1996), Shi et al. (2007), Tang et al. (2014) and http://e-monocot.org/; http://www.iucnredlist.org/search; http://www.efloras.org/flora_page.aspx?flora_id=2
67.	*P. insigne*	Terrestrial	October–December (flowering)	Brood-site/ generalized food deception	EN[a]	Bänziger (1996), Shi et al. (2007), Tang et al. (2014) and http://www.iucnredlist.org/search; http://www.efloras.org/flora_page.aspx?flora_id=2
68.	*P. spicerianum*	Lithophyte/ terrestrial	September–November (flowering)	Brood-site/ generalized food deception	EN[a]	Bänziger (1996), Shi et al. (2007), Tang et al. (2014) and http://e-monocot.org/; http://www.iucnredlist.org/search; http://www.efloras.org/flora_page.aspx?flora_id=2
69.	*P. villosum*	Epiphyte/ lithophyte	September–November	Brood-site/ generalized food deception	VU[a]	Bänziger (1996), Gogoi (2015), Shi et al. (2007), Tang et al. (2014) and http://e-monocot.org/; http://www.iucnredlist.org/search
70.	*Papilionanthe subulata*	Epiphyte	March–April	Not available	C	Karuppusamy et al. (2009) and http://e-monocot.org/

71.	*Pholidota articulata*	Epiphyte/ lithophyte	June–August (flowering) and October–December (fruiting)	Not available	C	Gogoi (2012) and http://e-monocot.org/; http://www.efloras.org/flora_page.aspx?flora_id=2
72.	*P. imbricata*	Epiphyte	September–March	Not available	C	Gogoi (2012) and http://www.indiabiodiversity.org/
73.	*P. pallida*	Epiphyte	September–November	Not available	C	Karuppusamy et al. (2009) and http://e-monocot.org/
74.	*Pinalia polystachya*	Epiphyte	October–November	Not available	C	Karuppusamy et al. (2009), Li (2010) and http://e-monocot.org/
75.	*P. stricta*	Epiphyte/ lithophyte	November–February (flowering) and April–May (fruiting)	Not available	Not available	http://e-monocot.org/; http://www.efloras.org/flora_page.aspx?flora_id=2
76.	*Polystachya concreta*	Epiphyte	July–August	Not available	C	Karuppusamy et al. (2009) and http://www.indiabiodiversity.org/
77.	*Stanhopea wardii*	Epiphyte	Not available	Not available	Not available	http://e-monocot.org/
78.	*Sirhookera lanceolata*	Epiphyte	August–November	Not available	NT	http://www.indiabiodiversity.org/ and MOE (2012)
79.	*S. latifolia*	Epiphyte	August–September	Not available	CR	http://www.indiabiodiversity.org/ and Averyanov and Averyanova (2003)
80.	*Taeniophyllum scaberulum*	Epiphyte	February–June	Not available	EN	http://www.indiabiodiversity.org/ and Kumar et al. (2001)
81.	*Thunia alba var. bracteata*	Epiphyte	March–May	Not available	SP	http://www.indiabiodiversity.org/ and Jalal (2012)
82.	*Trias stocksii*	Epiphyte	December–January	Not available	CR	http://www.indiabiodiversity.org/; http://e-monocot.org/ and Kumar et al. (2001)
83.	*Vanda spathulata*	Epiphyte	September–January	Not available	VU[a]	http://www.indiabiodiversity.org/; http://e-monocot.org/; http://www.iucnredlist.org/search
84.	*V. testacea*	Epiphyte	April–May	Not available	C	http://www.indiabiodiversity.org/; http://e-monocot.org/ and Karuppusamy et al. (2009)
85.	*V. thwaitesii*	Epiphyte	September–April	Not available	CR (PE)	http://www.indiabiodiversity.org/; http://e-monocot.org/ and MOE (2012)
86.	*Xenikophyton smeeanum*	Epiphyte	May–August	Not available	EN	http://www.indiabiodiversity.org/ and Kumar et al. (2001)
87.	*Zeuxine longilabris*	Geophyte	February–March	Not available	C	http://www.indiabiodiversity.org/; http://e-monocot.org/ and Karuppusamy et al. (2009)

C common, *CR* critically endangered, *CR (PE)* critically endangered (possibly extinct), *EN* endangered, *LR* lower risk, *NT* near threatened, *O* occasional, *R* rare, *SP* sparse, *VR* very rare, *VU* vulnerable

[a]denotes the species reported in "The IUCN Red List of Threatened Species™" (http://www.iucnredlist.org/search)

pollination biology in the near future to unravel several mysteries present in the evolution of deception in orchids and also for its better conservation.

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Evolution of Organismal Female Wasp Mimics in Sexually Deceptive Orchid Genus *Chiloglottis* (Orchidaceae)

19

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Abstract

Orchids are fascinating to the evolutionary biologists because of their spectacular flower structure, colours and specialized strategies in pollination. In 30,000 species of orchids, one third accomplishes pollination through deception by mimicking food, shelter and sexual partner of model insects. In sexual deception, orchids lure their pollinators by mimicking sex pheromone and by depicting the morphology of their model insects on the flower labellum. In Australian orchid genus *Chiloglottis*, occurrence of model's (female sex of pollinator) sex pheromone mimicry for pollination through sexual deception is well established. However, the morphological evolution of calli structures on labellum representing model insect mimic in *Chiloglottis* is still a mystery. In this study, we qualitatively analysed the calli structures on the labellum in 21 species of the genus *Chiloglottis* and propose its evolution progressing from organ to organism level of the model with a few intermediate mimics in the 'Darwinian Style'. From the qualitative analysis, we distinguish 3 groups of female wasp mimic evolution amongst 21 species as organ (parts of head and thorax, 8 species), intermediate (imperfect head and thorax, 8 species) and organism (head, thorax and abdomen, 5 species) level based on the arrangement of calli structures on the labellum. The labellum shape is found to have an association in the evolution of mimic structure, depicting cordate in organ level and spatulate or obovate or diamond shape

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in intermediate and organismal forms. Our results clearly indicate that the organismal form of insect mimics might have evolved from organ level in sexually deceptive orchids.

Keywords
Calli structure · Chiloglottones · Mimic on labellum · *Neozeleboria* · Organismal mimic

19.1 Introduction

In Orchidaceae, there are 18 genera which achieve pollination through sexual deception (Jersáková et al. 2006). Earlier studies on pollination in sexually deceptive orchids have paid attention mainly to the olfactory stimuli-based mimicry (Peakall et al. 2010; Peakall and Whitehead 2014; Schiestl et al. 2003) over the calli-mediated insect-form mimicry. In deceptive orchids, similarity between model and mimics in visual signals such as shape, colour and hairiness of calli is established (Ågren et al. 1984; Dafni 1984). Amongst the sexually deceptive orchids, the Australian genus *Chiloglottis* holds both prominent olfactory stimuli (chiloglottones) and deceiving tactile calli structures. The role of chiloglottones in sexual deception is well documented (Peakall et al. 2010; Peakall and Whitehead 2014; Schiestl et al. 2003). But the quantitative variations in numbers and shapes of calli have been merely interpreted as a taxonomic trait for classification of *Chiloglottis* species (Jones 1991, 1997). Visual signals allure pollinators to the flower in proximal distance, whereas tactile stimuli are also essential to enable successful pollinia transfer (Dafni 1984). We noticed a diverse range of calli structures at various levels of mimicry development, ranging from simple organ to complex organism structures in *Chiloglottis*. The evolution of complete model (female sex *Neozeleboria*) insect mimic could not be unlinked from lineages within the genera, thus in this paper, to the best of our understanding after the qualitative analysis of calli structures on the labellum, we have tried to trace the path of evolution of organismal mimic in *Chiloglottis* and propose that the evolution of female insect mimics in *Chiloglottis* might have occurred starting from organ level model insect mimicry to the organismal level in a stepwise manner with few intermediate mimics in a 'Darwinian Style'.

19.1.1 Species Selection and Species List

In order to understand the evolution of organ to organismal mimicry in sexual deceptive orchids, the genus *Chiloglottis* was considered for our study. There are 24 accepted *Chiloglottis* species listed in the plant list database (http://www.theplantlist.org/tpl1.1/search?q=Chiloglottis).

19.1.1.1 Picture Selection for the Study

The images of the respective *Chiloglottis* species was retrieved by using the binomial name of each species on Google image search engine. Out of the 24 species, authorized images for 16 species were obtained from RetiredAussies website (www.retiredaussies.com), and for the following species, *Chiloglottis cornuta*, *C. formicifera*, *C. longiclavata* and *C. platyptera*, images were obtained from websites of the University of Tasmania (http://www.utas.edu.au/dicotkey/dicotkey/orchids_this/gChiloglottis) and PBase (http://www.pbase.com/image/107517272) and from Flickr photo feed (https://www.flickr.com/photos/jvinoz/ 7,335,911,698/sizes/l), (https://www.flickr.com/photos/jvinoz/4956225936/sizes/l), respectively. The image of *C. anaticeps* is not given due to unavailability of high-resolution pictures even though it was considered for the study. *Chiloglottis* x *pescottiana*, *C. turfosa* and *C. palachila* were excluded from the study due to lack of information and images from authorized sources.

19.2 Organ to Organismal Insect Mimic Lineage

The labellum shape and calli numbers were noted visually from the studied images and matched to the respective species provided in the keys of *Chiloglottis* (Harden 1993). The pollinator taxa and sex pheromone emission details of *Chiloglottis* were noted from Mant et al. (2005a, b), Bower and Brown (2009) and Peakall et al. (2010). The studied species were categorized into organ, intermediate and organismal level based on their resemblance to the model insect.

19.3 Evolution of Organismal Female Wasp Mimics in *Chiloglottis*

Upon observation of flowers in *Chiloglottis*, we segregated them into three categories (organ, intermediate and organism) based on their levels of resemblance to the model insect forged by the calli structures on the labellum (Table 19.1). Mimics in organ level exhibit cordate labellum, whereas spatulate and diamond-shaped labellum is seen in intermediate mimics; organismal mimics display obovate and diamond-shaped labellum. Of the eight organ-level mimics, *C. valida*, *C. jeanesii*, *C. chlorantha*, *C. triceratops*, *C. pluricallata* and *C. gunnii* are pollinated by species of *Neozeleboria*; *C. grammata* is pollinated by *Eirone leai*, while self-pollination was recorded in *C. cornuta*. In eight species at intermediate level, five species are pollinated by species of *Neozeleboria*; *C. diphylla* was pollinated by *Arthrothynnus latus*, and for the rest of the species, sufficient information is not available. Four out of five organismal level mimics are pollinated exclusively by *Neozeleboria* species, where adequate information is not available for the other. Out of 21 studied species of *Chiloglottis*, 12 species produce one or more chiloglottones, where *C. diphylla* does not produce chiloglottone; enough data is not available for the rest of the eight species (Table 19.1).

Table 19.1 Name of the *Chiloglottis* species, its pollinator, labellum shape, types of chiloglottones and level of mimic (in terms of head, thorax, waist and abdomen)

S. no.	Name of the *Chiloglottis* species	Pollinator	Labellum shape	Chiloglottones	Mimic level, organ to organism			
					Head	Thorax	Waist	Abdomen
1.	*C. valida*	*Neozeleboria cryptoides* and *N. monticola*[a]	Broadly cordate	Chiloglottone 1[a]	× Parts of head	×	×	×
2.	*C. jeanesii*	*Neozeleboria* sp. (*aff. impatiens*)[a]	Broadly cordate	Chiloglottone 3[a]	× Parts of head	×	×	×
3.	*C. chlorantha*	*Neozeleboria aff. impatiens*[b] and *N. impatiens*[c]	Cordate	Chiloglottone 3[a]	× Parts of head	× Parts of thorax	×	×
4.	*C. triceratops*	*Neozeleboria monticola*[c], *N. monticola* 3[b] and *N. carinicollis*[a]	Cordate	Chiloglottone 4[a]	× Parts of head	× Parts of thorax	×	×
5.	*C. pluricallata*	*Neozeleboria impatiens*[b]	Broadly cordate	Chiloglottone 1 and chiloglottone 2[a]	× Parts of head	× Parts of thorax	×	×
6.	*C. grammata*	*Eirone leai*[a]	Broadly cordate	Chiloglottone 2[a]	× Parts of head	× Parts of thorax	×	×
7.	*C. gunnii*	*Neozeleboria* sp.[d]	Narrowly cordate	Chiloglottone 2[a]	× Parts of head	× Parts of thorax	× Partial waist	×
8.	*C. cornuta*	Self-pollination[b]	Narrowly cordate	Not available	× Parts of head	× Parts of thorax	× Partial waist	×
9.	*C. sylvestris*	*Neozeleboria* sp. 50[c]	Diamond	Not available	✓	✓	✓	×

10.	*C. seminuda*	*Neozeleboria* sp. (proxima2)[a] and *N.* sp. 29[b]	Diamond	Chiloglottone 1[a]	✓	✓	✓	✓
11.	*C. sphaerula*	Not available	Broad spatulate	Not available	✓	✓	✓	✓
12.	*C. longiclavata*	Not available	Diamond	Not available	✓	✓	✓	✓
13.	*C. diphylla*	*Arthrothynnus latus*[a]	Diamond	No chiloglottone[a]	✓	✓	✓	✓
14.	*C. reflexa*	*Neozeleboria* sp. 30[c] and *N. nr. proxima*[d]	Diamond	Not available	✓	✓	✓	✓
15.	*C. sphyrnoides*	*Neozeleboria* sp. 3[a]	Broad spatulate	Chiloglottone 5[a]	✓	✓	✓	×
16.	*C. trapeziformis*	*Neozeleboria cryptoides* and *N. monticola*[a]	Spade to diamond	Chiloglottone 1[a]	✓	✓	✓	×
17.	*C. anaticeps*	*Neozeleboria* sp. 33[c]	Obovate	Not available	✓	✓	✓	✓
18.	*C. platyptera*	*Neozeleboria* sp. 40[c]	Obovate	Chiloglottone 1 and chiloglottone 2[a]	✓	✓	✓	✓
19.	*C. trullata*	Not available	Obovate	Not available	✓	✓	✓	✓
20.	*C. truncata*	*Neozeleboria aff. ursitatum*[c]	Diamond	Not available	✓	✓	✓	✓
21.	*C. formicifera*	*Neozeleboria* sp. 41[b]	Diamond	Chiloglottone 1 and chiloglottone 4[a]	✓	✓	✓	✓

× indicates absence and ✓ indicates presence

[a]Peakall et al. (2010), [b]Mant et al. (2005a), [c]Mant et al. (2005b), [d]Bower and Brown (2009)

In *C. valida*, a large calli looks like the parts of the head of the model in the centre and it is flanked by two sets of smaller calli. In *C. chlorantha*, 12 calli are collectively arranged as parts of head and outer margin of the model's thorax. *C. triceratops* exhibits 11 calli representing parts of the head and an elongated thorax. In *C. pluricallata*, 12 calli form parts of the head and complete margin of the thorax. In *C. grammata*, 19 calli form parts of the head and prominent thorax with a few additional calli. A total of 14 calli changed their shape and appear as protuberances of the head, thorax and a primitive waist in *C. gunnii*. In *C. cornuta*, an incomplete skeleton of model insect is formed by eight protuberance calli structures (Fig. 19.1a–h).

Of the eight species at intermediate level of resemblance to model's structure, *C. sylvestris* has a prominent head formed by a calli, and numerous other calli form a compact waist and thorax. The protuberance calli structures resembling head, thorax, waist and a primitive abdomen are noticed in both *C. seminuda* and *C. sphaerula* with minor variations. In *C. longiclavata*, the mimic is like *C. seminuda* but with additional protuberance calli forming an incomplete abdomen. In *C. diphylla*, a depression on the calli forms the model's head which gives rise to eye-like structures and poses a narrow abdomen. The eye-like calli is wider and poses hairlike extensions bordering narrower thorax and an abdomen in *C. reflexa*. In *C. sphyrnoides*, the eyes are further separated apart and sporting a visible thorax and waist. In *C. trapeziformis*, the head with prominent eyes and thorax are joined together to form two body segments with a distinct waist (Fig. 19.2a–h).

In organism level of evolution, almost a whole model insect is achieved in five species. In *C. anaticeps* (not shown in the figure), in addition to head, thorax and waist, a few bulging calli give the outline of a complete abdomen. A head with two distinct eyes, an elongated thorax and a prominent abdomen are seen in *C. platyptera*. In *C. trullata*, the body parts are further separated from each other when compared to *C. platyptera*. In *C. truncata*, the body parts are even more separated and trimmed down, forming an enhanced mimic of the model organism. An almost complete mimic of the female model is portrayed in *C. formicifera* (Fig. 19.3a–d). The evolution of insect mimicry in *Paracaleana* and *Drakaea* trails a similar path, initiating from *P. dixonii* and *D. elastica* in each and achieving a higher morphological resemblance to their respective model insects in *P. minor* and *D. livida* (Fig. 19.3e–h and for futher details see supporting information).

We assume that selection may favour different lineages of mimics independent of each other during the rapid diversification of species in this genus. Existence of separate lineages is partly confirmed by the lack of frequent hybridization between these species (except *Chiloglottis* x *pescottiana*) (Peakall et al. 1997) and occurrence of sympatric species (Mant et al. 2005c). In the genus *Chiloglottis,* the modus operandi of selection appears to be by varying the number, shape, colour and position of calli structures in each species as variable mimics. We deduce that mimicry of model insects must have refined from organ-level mimic on a cordate labellum, where eventually, the labellum ran out of space to accommodate the whole insect parts and thus ending up in forming a 'stick figure' model of the insect in *C. cornuta*. The fact that the 'stick figure' model in *C. cornuta* is self-pollinated provides evidence that it was

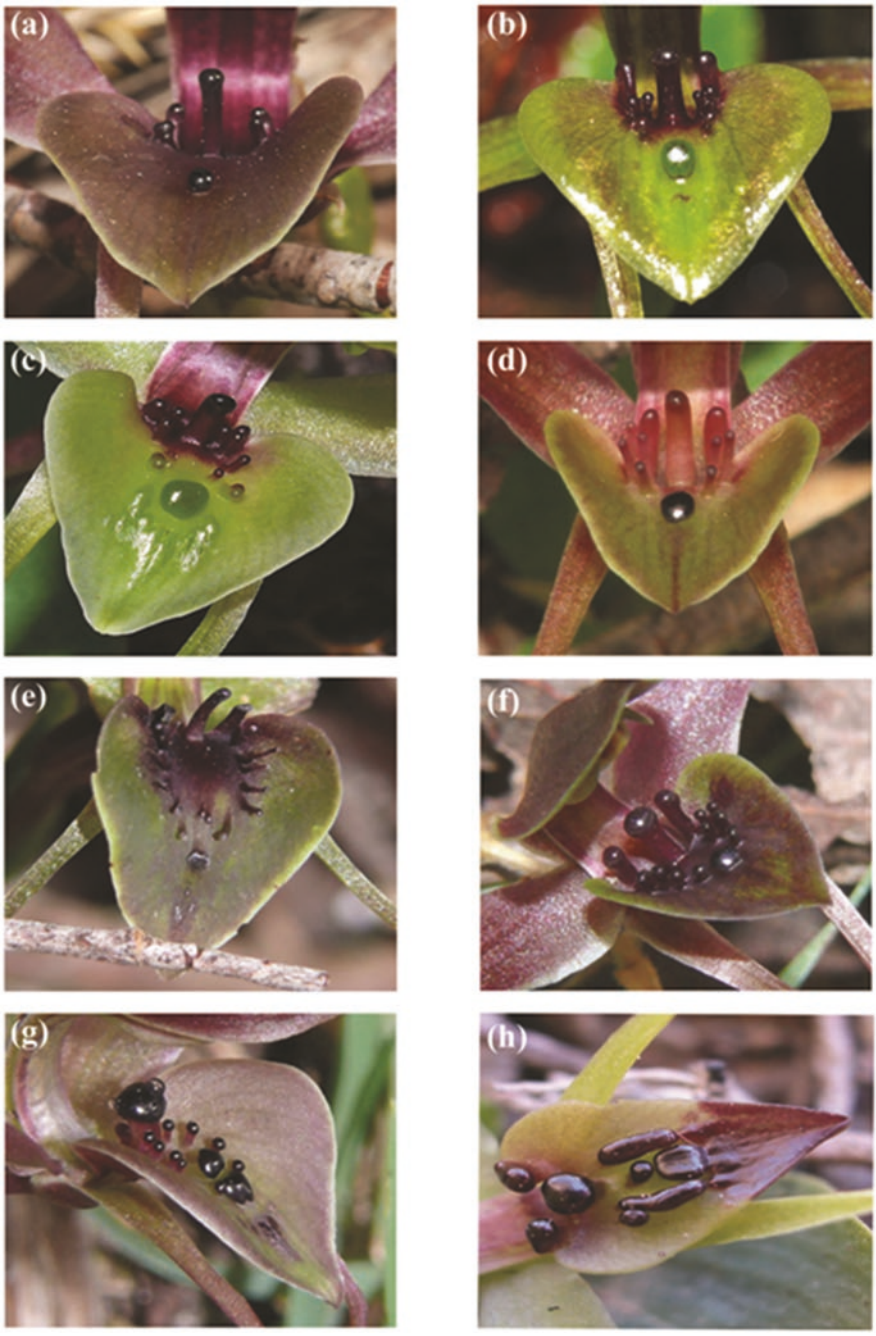

Fig. 19.1 Evolution of organ level mimics in *Chiloglottis* species. (**a**) *Chiloglottis valida*, (**b**) *C. jeanesii*, (**c**) *C. chlorantha*, (**d**) *C. triceratops*, (**e**) *C. pluricallata*, (**f**) *C. grammata*, (**g**) *C. gunnii* and (**h**) *C. cornuta*

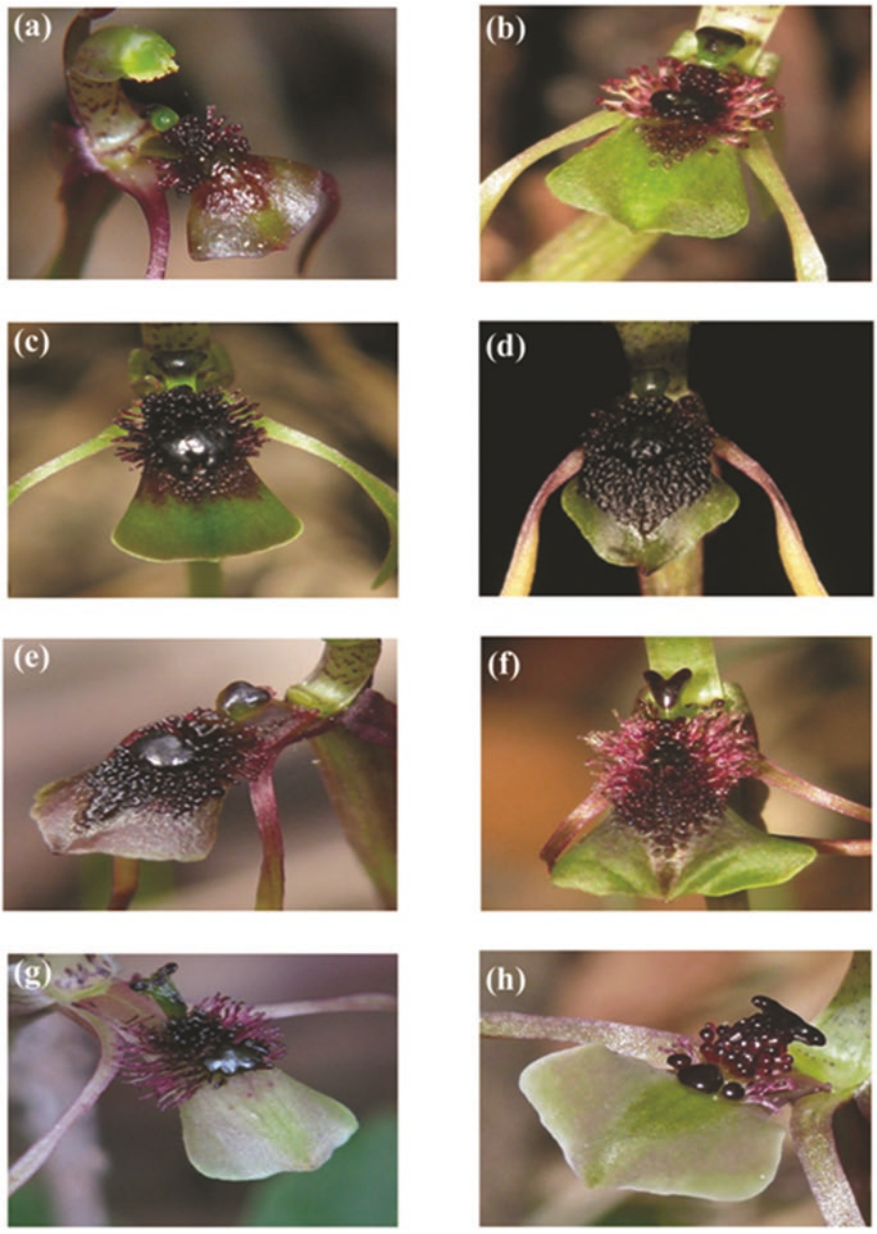

Fig. 19.2 Evolution of intermediate level mimics in *Chiloglottis* species. (**a**) *Chiloglottis sylvestris*, (**b**) *C. seminuda*, (**c**) *C. sphaerula*, (**d**) *C. longiclavata*, (**e**) *C. diphylla*, (**f**) *C. reflexa*, (**g**) *C. sphyrnoides* and (**h**) *C. trapeziformis*

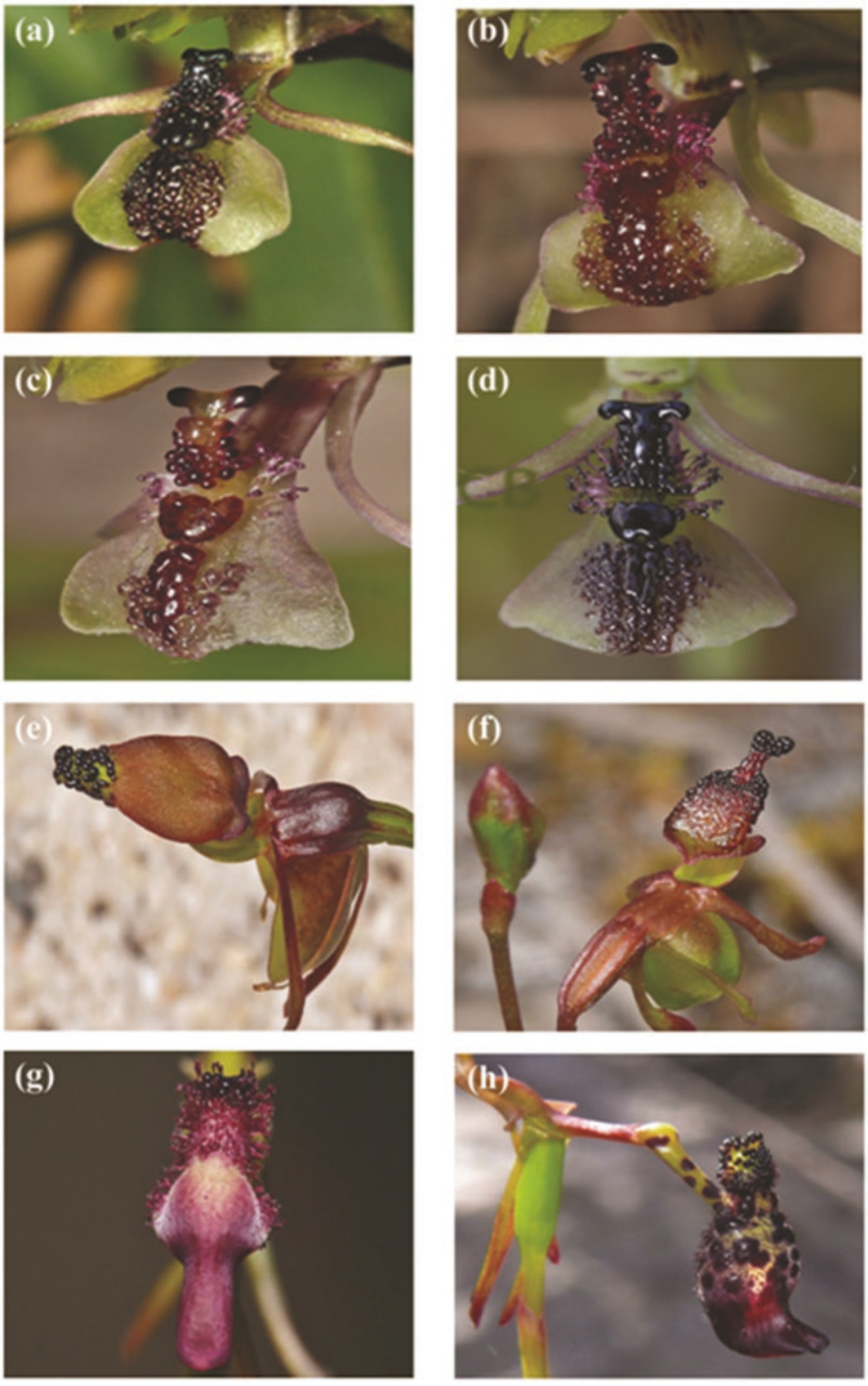

Fig. 19.3 Evolution of organismal level mimics in *Chiloglottis* species (**a**–**d**), *Paracaleana* and *Drakaea* species (**e**–**h**). (**a**) *Chiloglottis platyptera*, (**b**) *C. trullata*, (**c**) *C. truncata*, (**d**) *C. formicifera*, (**e**) *Paracaleana dixonii*, (**f**) *P. minor*, (**g**) *Drakaea elastica* and (**h**) *D. livid*a

not successful in pollination by mimicry, whereas further development has been observed in spatulate and diamond-shaped labellum where the whole body of the mimic could be accommodated. Thus, it is evidenced in the five species which have evolved striking resemblance to the model female wasp. Extensive calli restructuring in intermediate-level mimics must have resulted in the improved mimicry observed in organismal-level mimic. These variations are principally aimed to provide subtle visual and tactile stimuli by mimics to the male pollinators. From an evolutionary point of view on *Chiloglottis*, the primary focus is shed chronologically on the head and thorax regions of the mimic, where we believe that the head and thorax regions are adequate for successful pollinia transfer.

During the process of evolution in *Chiloglottis*, the mimic head, thorax, waist and abdomen have been put through various transformations to improve their degree of imitation. Formation of model's eye by mimics in many *Chiloglottis* species that occurs in multiple steps with extensive reshuffling of calli suggests that it could be one important visual signal to the males, which may help in orienting its body to that of the female mimic. The *Chiloglottis* species-pollinator relationship is clearly unilateral; orchids depend on their pollinators, but there is no evidence for a reverse trend (Nilsson 1992). Pollinator specificity and field pollinator choice test identified two cryptic species within *C. valida* and three within *C. pluricallata* which suggest selection continues its efforts to improve the fine variations in mimics of the species in this genus (Bower and Brown 2009; Mant et al. 2002). Interestingly, the three groups (organ, intermediate and organismal) segregated in the present study greatly coincide with the earlier groupings performed in this genus with the aid of molecular (Ågren et al. 1984) and chemical pheromone (Peakall et al. 2010) based tools except for some species. The *Chiloglottis* species at organ and intermediate levels are pollinated through self, *Neozeleboria* and other wasp species, whereas in organismal level, it is pollinated exclusively by species of *Neozeleboria*. We concur that, with the level of resemblance to the female wasp, the relative species specificity of the pollinator also increases drastically.

In *Chiloglottis*, it has been observed that it is not just the floral parts that undergo variations in the process of evolution, but even the minute mimic structures resembling the head, thorax and abdomen of the model insect undergoes transformation to improve its degree of mimicry, which would have paved way for the evolution of many species. Thus, we conclude that this type of multimodal evolution must have led to the existence of very many species in a relatively short period of time in angiosperms described by Charles Darwin (Davies et al. 2004) as 'an abominable mystery', for which *Chiloglottis* is a standing example.

19.3.1 Supporting Information

19.3.1.1 Evolution of Insect Mimics in Genus *Paracaleana*

In *Paracaleana dixonii*, a few protuberance calli are initiated at the tip of the labellum to mimic the body parts of the model insect. A smooth layer of calli is extending at the edges towards the base in *P. terminalis*. In *P. triens* and *P. granitica*, additional numbers of bulged calli are present at the tips, progressing towards the base of the labellum. Notched calli-covered labellum tip is slender and extends towards the base in *P. gracilicordata*. In *P. hortiorum*, the labellum towards the tip is slender, and calli cover about half of the labellar surface. A major area of *P. nigrita* labellum is covered by calli, featuring notched bulks of calli towards the slender tip of the labellum. In *P. minor,* the slender tip of the labellum is modified to form a head and neck-like projections; calli cover of the labellum on surface is almost complete (Fig. 19.4a–h).

19.3.1.2 Evolution of Insect Mimics in Genus *Drakaea*

In genus *Drakaea*, the general labellar structure resembles that of an insect's body parts. In *Drakaea elastica,* the calli formation begins at the base of the labellum with a few sparsely arranged calli surrounded by hairlike projections. More concentrated calli formation that is localized at the base of the labellum with a dense tuft of hairlike calli surrounding it is observed in *D. concolor*. In *D. glyptodon*, hairlike calli are spread all over the labellum except the tip; a clump of bulged calli is held by a thin stalk at the labellar base forming an insect head-like structure. A long narrow labellar tip and a concentrated mass of calli are seen at the base of the labellum in *D. micrantha*. In *D. thynniphila*, the entire labellar surface is covered by a tuft of hairlike calli with protuberances near the base of the labellum. The labellar calli coverage is complete, and the structure resembles much like an insect in *D. confluens*. Prominent isolated calli structures are distributed randomly all over the labellar surface with sparsely distributed minute hairlike projections in *D. livida.* In *D. gracilis*, the labellum resembles much of an insect with the head-like projection at the base and is covered by dense tufts of hairy calli. In *Drakaea* the different structural variations observed indicate that it could have evolved from diverse lineages (Fig. 19.5a–h).

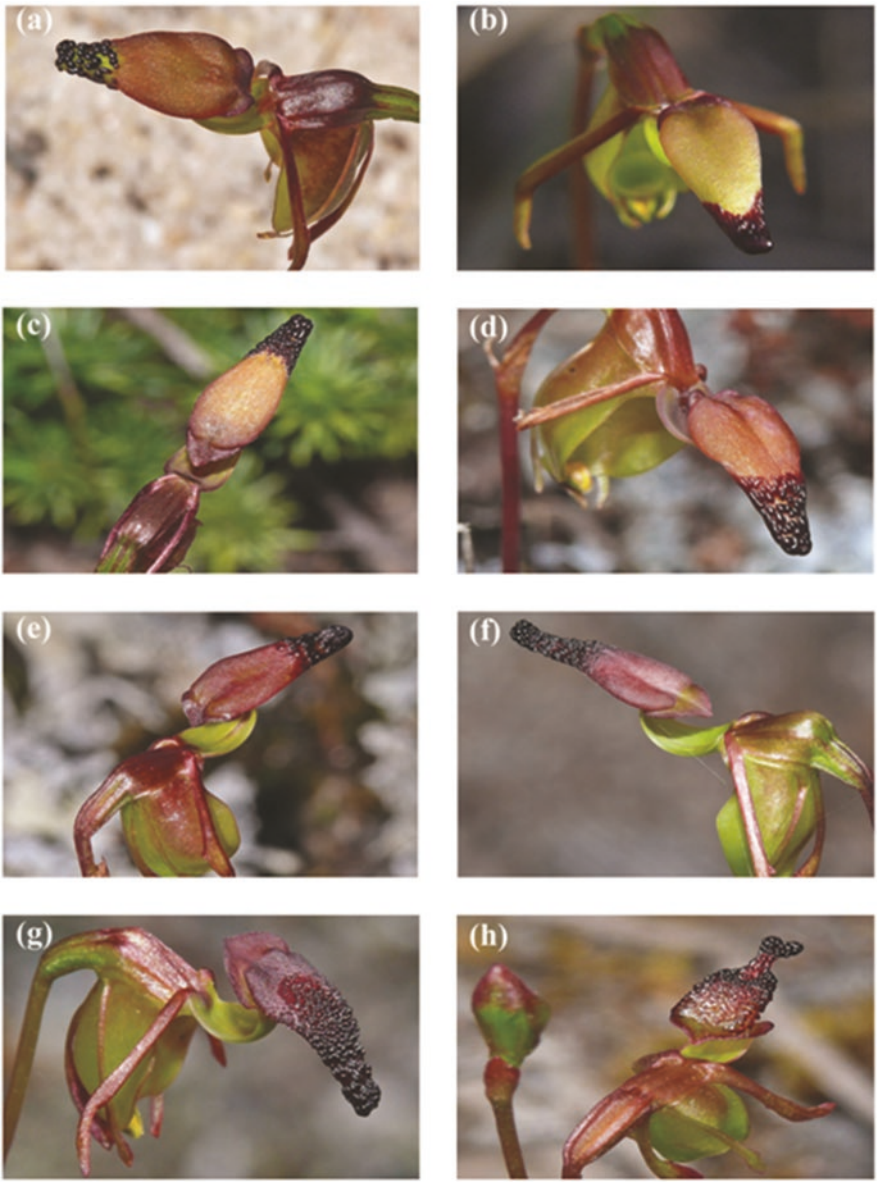

Fig. 19.4 Evolution of insect mimics in *Paracaleana* species. (**a**) *Paracaleana dixonii*, (**b**) *P. terminalis*, (**c**) *P. triens*, (**d**) *P. granitica*, (**e**) *P. gracilicordata*, (**f**) *P. hortiorum*, (**g**) *P. nigrita* and (**h**) *P. minor*

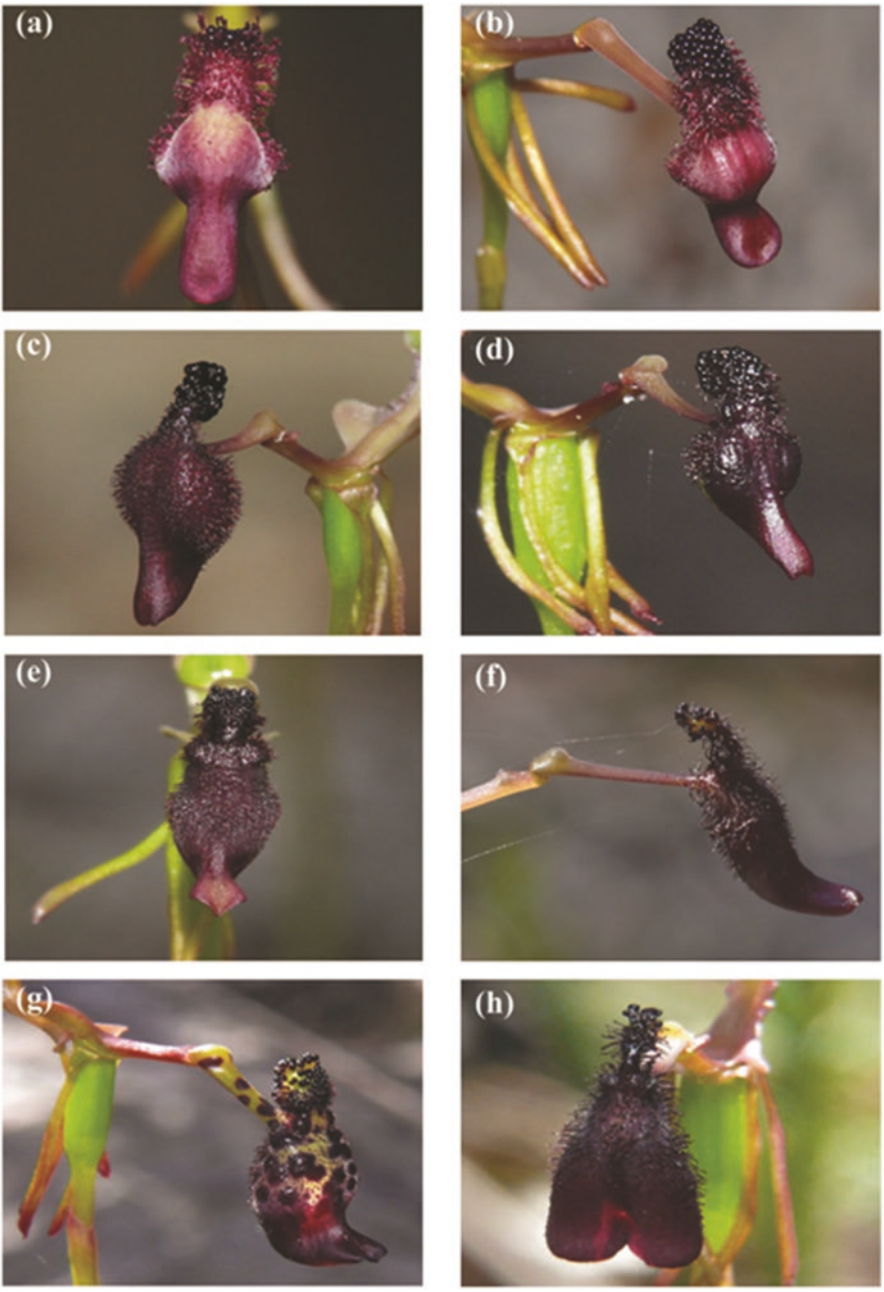

Fig. 19.5 Evolution of insect mimics in *Drakaea* species. (**a**) *Drakaea elastica*, (**b**) *D. concolor*, (**c**) *D. glyptodon*, (**d**) *D. micrantha*, (**e**) *D. thynniphila*, (**f**) *D. confluens*, (**g**) *D. livida* and (**h**) *D. gracilis*

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Pollination Studies in the Genus *Habenaria* Willd. (Orchidaceae) from Western Ghats, India

20

B. T. Dangat and R. V. Gurav

Abstract

Orchidaceae is one of the largest families of angiosperms with diverse reproductive mechanisms and an ideal plant group for pollination studies. In the genus *Habenaria*, structure of flowers is designed in such a way that it permits access to nectar in long, narrow tubes called as spur that project away from the lip. Insects with proboscis equaling the length of spur are able to withdraw nectar from such spur. During the present study, nine species of *Habenaria* were studied for pollination biology, out of which pollination of *H. longicorniculata* by *Agrius convolvuli* and diurnal pollination in *H. foliosa* var. *foetida* by blue tiger butterfly and hawk moth belonging to the genus *Dysgonia* and *H. furcifera* by *Parotis marginata* and *Hydriris sp.* have been carried out successfully, which undoubtedly forms new pollination record for India.

Keywords

Habenaria · Orchidaceae · Pollination biology · Western Ghats

20.1 Introduction

Orchidaceae shows incredible range of diversity in habit, shape, size, colour, fragrance of flowers, etc. Orchid flowers have developed interesting characters such as shape, colour, opening of flower, nectariferous spur and species-specific scent that attract various pollinators and visitors. This shows their reliance on cross-pollination

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by specific pollinators to enhance reproductive success. Orchidaceae is one of the largest families of angiosperms with diverse reproductive mechanisms and an ideal plant group for pollination studies. Orchid pollination and evolution are common topics in evolutionary biology. Perhaps the highly specialized mechanisms by which orchids are fertilised have been the subject of many studies, with the most well known being *The Various Contrivances by which Orchids are fertilised by Insects* by Darwin (1862). Darwin in his book famously argued that the long spurs (ca. 32 cm) of *Angraecum sesquipedale*, a Madagascan orchid species, represent an example of floral specialization for pollination by a long-tongued hawk moth. Arditti (1992) and Dressler (1993) suggested that the orchid floral structure is generally specialized to avoid spontaneous self-fertilization and promote insect-mediated outcrossing. From various reports, it is confirmed that the settling moth and hawk moth pollination in the Orchidiinae is widespread (Nilsson and Jonsson 1985; Nilsson et al. 1992; Johnson and Liltved 1997; Singer and Cocucci 1997; Singer 2001; Singer et al. 2007; Peter et al. 2009; Pedron et al. 2012; Dangat and Gurav 2014).

Habenaria is one of the largest terrestrial orchid genera represented in both the New and Old World tropics and subtropics, with a few species extending into temperate areas, particularly in eastern Asia (Pridgeon et al. 2001). The genus has a temperate and pantropical distribution with the major and main centres of diversity in Brazil, southern and central Africa and East Asia (Kurzweil and Weber 1992). The number of species is assessed earlier by various researchers, but due to continuous additions and deletion of several species, it is quite difficult to estimate exact number of the species; current estimates are about 835 species in the world (WCSP 2018). Brazil, with approximately 170 species (Hoehne 1940; Pabst and Dungs 1975), is the country with the largest number of *Habenaria* species in the New World and possibly also worldwide. The genus is distributed throughout India and represented by ca. 60–90 species including 35–40 endemic ones. In Western Ghats, it is represented by ca. 50 species with 25–30 endemic species (Misra 2007; Choudhury et al. 2011; Jalal and Jayanthi 2012).

The structure of flowers is designed in such a way that it permits access to nectar in long, narrow tubes called ‘spur’ that project away from the lip. Insects with proboscis equaling the length of spur are able to withdraw nectar from such spur. The insects with mouthparts capable of reaching into such long, narrow tubular flowers belong to Lepidoptera and long-tongued hawk moths. The proboscis of the hawk moths (Sphingidae) is among the most highly developed of Lepidoptera and adapted to probing and accessing nectar from the flowers with long tubes (Miller 1997). The size and flight patterns of long-tongued hawk moths combine to make them ideal agents for carrying pollen or pollinaria rapidly and efficiently over long distances between individual plants (Grant 1985; Nilsson et al. 1992; Johnson 1995; Johnson and Liltved 1997). In most of the moth-fertilised species pollinaria of *Habenaria* species have been reported to adhere to various smooth body parts of pollinators, such as the surface of the eye, proboscis and distal parts of the forelegs.

In many floristic works, species of *Habenaria* are poorly represented as they escape attention of workers due to their short lifespan. Due to the same reason,

Table 20.1 Pollination biology studies of *Habenaria* species in the world

Sr. no.	Name of the species	Name of the pollinators	Name of the authors	Year of studies
01	*Habenaria obtusata*	Mosquito	Thien	1969
02	*H. obtusata*	Mosquito	Thien and Utech	1970
03	*H. pleiophylla*	Passion vine butterfly	Moreira et al.	1996
04	*H. gourlieana*	Hawk moth	Singer and Cocucci	1997
	H. hieronymi			
	H. paucofilia	Settling moth		
	H. rupicola	Small moths or mosquitoes		
	H. montevidensis	(postulated)		
05	*Habenaria parviflora*	Crane-fly females and Pyralid moths	Singer	2001
06	*H. epipactidea*	Hawk moth	Peter et al.	2009
07	*H. johannensis*	Butterfly (Genus *Urbanus*)	Pedron et al.	2012
	H. macronectar	*Manduca rustica*		
	H. megapotamensis	*M. sexta*		
	H. montevidensis	*Eumorpha labruscae*		
		M. cf. *lucetius*		

aspects such as floral morphology, pollination biology and adaptive strategies are very poorly studied. Unfortunately, pollination biology of *Habenaria* has remained a neglected branch of study. Till date, a detailed study on pollination biology of 13 species of *Habenaria* from abroad has been carried out (Table 20.1), while in India there is not a single report (excluding Dangat and Gurav 2014) on the pollination biology of this beautiful genus. *Habenaria* is variously assessed and claimed to be a very large polymorphic genus comprising ca. 800 species, but unfortunately not more than 2% of the total species in the world are studied for pollination and pollination biology.

During various field study tours to different localities; size of area, accessibility to the area, other vegetation around the population, number of population, number of flowers per plant, opening and closing of flower, scent or odour of the flower, etc. were studied and noted in field notebook. For a detailed study of pollination biology, the method of Peter et al. (2009) was slightly modified and used during the present study, which is described below.

20.1.1 Selection of Study Site

Study site was selected depending upon observations on number, type and area of population including probable visitors and pollinators for that orchid population. A careful observation was done especially on the flowers for pollinaria removal or adhering of pollinia to stigma. The population showing removal of pollinaria or adhering of pollinaria to the stigma was selected at first sight; e.g. in the case of *Habenaria longicorniculata,* it was observed that in many of the flowers, stigma get

Table 20.2 List of *Habenaria* species selected for pollination biology study

Sr. no.	Name	Area of study	Year of studies	No. of days observation was carried out
1	*H. crinifera*	Amba, Maharashtra	2012–2016	25 25
2	*H. foliosa var. foetida*	Botanical garden, Department of Botany, SUK, Maharashtra	2013–2016	26
3	*H. furcifera*	Kasar-Kandgaon, Ajara, Maharashtra	2013–2016	16
4	*H. suaveolens*	Chowkul, Amboli, Maharashtra, Morjai Plateau, Gaganbawda, Maharashtra	2012–2016	25 18
5	*H. grandifloriformis*	Masai Plateau, Panhala, Maharashtra Shantiniketan, Morewadi, Kolhapur, Maharashtra	2012–2016	29 45
6	*H. heyneana*	Masai Plateau, Panhala, Maharashtra	2012–2016	17 22
7	*H. longicorniculata*	Bugate Alur, Karnataka	2012–2016	25
8	*H. rariflora*	Chowkul, Amboli, Maharashtra, Morjai Plateau, Gaganbawda, Maharashtra	2012–2016	27 18
9	*H. roxburghii*	Suttgati Ghat, Karnataka	2013–2016	25

fertilised or pollinaria were deposited on the stigma naturally at Bugate Alur, Ajara-Amboli Road, Karnataka, India. Population studied for present work was monitored since 2009 for its phenological details. As most of the species were white coloured, these were nocturnally fertilised therefore upon selection of final site, depending upon climatic conditions; i.e. without rain or in slightly drizzling conditions around 6:00 pm, sites were screened for the species morphology.

The present study includes nine species (Table 20.2) selected depending upon their population details and accessibility to the location.

(i) ***Habenaria longicorniculata***: The 12–15 cm-long spur of this species was the major reason and fascination for selection of this species for pollination studies. Earlier work suggested that such long spur species was fertilised by the long-tongued hawk moth only. Other criteria such as white colour of flower, nocturnal opening of flower and nocturnal emission of sweet scent were also the reasons for selection of this species.

(ii) ***H. foliosa var. foetida***: The typical foetid odour was the major reason for selection of this species. During frequent visit to the botanical garden where this species is growing in earthen pot, it has been observed that in sunny days around 10:00 to 11:00 am, the odour of the flower was very strong and lots of butterflies get attracted towards this species.

(iii) ***H. furcifera*:** During routine field tours while collecting this species, it has been observed that one green-coloured moth got stuck in a spiderweb. After critical investigation, it has been seen that pollinaria get attached to the proboscis of this moth. Green-coloured flowers, 2–3 cm-long nectariferous spur and camouflage of moth were the main reason for selection of this species.

(iv) ***H. rariflora* and *H. suaveolens*:** White-coloured large flowers, nocturnal emission of sweet scent, 4–5 cm-long nectariferous spur and both species growing together were the main reason for selection of this species.

(v) ***H. crinifera*:** This species also commonly known as dancing doll due to its incredible shape of flower was the major fascination for selection of this species along with other criteria such as white colour and long nectariferous spur for the study of pollination biology.

(vi) ***H. grandifloriformis*:** After the June–July rain, this was the first species which emerged out and grew profusely covering the entire ground. Nocturnal opening of the flower, white colour of the flower and long nectariferous spur were the main criteria behind selection of this species.

(vii) ***H. heyneana*:** Commonly known as toothbrush orchid, whitish yellowish small flowers with about 2 cm long nectariferous spur attracts many butterflies and small insects. This was the major reason behind selection of this species.

(viii) ***H. roxburghii*:** Small bright white-coloured flowers crowded in dense raceme with 3–4 cm-long nectariferous spur were the main reason behind selection of this species.

20.1.2 Species Morphology

Morphological data related to pollination biology such as colour, opening, size, smell or odour of flowers, labellum, spur size, number of population, number of flowers per plant, number of flowers carrying pollinaria or the number of flowers from which single or both pollinaria were removed was observed, and data was recorded in the field notebook. Observations were recorded from 6:00 pm to 8:00 am. This time schedule was adopted since previous observations indicated that flowers of most of the species (e.g. *Habenaria grandifloriformis*, *H. longicorniculata*, *H. suaveolens*, *H. rariflora*) open and emit sweet, pleasant fragrance at dusk (ca. 8:00 pm) suggesting nocturnal pollination. In the case of *H. foetida*, it emits foetid smell throughout the day; photographs were taken with a Nikon D90 digital SLR camera. Digital images were edited and assembled on plates using Adobe Photoshop 7.0 (San Jose, CA, USA).

20.1.3 Pollinator Behaviour

Pollinator and visitor behaviour towards inflorescence and a particular flower was recorded with the help of low-power torch/flashlight. After every 10–15 min.

Intervals, patches of study area were flooded with flashlight to detect probable visitors or pollinators. All available inflorescences were observed for the visits of pollinators. Since most of the observations were made in the dark, it directly refers to pollinator behaviour as was perceived. It was not possible to confirm when the insects arrived or how many inflorescences they had already visited until they were noticed. Once the activities of pollinators or visitors were sighted, careful observation was made on the activity of the probable pollinators or visitors with torch.

Its foraging, landing pattern, insertion of proboscis and removal of pollinaria were observed by doing photography in the field. Insect effectively removing pollinaria or the insect with adhered pollinaria on body part such as eye, head or proboscis were considered as potential pollinators, while those lacking all these features were considered as probable visitors. After photography, an effort was made to count the total number of pollinators; depending upon their number, two or three potential pollinators were captured with the help of a butterfly net, so that it would not hamper or disturb their natural population. Pollinators and visitors were captured with the help of butterfly net and observed for number of pollinaria attached, site of attachment and proboscis length. These observations were recorded with the help of field notes and photographs. Visits to flowers made by each visitor insect were recorded, along with their number, surrounding environmental conditions (local weather and cloud cover), number of individual open flowers and number of flowers visited. Upon capturing the potential pollinator, it was brought back to the tent and observed carefully for attachment site of pollinaria. In laboratory, it was treated with formaline and mounted on thermacol sheet with wing and proboscis spread out using blunt head pins. Measurement such as length and width of entire body, wing span, proboscis and colour of the potential pollinator was recorded.

20.1.4 Scent

Scent of fresh inflorescence was determined by the human nose at an interval of 10–15 min between varying times for different species, e.g. for *Habenaria longicorniculata* J. Graham from 6:00 pm to 8:00 am. Hundred individuals per species were sampled for this purpose.

20.1.5 Male Efficiency Factor

Male efficiency factor was calculated by Nilsson et al. (1992) method. Percentage of fertilised flowers divided by the percentage of flowers acting as pollen donors was calculated for 50 inflorescences. The main criteria for selection of the inflorescence were its freshness. Stigmatic surfaces have to be fresh in order to confirm that they are fertilised or the flower withered naturally.

20.2 Pollination in Genus *Habenaria*

During the present study, nine species of *Habenaria* were selected for pollination biology studies. Detailed list of target species, number of attempts made and sites of study is provided in Table 20.2. Among the nine species, pollination of three spp., viz. *Habenaria foliosa var. foetida* Blatt. and McCann, *H. furcifera* Lindl. and *H. longicorniculata* J. Graham, has been carried out successfully, which undoubtedly forms new pollination record for India.

(a) Pollination of *H. longicorniculata* J. Graham by *Agrius convolvuli* L.
(b) Diurnal pollination in *H. foliosa* var. *foetida* (Blatt. and McCann) Bennet by blue tiger butterfly (*Tirumala limniace*) and hawk moth belonging to genus *Dysgonia*.
(c) Pollination of *H. furcifera* Lindl. by *Parotis marginata* Hampson and by a moth (*Hydriris* sp.) was reported for the first time from India.

20.2.1 Pollination Biology of *Habenaria longicorniculata* J. Graham (Figs. 20.1 and 20.2)

H. longicorniculata flower is characterized by long nectariferous spur up to 15 to 20 cm in length and therefore locally called 'long-tail orchid'.

Floral Morphology *H. longicorniculata* is the only Indian species having the longest tube-like slender nectariferous spur ca.12–20 cm (Fig. 20.1a, b). Among the studied population, a range of 10–15 cm-long spur has been observed with a mean of 13 cm, frequently containing abundant nectar. The population shows inflorescence height up to ca. 70 cm, bearing maximum five flowers per inflorescence, with mean flower number of three. The white-coloured flowers with sweet scent, which is high during dusk compared to other times, open by evening from 5:30 to 6:30 pm. Individual flower remains open for a period of 2–3 weeks until they are either fertilised or eventually wilt and drop down from the inflorescence stalk, which also dries up if there is no fruit set. The number of flowers open on a single plant is generally 3–4. The flowering pattern was found to be inconsistent at different localities, which depends on rainfall. Plants start to grow in late July and bloom in the month of August which may remain up to mid-September according to climatic conditions.

A typical flower shows entirely white petals with tri-lobed lip (Fig. 20.1c, e), lateral lobes of the lip broader than the middle with entire margin. Sepals are green coloured forming a dome-like structure, consisting of a column (Fig. 20.1c, d) on which sticky green stigma is raised; stigma is divided into two lobes situated on

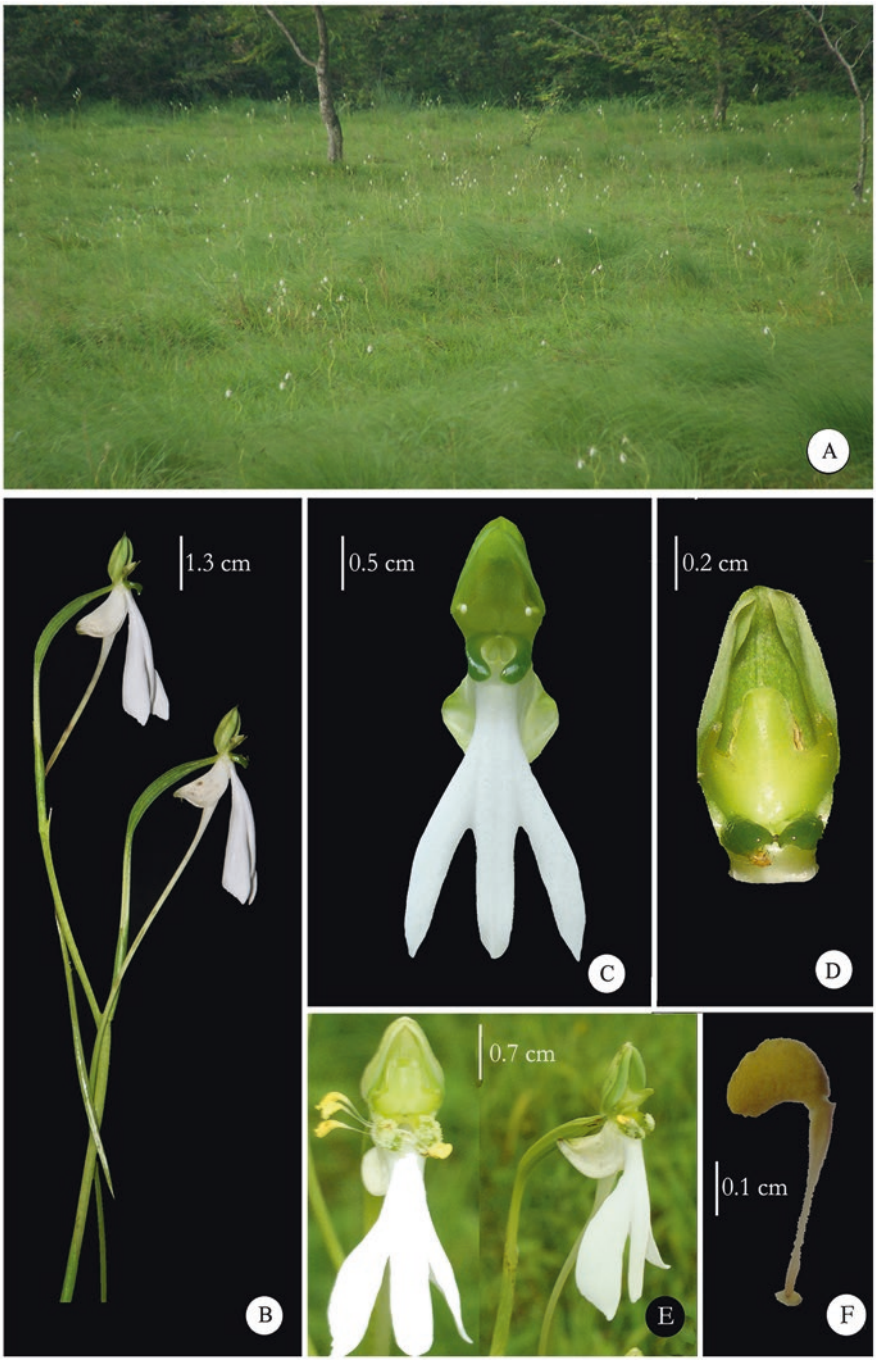

Fig. 20.1 Morphology of *Habenaria longicorniculata* J. Graham. (**a**) Habitat. (**b**) Inflorescences. (**c**) Entire flower. (**d**) Column view. (**e**) Fertilised flowers (front and lateral view). (**f**) Single pollinarium

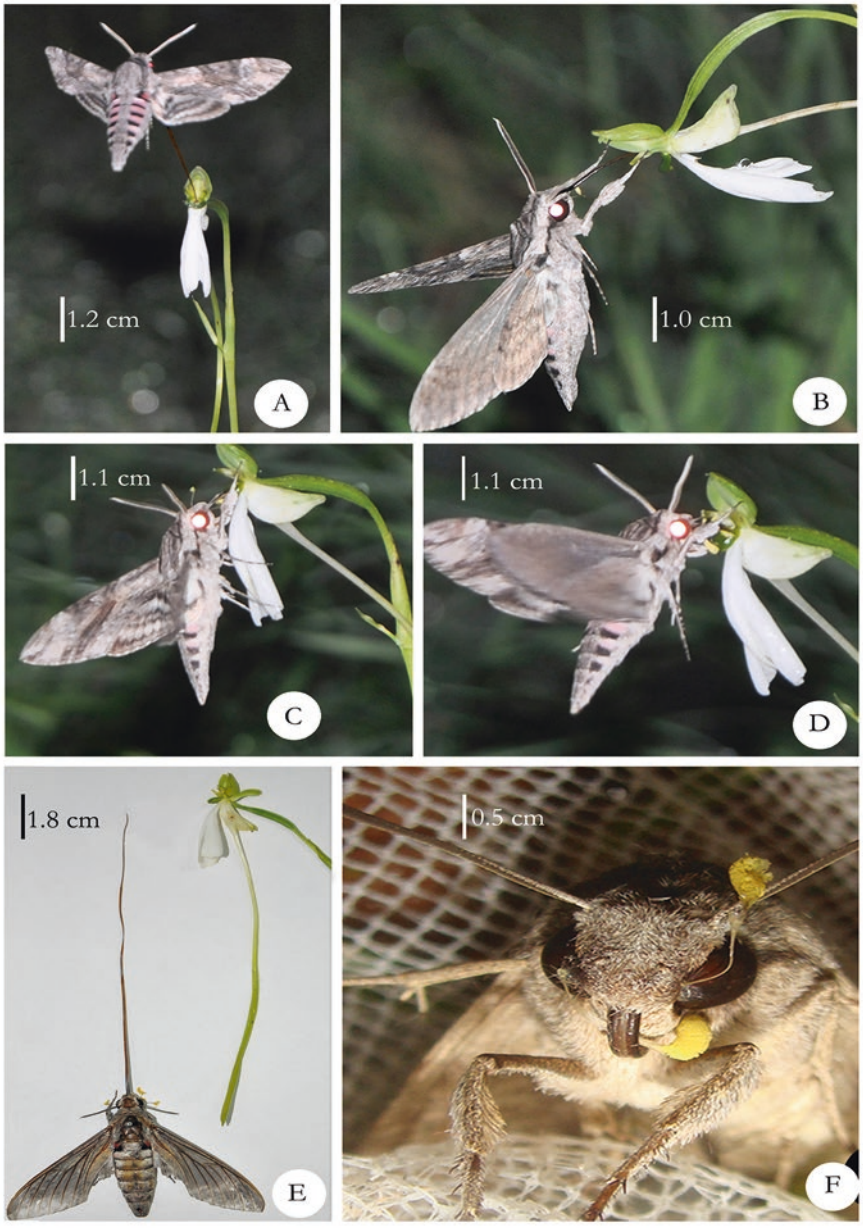

Fig. 20.2 Nocturnal activity of hawk moth during pollination. (**a**) *Agrius convolvuli* probing flower of *Habenaria longicorniculata.* (**b**) *A. convolvuli* with proboscis fully inserting into the spur. (**c**) *A. convolvuli* landing on labellum. (**d**) Transfer of pollinaria on stigma. (**e**) Comparison of proboscis and spur. (**f**) An individual of *A. convolvuli* bearing pollinaria

both sides of the opening to the spur, which is very narrow and therefore affords a passage for the proboscis of Lepidopterids only. Rostellum is present on the opening of the long slender nectariferous spur and is distinctive with the equidistantly separated viscidia from each other. Anther sacs containing the pollinaria are concealed behind the rostellum. Pollinaria (Fig. 20.1f) length varies from 0.5 to 1 cm, yellowish, consists of pollinia and composed of numerous massulae with numerous tetrahedral to rounded pollen grains. Pollinia are attached with elastic thread-like caudicle with a disc-shaped sticky viscidium at the base.

Pollinator Behaviour (Fig. 20.2) After about 120 h of critical and careful observation, 11 individual hawk moth visiting bouts to flowers were recorded. Duration of visit of the hawk moth varies greatly with rainfall and local climatic conditions. They generally are not in flight in heavy rains and strong winds. During overcast, cloudy and drizzly conditions without rain and cool breeze, 12 hawk moths came out to visit the flowers for food. Maximum activity was seen between 8:30 and 11:00 pm, while minimal activity was seen beyond midnight onward up to 3:00 am. An individual visit of a hawk moth to a particular flower ranges between 2 and 10 s. Cold breeze spreads sweet scent in the air and attracts the hawk moths.

Upon arriving on the flower, hawk moths hover in front of the flowers twice or thrice, with coiled proboscis. Then, the moth bends slightly to probe the entrance of an individual flower (Fig. 20.2a, b). It uncoils the proboscis and inserts it gently into the flower. For complete insertion of proboscis into the spur, it lands on the labellum. During this course of probing, it lands its forelegs on the labellum, then for complete insertion of proboscis, it anchors the hind legs on the connate head of sepals of flowers (Fig. 20.2c, d). This action of foraging takes about 2–5 s. During this action, the inflorescence axis bends at an angle of nearly 40°–60° towards the ground (Fig. 20.2b). After foraging and feeding on the available nectar, the hawk moth withdraws its proboscis and flies away. Upon continuous flashing torch lights, it flew up into sky to a height of about 30 m.

Pollinator Morphology and Identity Hawk moth caught on flowers with the help of butterfly net was identified as *Agrius convolvuli* L. with a common name hawk moth. Normally it keeps the proboscis coiled under the head and extends it when probing flowers for feeding. The proboscis length was 13 cm in the female, while in the male it was up to 10 cm (Fig. 20.2e). Due to limited number of moths, only three individuals were collected, so that their population will not be disturbed. During this study other visitors observed are *Leucophlebia lineata*, *Leucophlebia emittens*, *Pygospilatyres*, *Syntomoides imaon*, *Trigonodes hyppasia*, *Neoscona rumpfi*, *Oxyopes birmanicus* and *Hesperotettix speciosus*.

Pollination Mechanism Hawk moth hovers on to the flowers to insert proboscis in the spur; at the same time it presses the sticky viscidium with the base of its head. The pressure of head and sticky glue on the viscidium detaches the pollinaria by

pulling them out on the attached stalks. This involves the momentary lifting of the 'cap' that covers the pollinaria as they detach. Viscidium was found attached to the surface of the eyes also in one case (Fig. 20.2c, f). Once attached to the hawk moth, the pollinaria rest against the head or base of the proboscis. When a hawk moth carrying pollinaria probes another flower, the head comes up and flushes against the lower inner part of the column. Here the pollinaria are easily transferred and massulae comes in contact with the sticky stigma (Fig. 20.2d).

Scent At evening near about 6:00 pm, flowers start emitting a sweet and pleasant scent which is at maximum during 9:00–11:00 pm. As time passes, gradually it decreases and around 3:00 am it reduces greatly.

Male Efficiency Factor To calculate male efficiency factor, 220 fresh flowers from 50 inflorescences were screened. About 169 (77%) flowers had their stigma fertilised, while 127 (58%) flowers were found to act as pollen donors. It was also observed that 97 (44%) flowers had only one pollinarium removed, while 69 (31%) flowers had both pollinaria removed. The male efficiency factor was ca.1.33, i.e. 1.3 flowers fertilised per pollinarium removed.

20.2.2 Pollination Biology of *Habenaria foetida* (Blatt. and McCann) Bennet

This species is characterized by foul/foetid smell of flowers throughout the day. The present study involved observations of population of *Habenaria foetida* growing in the Botanical garden, Shivaji University Campus, Kolhapur, from the emergence of inflorescence up to fruit setting, both during the day and night.

Species Morphology (Fig. 20.3) *H. foetida* is a tuberous, perennial, robust herb, widespread throughout Western Ghats. Stem is erect and stout, up to 50 cm; leaves scattered along the stem; inflorescence lax, 35–50 cm (Fig. 20.3a, b); length of spur ca. 4 cm; flowers 12 per inflorescence, white with green tinge, subsessile, foetid smell during the day; sepals unequal, 3-nerved, green; petals white, 2-partite, 3-nerved, lip greenish white, 3-partite to the base, spurred, up to 4 cm (Fig. 20.3c, d); pollinia 2, obliquely ovate in outline, caudicle slender, dilated towards the base and attached to a small, yellow, sticky viscidium (Fig. 20.3e); stigma sub-cylindrical, blunt, recurved; and entrance to the spur lies between their base. Flower remains open for a period of 12–15 days until pollination or finally wilting. It was observed that in different localities, due to variation in climate, period of vegetative growth and flowering slightly varies. Generally plants start to grow in early August and are in full bloom after a month and half up to mid-September.

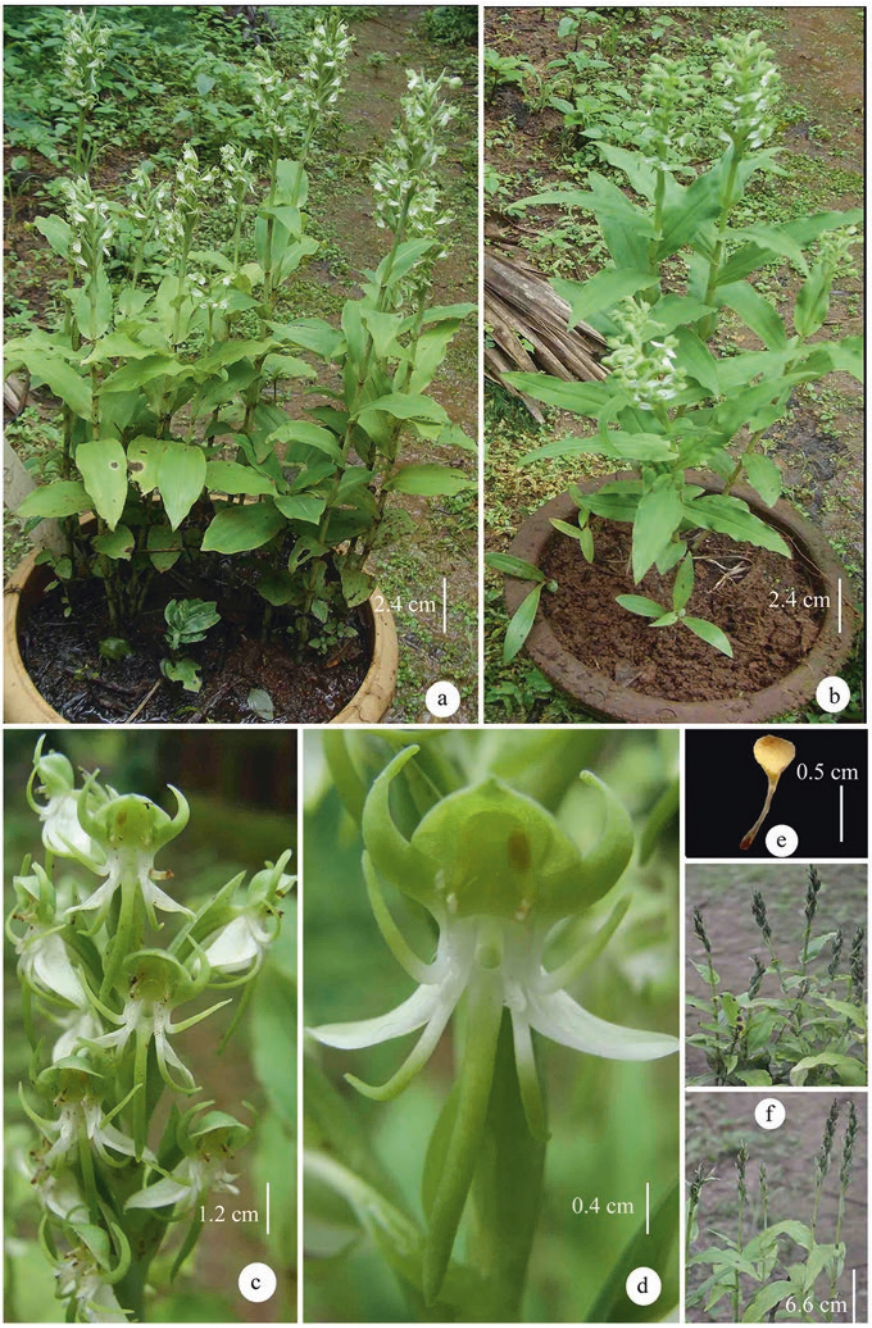

Fig. 20.3 Morphology of *Habenaria foetida* Blatt. and McCann. (**a** and **b**) Habit. (**c**) Inflorescence. (**d**) Entire flower. (**e**) Single pollinarium. (**f**) Capsule formation after pollination

Pollinators Present study confirmed that (hawk moth) *Dysgonia* sp. and (blue tiger butterfly) *Tirumala limniace* Cramer are the pollinators of *Habenaria foetida*. The length of proboscis in blue tiger is 4 cm and in hawk moth 3.6 cm.

Pollination Mechanism (Fig. 20.4) Blue tiger butterfly and moth visits were recorded. The time of visits vary greatly. They were not seen in heavy rain or strong wind. Butterfly visited in sunny condition, but hawk moth even visited during overcast and cloudy conditions. Visitors such as *Danaus chrysippus*, *Euploea core*, *Neoscona rumpfi* and *Hesperotettix speciosus* were also observed. Activity of blue tiger butterfly was from 10:30 to 11:30 am. In the case of moth, activity was observed during 8:30 to 9:30 pm. A single visit to a flower by blue tiger butterfly took between 10 and 30 s, whereas visit by the moth took 2–6 s. Both pollinators show similar activity like hovering around the inflorescence with coiled proboscis, landing on the labellum, uncoiling of proboscis and inserting into the spur.

While inserting the proboscis, insects bend their head which nearly enters into the connate hood of sepals (Fig. 20.4a–g). The foraging lasts for 10–30 s; blue tiger butterfly withdraws its proboscis and moves to the second flower by walking or flying. Hawk moth withdraws its proboscis and hovers around the inflorescence again. Moth hovers, while blue tiger butterfly sits on the flower to insert proboscis in the spur. Head of the insect presses the sticky viscidium at the base, detaches and pulls the pollinaria out on the attached stalks. It involves momentary lifting of the 'cap' that covers the pollinaria. Viscidium with pollinarium was found on the proboscis and sometimes on other parts of the head in hawk moths (Fig. 20.4n) and to the eyes or antennae in the blue tiger butterflies (Fig. 20.4f, g). When a moth carrying a single or many pollinaria probes another flower, the head comes up and flushes against the lower inner part of the column. The pollinarium/pollinaria gets transferred onto the sticky stigma (Fig. 20.4m). The butterfly carried more pollinia than the moth, i.e. it may be a more efficient pollinator than the moth.

Male Efficiency Factor Fresh flowers about 160 collected from 22 inflorescences were screened, of which 145 (90%) flowers were fertilised. 112 (70%) flowers had lost their pollinia, of which 87 (54%) flowers had only one pollinarium removed and 67 (35%) had both removed. The male efficiency factor is 1.29.

20.2.3 Pollination Biology of *Habenaria furcifera* Lindl

This species is characterized by robust habit, with several large cauline leaves, densely lax raceme up to 40–45 cm. Flowers are dark greenish to yellowish green with equally tri-lobed lips with deflexed side-lobes and thin lip; spurs are longer than the ovaries. This species is often confused with *H. ovalifolia*. But it has a distinctive species status on the basis of midlobe of the lip which is touching the sepals in *H. ovalifolia* while spreading and deflexed in *H. furcifera* (Plate – 21). Present

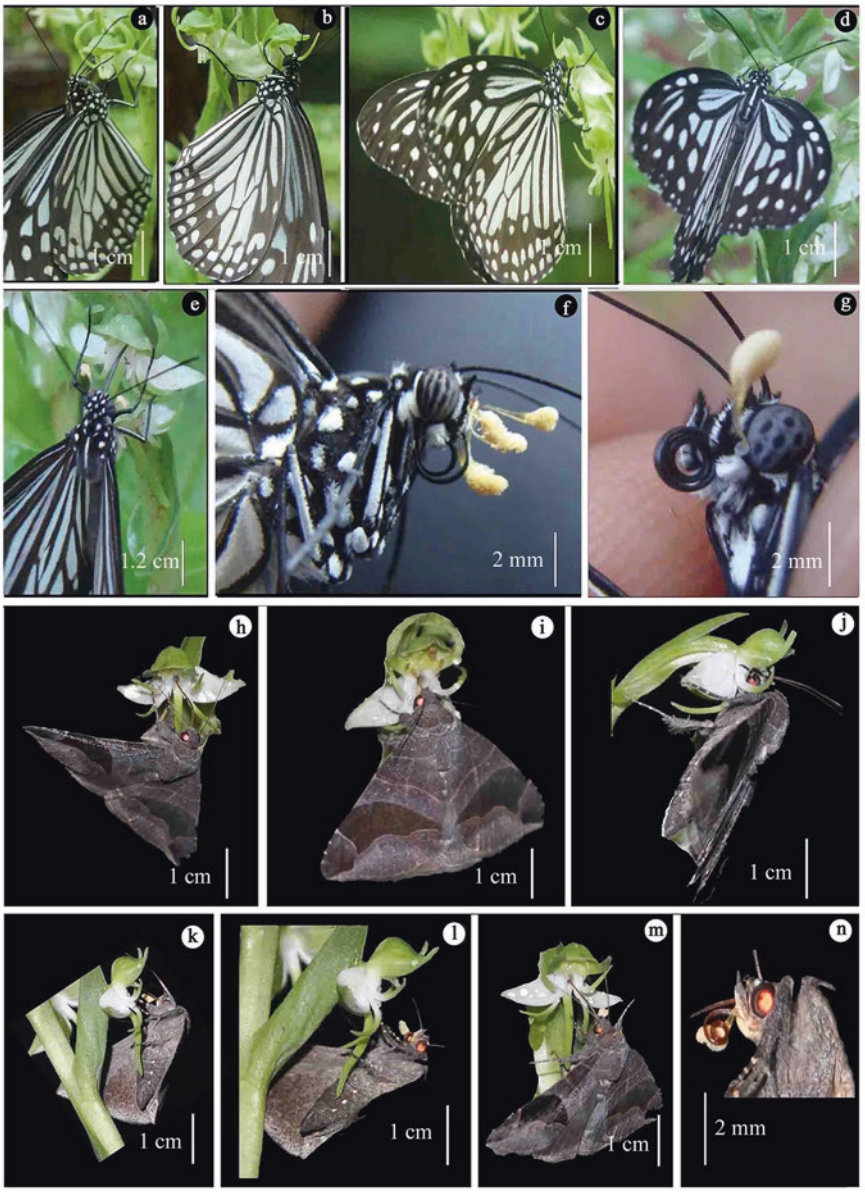

Fig. 20.4 Activity of butterfly and hawk moth during pollination. (**a**–**g**) *Triumala limniace* Cramer. (**a**–**d**) Probing flower of *Habenaria foetida.* (**e**) Removal of pollinaria on its eyes. (**f**) An individual bearing pollinaria on its eyes. (**g**) Eye attachment of single pollinarium. (**h**–**n**) *Dysgonia* sp. (**h**–**j**) Probing flower. (**k**–**l**) Pollinarium removed from the flower. (**m**) Transfer of pollinarium to another flower. (**n**) Pollinarium attached to head region

study involved observations of population of *H. furcifera* growing under dense forest or under the canopy of trees, at Kasar-Kandgaon, Tal-Ajara, Dist.-Kolhapur, Maharashtra, India, from the opening of flower up to fruit setting, throughout day and night. Flowers are dark green to yellowish green, flowering starts late in the month of September and fruiting up to December (highly dependent on local rainfall). Flower remains open for a period of 10–15 days until fertilised or up to natural withering or wilting.

Species Morphology (Fig. 20.5) Plants are 30–70 cm tall, green, erect, terete, slender, and glabrous with four to seven leaves which are long, spreading, cauline and in a cluster in the basal one-third portion of the stem (Fig. 20.5a). Inflorescence is long, stout, erect, bracteate, glabrous, densely lax, raceme with many flowers and rachis is 20–40 cm long (Fig. 20.5b). Flowers are small, 6–15 mm in size, pale to dark greenish in colour, shortly pedicellate and bracteate and in long stout, erect, densely lax inflorescence (Fig. 20.5c). Sepals are spreading or reflexed, subequal or unequal, 3-nerved, rounded to sub acute while petals are long, erect, broadly oblong-lanceolate, retuse, sub-falcate, slightly broader than the sepals as long as the dorsal sepal, forming a hood with middle and dorsal sepal over the column. Lip is longer than the sepals, linear, deeply divided at the base into three lobes and distinctly trilobed. Spur is very slender, cylindric, pendulous, 1.7–3.0 cm long, longer than the ovary, laterally compressed and curved at the apex. In present analysis 3 cm is the longest length of the spur.

Pollinator Morphology and Identity (Figs. 20.6 and 20.7) Two moths were captured pollinating different flowers of the present species. After critical examination and consulting with experts, one is identified as *Parotis marginata* Hampson (green-coloured moth) and *Hydriri ssp*. (brown colour). Both are small in size having wing span about 4–4.5 cm. Proboscis in green-coloured moth is up to 3–4 cm, while in brown-coloured moth, it is 3–3.6 cm. Seven green-coloured moths and five brown-coloured moths were observed pollinating different flowers on the same or different inflorescences. Considering orchid conservation and current threats to orchids and their pollinators due to various anthropogenic pressures, only one moth pollinator is captured for detailed study such as site of attachment of pollinia, spur length, etc. No visitors were observed.

Pollinia Transfer by Hawk Moth (Fig. 20.6) (i) ***Parotis marginata*** – It gets camouflaged and is very difficult to locate in the dark. It shows activity in the evening or dusk; at about 7:00–7:30 pm, it comes out for foraging. This activity continues up to 11:30 pm. Only one moth was observed foraging on plants at 1:40 pm. Maximum activity of this moth is observed between 7 and 9 pm. As number of plants was more, i.e. 14 plants growing in vicinity of each other, and number of moths foraging on this plant was also large, i.e. 7, it was quite difficult to identify the individual visitation

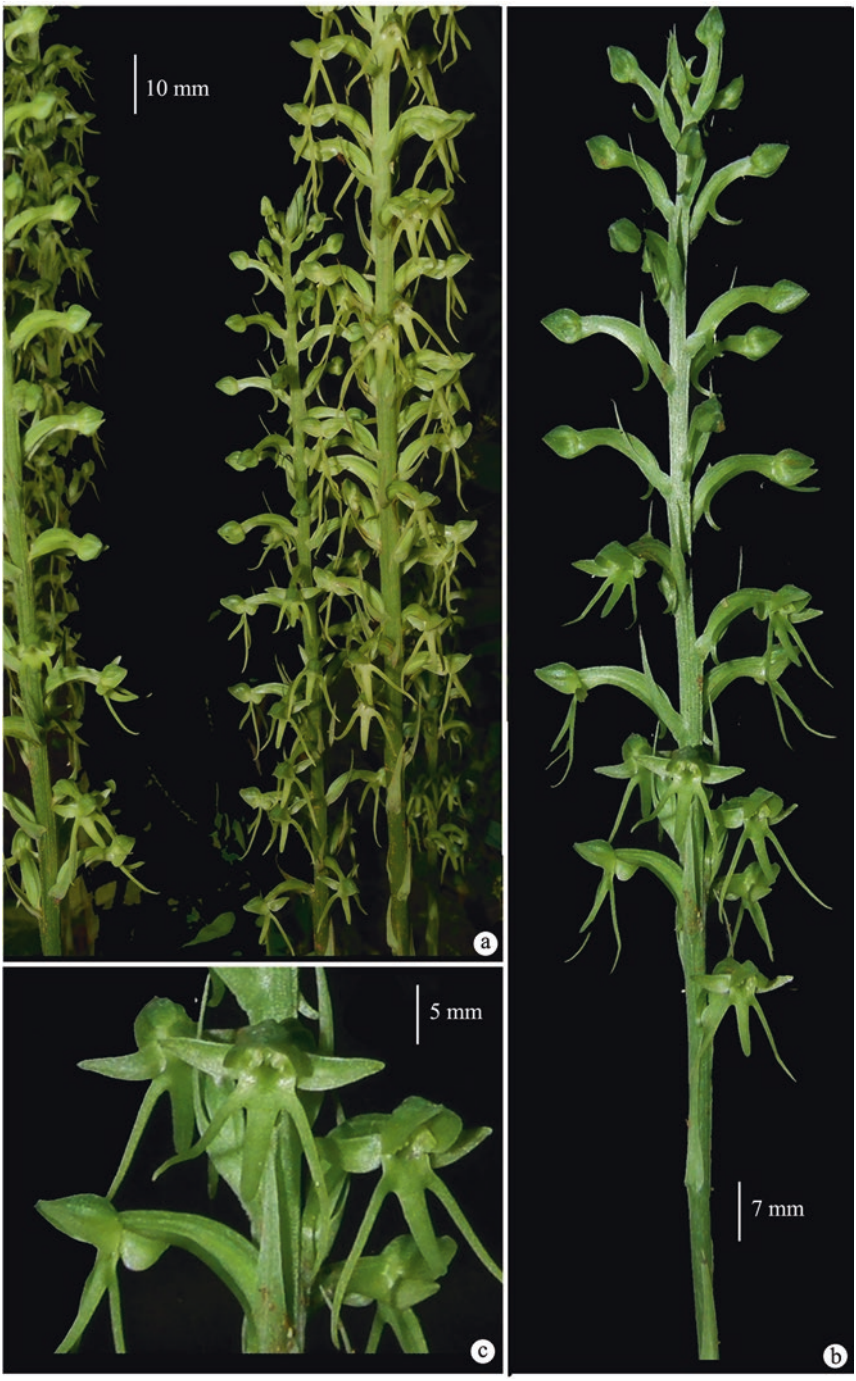

Fig. 20.5 Morphology of *Habenaria furcifera* Lindl. (**a**) Habit. (**b**) Inflorescence. (**c**) Close-up of flowers

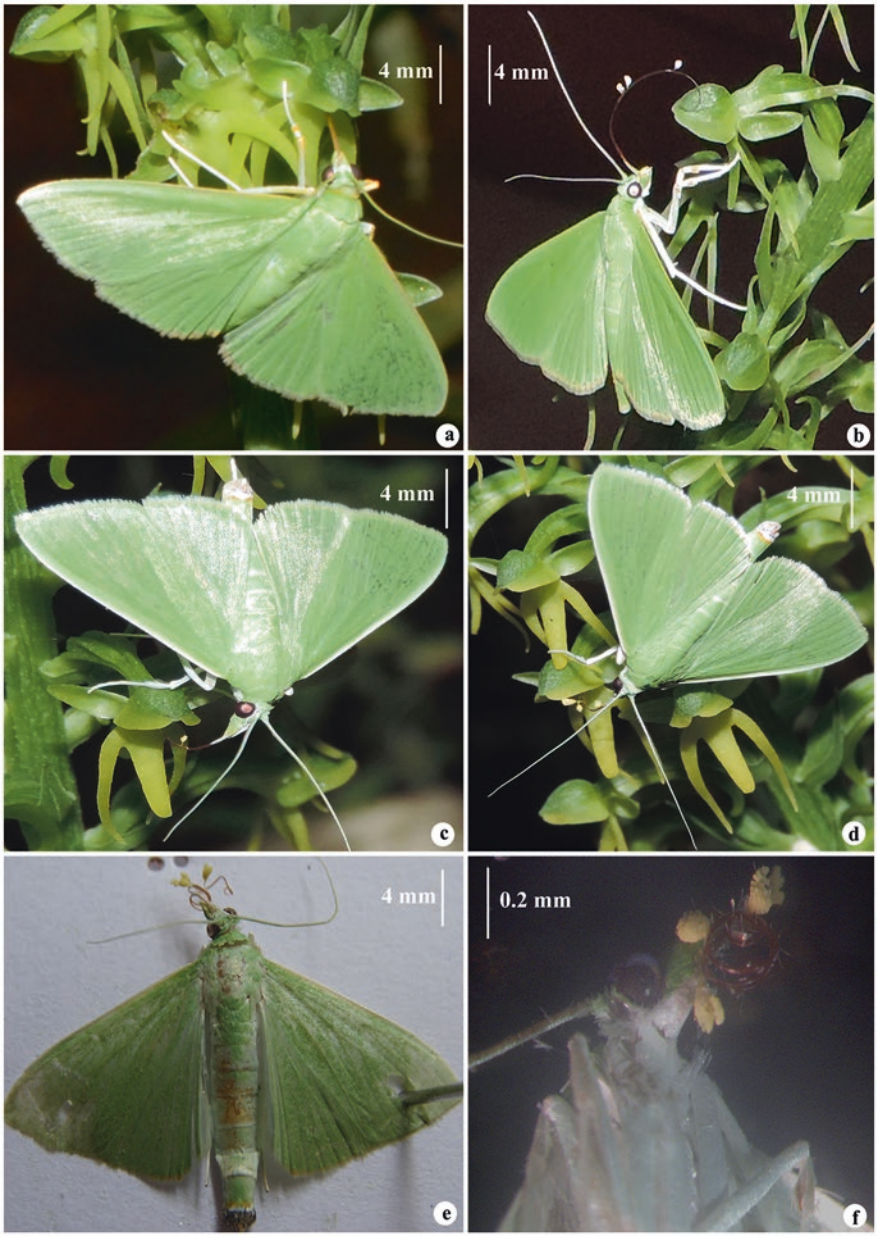

Fig. 20.6 Nocturnal activity of *Parotis marginata* Hampson. (**a**) Moth probing flowers of *Habenaria furcifera* with proboscis completely inserted into the spur, (**b**) removal of pollinaria, (**c**) transferring pollinaria to other flowers (cross-pollination), (**e**) entire moth with pollinaria attached to proboscis, and (**f**) proboscis attachment of pollinaria

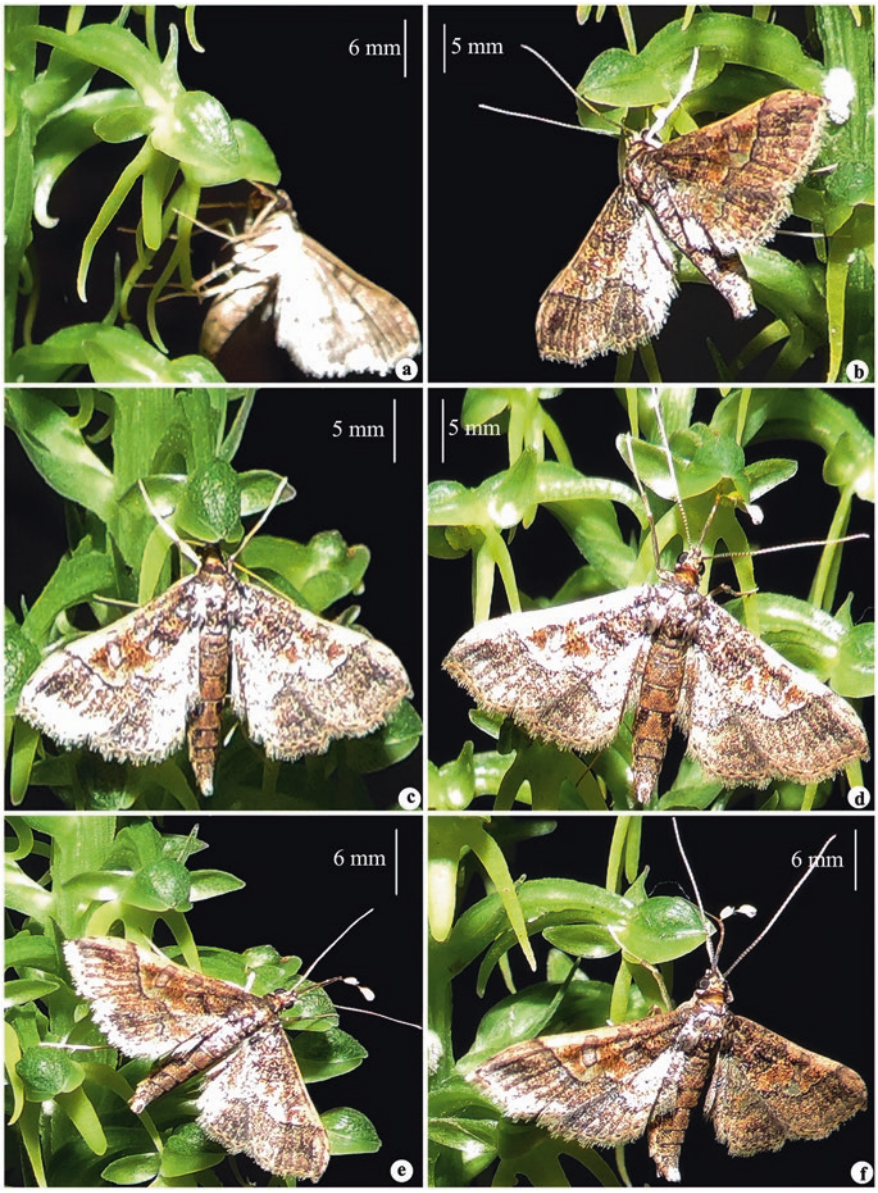

Fig. 20.7 Nocturnal activity of moth (*Hydriris* sp.). (**a**–**c**) Moth-probing flowers of *Habenaria furcifera*, (**d**) removal of pollinarium, (**e**) transferring pollinaria to other flower (cross-pollination), and (**f**) proboscis attachment of pollinaria

bouts. This moth flies over the inflorescence with coiled proboscis and lands on the flower, sometimes upside down, uncoil its proboscis and probe the flower. Probing of flower continues from a few seconds to 1 min, and then it walks on the inflorescence and probes another flower (Fig. 20.6a–f). As the inflorescence is dense, compact and many-flowered, it is quite difficult to reveal whether it probes the flower sequentially or arbitrarily. As length of its proboscis is longer than the spur, it simply inserts proboscis into the spur without inserting its head into the connate hood, which generally occurs in case of *H. longicorniculata*. Since there is no contact of head region to the column while probing, only pollinia get attached to the proboscis. At a time one to two pollinia get attached near the middle of the proboscis, and a maximum four pollinia were observed adhering to the proboscis. Once pollinia get attached, and the moth tries to forage another flower, they get transferred to the other flower, ensuring effective cross-pollination. Pollination and foraging activity is strictly restricted during night. This moth is a little phototactic; when torch light is flashed, it flies away for a few minutes and comes back immediately after 5–10 min when it is dark. As the flowers do not have any smell, this moth is attracted for the nectar only. Hence, food deception plays important role in this studied species.

(ii) ***Hydriris sp.*** (Fig. 20.7) – This moth shows activity at night, i.e. strictly nocturnal; it arrives at the inflorescence around 9–9:30 pm. It hovers over the inflorescence with coiled proboscis and lands on the flower, where labellum plays a role of landing platform. After arriving at the flower, it uncoils the proboscis to insert it into the spur. As length of the proboscis is same or little smaller than the spur, it partially inserts its head into the connate hood formed by sepals and petals. While probing, pollinia get attached to the distal end or sometimes to the middle of the proboscis. Individual visitation bouts range four to five per moth in between 9 and 11 pm. Maximum activity of this moth is observed during 9–9:30 pm; as time passes, activity gradually decreases, and after 11 pm, activity of this moth completely stops. Foraging of single flower lasts for a few seconds, i.e. 2–10 s (Fig. 20.7a–f). After this, it flies away and comes back again in 5–10 min. It is highly negatively phototactic; upon illumination of light, it immediately flies away and comes back after 10–15 min. Once pollinia get attached while probing one flower, when it comes for probing another flower, pollinia effectively get transferred to the other flower; thereby efficient and effective cross-pollination takes place.

Male Efficiency Factor 340 fresh flowers from 12 inflorescences were screened, among which 207 (61%) flowers confirmed pollination, while 122 (36%) flowers were pollen donors. It was also observed that 97 (44%) flowers had only one pollinarium removed, while 69 (31%) flowers had both pollinaria removed. The male efficiency factor was ca.1.69, i.e. 1.7 flowers fertilised per pollinarium removed.

During the present study, nine species were targeted for pollination biology studies, of which pollination biology of three species, viz. *Habenaria longicorniculata*,

H. foliosa var. foetida, and *H. furcifera*, was successfully carried out. The remaining six species, viz. *H. crinifera*, *H. suaveolens*, *H. grandifloriformis*, *H. heyneana*, *H. rariflora* and *H. roxburghii*, were also observed. Visitors, such as butterflies and unidentified moths, were observed visiting flowers of *H. heyneana*, *H. roxburghii* and *H. crinifera*. In the case of *H. grandifloriformis*, *H. rariflora* and *H. suaveolens*, no visitors and pollinators were observed maybe because of climatic conditions, as all of these species grow during heavy monsoon. During this period, either there is heavy rainfall or heavy fog which directly affects the activity of pollinators and visitors, so it is quite difficult to observe probable visitors and pollinators. It has been observed in previous studies that during heavy rainfall moths or butterflies never come out for foraging. This may be the reason leading to unsuccessful studies in pollination biology of these species.

During the present studies, it has been observed that either visual cues such as colour, shape and size or olfactory cues such as scent/odour play an important role in pollination biology of *Habenaria*. Food deception is the main reason behind pollination of all the species studied; nectar is rewarded for the pollinator as a food.

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Part V

Orchid Chemicals and Bioactive Compounds

Phytochemical Analysis, Antioxidant and Anti-inflammatory Activity of *Eria tomentosa* (Koen.) Hook. f.

21

Most. Tanzila Akter, Mohammed Kamrul Huda, Mohammed Mozammel Hoque, and Minhajur Rahman

Abstract

Eria tomentosa (Koen.) Hook. f. belonging to the family Orchidaceae is a medicinal plant which has been investigated for its medicinal efficacy. The present investigation was addressed by determining phytochemical analysis, antioxidant activity and anti-inflammatory activity of the studied species. The dried samples of leaf, bulb and root of *E. tomentosa* were extracted with hexane, dichloromethane (DCM), methanol and butanol fractions. Phytochemical analysis of secondary metabolites, viz. phlobatannins, saponins, tannins, terpenoids, steroids, glycosides, anthraquinone, quinine and coumarin properties, were screened out for its medicinal value. Among the three studied parts, root extract was found to have disease-resisting secondary metabolites. Antioxidant activity of crude extract of *E. tomentosa* was determined at four fractions of leaf, bulb and root extract, where leaf part was found to have the most potential. Butanol fraction of leaf revealed the highest scavenging activity which was 94.88% (at concentration 100 µg/ml). However, a notable percentage of antioxidant activity was 93.79%, found in butanol fraction of bulb (at concentration 100 µg/ml) and DCM fraction of root (at concentration 250 µg/ml). Anti-inflammatory activity was evaluated using heat-induced albumin denaturation assay. The highest anti-inflammatory activity (97.26%) was found in hexane fraction of bulb of *Eria tomentosa* against egg albumin denaturation. On the other hand, the least anti-inflammatory activity (72.45%) was found in butanol fraction of root. The present study concludes that the *Eria tomentosa* (Koen.) Hook. f. has good medicinal properties including antioxidant and anti-inflammatory activities.

Keywords

Eria tomentosa · Secondary metabolites · Antioxidant and anti-inflammatory activity

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21.1 Introduction

Orchids are the most fascinating and diverse group of flowering plants distributed all over the world from tropics to high alpine (Pant 2013). About 30,000 to 70,000 plants are used medicinally across the world, and 80% of the world's rural people meet their needs of preliminary health care from such plants (Sarkar 1996; WHO 2002). Bangladesh is rich in orchids, with 177 species reported under 70 genera (Huda 2007). About 26 species of orchids have also been used by the tribal people of Bangladesh to treat different diseases (Huda et al. 2006).

Medicinal plants that possess bioactive properties especially their thrombolytic, antibacterial, antifungal activities, etc., can manage the infectious diseases, fever, premature aging, cancer and others (Rahman 2011). *Bulbophyllum lilacinum* Ridl. is a member of the family Orchidaceae having beautiful flowers and also medicinal value. Extracted juice from the pseudobulbs of *Bulbophyllum* species is being used for restoration of adolescence and also as tonic (Deorani and Naithani 1995; Chowdhery 1998). It also plays an important role in the medicinal sector, and hill people of Bangladesh use orchids for preparing their traditional medicines. Phytochemical investigations of orchid family were performed for alkaloid constituents (Luning 1974). Many orchids play significant role in traditional systems of medicine because they are rich in alkaloids, flavonoids, glycosides, carbohydrates and other phytochemical contents (Rahman and Husen 2003). Pharmacological studies conducted on orchids indicate the immense potential of these plants in treating different diseases such as neurodegenerative disorders, convulsion, cancer and diabetes (Gutierrez 2010). Orchids are considered as an antioxidant agent. Recently there has been an observed increase of interest in the therapeutic potential of medicinal plants as in reducing of free radical-induced tissue injury (Pourmorad et al. 2006; Stajner et al. 2009). A result from biological and phytochemical studies indicates that medicinal plants have profound antioxidant potential that can be exploited further in the prevention and treatment of these devastating disorders (Mazumder and Rahman 2008). *E. tomentosa* (Koen.) Hook. f. is used to treat different diseases among tribal people of Bangladesh (Akter et al. 2017), but it has not yet been investigated for its efficacy in relation to medicinal properties. With this view in mind, the present experiment has been done to screen out the phytochemical properties as well as the bioactivity of the medicinal orchid *E. tomentosa* (Koen.) Hook. f.

Materials: The root, leaf and stem of *E. tomentosa* (Koen.) Hook. f. were collected from Kaptai National Park, Rangamati, Bangladesh. Samples were thoroughly washed with water and dried in oven at 65 °C for 48 h. It was then ground into coarse powder by using grinding machine and stored in airtight container for further investigation. Mixing of one part with another was carefully avoided. The voucher specimen of the orchid species is preserved at the Herbarium of Chittagong University.

Samples of 25 g from each part were taken for further analysis. Methanol of 50 ml was added to the 25 g of samples in a conical flask. It was shaken vigorously for 30 min and kept overnight and then shaken again and sonnicated for 10 min and filtered using Whatman filter paper No 1. The process was repeated for three times

with methanol, and the extract was then rotavaporated. The dried sample was kept as crude sample for each part. The concentrated crude extract was fractionated into four different solvent systems, viz. methanol, n-hexane, butanol-1 and dichloromethane (DCM), by following the method of Kupchan (1969).

21.2 Phytochemical Tests

Qualitative tests were carried out on the fresh sample, powdered specimens and methanol-extracted crude using standard procedures to identify the constituents as described by Sofowara (1993), Trease and Evans (1989) and Harborne (1973).

21.2.1 Test of Alkaloids

For qualitative test of alkaloid, the most reliable and rapid testing method was developed by Webb (1952), and the method was slightly modified by Aplin and Canon (1971). For the qualitative test of alkaloid, five alkaloid detecting reagents were used. These are Dragendorff's reagent (D), Hager's reagent (H), Mayer's reagent (M), Wagner's reagent (W) and Tannic acid reagent (T). These reagents were prepared following the methods of Cromwell (1955).

Fresh finely chopped 5 g of pasted plant material was mixed up to moisten with 10 ml of 2% HCL and heated in water bath of 60 °C for 1 h. After cooling the extract was filtered through Whatman No. 1 filter paper. Two drops of extract were put on a microscopic groove slide with one drop of the alkaloid detecting reagent. The relative abundance of precipitate, if any formed in the plant extract with the reagent, was considered as an index of the quality of the presence of alkaloid and was expressed by '+', '++' and '+++' signs which mean slight, moderate and substantial to heavy amount, respectively. No precipitate was indicated by '−' (negative sign) and stood for the absence of alkaloid in the plant extract.

21.2.2 Test for Phlobatannins

Deposition of a red precipitate when an aqueous extract of each plant sample was boiled with 1% aqueous hydrochloric acid (HCL) was taken as evidence for the presence of phlobatannins (Edeoga et al. 2005).

21.2.3 Test for Flavonoids

A portion of the crude powdered plant sample was heated with 10 ml of ethyl acetate over a stem bath for 3 min. The mixture was filtered, and 4 ml of filtrate was shaken with 1 ml of dilute ammonia solution. A yellow colouration was observed indicating a positive test for flavonoids (Edeoga et al. 2005).

21.2.4 Test for Saponins

About 2 g of crude powder was boiled with 20 ml of distilled water in a water bath and filtered. 10 ml of filtrate was mixed with 5 ml of distilled water and shaken vigorously for a stable persistent froth. The persistence of froth indicates the presence of saponins (Kapoor et al. 1969).

21.2.5 Test for Tannins

About 0.5 g of the crude powdered samples was boiled in 10 ml of distilled water in a test tube and filtered. A few drops of ferric chloride reagent were added to the filtrate. A blue-black precipitate was taken as evidence for the presence of tannins (Harborne 1973).

21.2.6 Test for Terpenoids

Crude powder of 0.5 g was dissolved in 5 ml of methanol. 5 ml of the extract was treated with 2 ml of chloroform in a test tube. 3 ml of concentrated sulphuric acid was carefully added to the mixture to form a layer. An interface with a reddish brown colouration is formed if terpenoid constituent is present (Kolawole et al. 2006).

21.2.7 Test for Steroids

0.5 g of crude powder was dissolved in 5 ml of methanol, 1 ml of the extract was dissolved in 10 ml of chloroform and equal volume of concentrated sulphuric acid was added by sides of the test tube. The upper layer turns red, and sulphuric acid layer showed yellow with green fluorescence. This indicated the presence of steroids (Kolawole et al. 2006).

21.2.8 Test for Glycosides

Crude powder of 0.5 g was dissolved in 5 ml of methanol. 10 ml of 50% HCL was added to 2 ml of methanolic extract in a test tube. Then it was heated in a boiling water bath for 30 min. 5 ml of Fehling's solution was added to the mixture, and the mixture was boiled for 5 min. A brick-red precipitate was taken as evidence for the presence of glycosides (Harborne 1973).

21.2.9 Test for Anthraquinone

2 ml of filtered extract solution was added with few drops of Magnesium acetate. Formation of pink color indicates the presence of Anthroquinone (Sofowara 1993).

21.2.10 Test for Quinine

1ml of extract, 1ml of concentrated Sulfuric acid was added and was allowed to and for some time to develop color. Development of red color shows the presence of Quinine (Sofowara 1993).

21.2.11 Test for Coumarin

1ml of extract, 1ml of 10% NaOH was added and was allowed to stand for some time development of yellow color shows the presence of Coumarin (Sofowara 1993).

21.3 Therapeutic Properties and Experimental Design

21.3.1 Antioxidant Activity

The antioxidant activity of the methanolic, n-hexane, butanol-1 and DCM extract of the root, leaf and stem of *E. tomentosa* and the standard antioxidant ascorbic acid were assessed on the basis of the free radical scavenging effect of the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH, MWt.394.32) free radical activity according to the method described (Cuendet et al. 1997) with slight modification.

DPPH Assay The reaction mixture contained 3 ml 0.004% DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) in 100% methanol and 5 ml crude extract or ascorbic acid solution in case of experiment or standard control, respectively. After 30 min incubation period at room temperature (19 °C) in the dark, the absorbance and optical density (OD) were measured against a blank at 517 nm in UV-visible spectrophotometer (Shimadzu, Japan). The degree of discolouration of DPPH from purple to yellow following reduction indicated the scavenging efficiency of the extract or ascorbic acid solution. Lower absorbance followed by the degree of discolouration of the reaction mixture indicated the free radical scavenging efficiency of the substances. The percentage of DPPH discolouration (scavenging) activity was calculated with the help of the following formula:

$$\%\text{of scavenging activity} = \left(\frac{A-B}{A}\right)\times 100$$

where *A* was the absorbance of control (DPPH solution without the sample) and *B* was the absorbance of DPPH solution in the presence of the sample. Values are presented as mean with ± SE of the mean of three replicates. The % scavenging activity was plotted against log concentration, and the IC_{50} (inhibition concentration 50 µg/ml) value of plant extract was calculated by using linear regression analysis.

21.3.2 Anti-inflammatory Activity

The ability of anti-inflammatory activity was assessed by the method of Mizushima and Kobayashi (1968) with slight modification. Three for standard, three for control and three for each plant extract were tested. The tubes were marked accordingly. 2 ml of 5% egg albumin solution were kept into all treatment tubes. 2 ml of the respective solvent was added to the control tubes. 2 ml acetylsalicylic acid (0.1 mg) was mixed for positive control group. On the other hand, for the test groups, 2 ml of (1000 mg/kg) extract was mixed to the test groups as marked. The pH (5.6 ± 0.2) of all reaction mixtures was adjusted by 1N HCl. All the reaction mixtures were heated at 57 °C for 20 min. After cooling and filtering, the absorbance was measured spectrophotometrically at 660 nm. The test was repeated three times for each extract as replicating. The anti-inflammatory activity was calculated by using the following equation:

$$\%\text{of inhibition} = \left(\frac{A-B}{A}\right)\times 100$$

where *A* = absorbance of control (5% egg albumin solution and methanol) and. *B* = absorbance of test group (5% egg albumin solution and plant extract) or *B* = absorbance of standard solution (5% egg albumin solution and acetylsalicylic acid).

21.4 Phytochemical Analysis

The result of Table 21.1 indicates that in respect to qualitative test for the determination of the presence of alkaloids, leaves of the studied orchid *E. tomentosa* were found to be highly positive in each test except the Wagner's reagent test. Bulbs were

Table 21.1 Qualitative test for alkaloids of *Eria tomentosa* (Koen.) Hook. f.

	Qualitative estimation of alkaloids by different reagents				
Plant parts used	D	H	M	W	T
Leaf	+++	+++	+++	++	+++
Bulb	++	+++	+++	++	+++
Root	+	++	++	+++	+++

D Dragendorff's reagent, *H* Hager's reagent, *M* Mayer's reagent, *W* Wagner's reagent and *T* Tannic acid reagent. + (slight), ++ (moderate), +++(substantial)

Table 21.2 Qualitative test for ten secondary metabolites of *Eria tomentosa* (Koen.) Hook. f.

	Secondary metabolites (% of coloration)									
Plant parts used	Phl.	Flv.	Sap.	Tan.	Ter.	Str.	Gly.	Anthr.	Qui.	Cou.
Leaf	–	+++	++	+++	+	++	+	+	+++	++
Bulb	–	++	+++	+++	++	+++	+++	++	++	+++
Root	+++	++	+	+++	+++	+++	+++	++	+++	+++

Phl., Phlobatannins, *Flv.*, Flavonoids, *Sap.*, Saponins, *Tan.*, Tannins, *Ter.*, Terpenoids, *Str.*, Steroids, *Gly.*, Glycosides, *Anthr.*, Anthraquinone, *Qui.*, Quinine, *Cou.*, Coumarin
+ (slight), (++ moderate), +++ (substantial)

found to be highly positive in Hager's reagent, Mayer's reagent and tannic acid reagent tests. In contrast, the presence of alkaloids was revealed to be high through Wagner's reagent and tannic acid reagent tests in roots.

The results of the qualitative test for ten secondary metabolites are presented in Table 21.2. The results demonstrate that flavonoids, tannins and quinine were present highly whereas saponins, steroids and coumarin were present moderately in the extract of leaves. On the other hand, saponins, tannins, steroids, glycosides and coumarin were found to be highly positive in the extract of bulbs. Furthermore, phlobatannins, tannins, terpenoids, steroids, glycosides, quinine and coumarin were remarkably present in the extract of roots of the studied orchid.

The present study corroborates with the findings of Shrestha et al. (2015) who investigated on phytochemical screening of Nepalese medicinal plants *Swertia chirayita* and *Dendrobium amoenum* and reported the presence of positive result for alkaloid, terpenoids, flavonoids, glycosides and tannins. Radhika and Murthy (2013) detected alkaloids, tannins, flavonoids, glycosides, phenols, saponins and coumarins in the extract of various plant parts of *Rhyncostylis retusa* Blume which is comparable with the present study. The present experiment also substantiates with the investigation of Willams and Saju (2016) who worked on phytochemical screening potential of a wild epiphytic orchid *Acampe praemorsa* (Roxb.) and reported the presence of saponins, phenol, terpenoids, tannins and glycosides.

21.5 Antioxidant Efficacy

It is evident from the results that among the five different concentrations used in the study (50, 100, 150, 200, 250 µg/ml), ascorbic acid showed 99.69%, 99.07%, 98.92%, 98.61% and 98.45% scavenging activity, respectively. In the case of the extract of leaf, the n-hexane fraction showed the highest scavenging activity of 93.95% at the concentration of 250 µg/ml and the lowest scavenging activity of 64.13% with the concentration of 50 µg/ml. DCM fraction part displayed maximum scavenging activity (91.30%) at the concentration of 250 µg/ml and minimum (87.11%) at 100 µg/ml, scavenging activity was found to be peaked (90.37%) at the concentration of 200 µg/ml and bottomed (70.34%) at the concentration of 250 µg/ml while the methanolic fraction is concerned. Likewise, butanol-1 fraction part demonstrated the greatest scavenging activity of 94.87% at concentration 100 µg/ml

and the lowest 92.70% at the concentration of 200 µg/ml (Fig. 21.1). However, pertaining to the leaf extract, butanol-1 fraction showed the maximum scavenging activity, whereas n-hexane showed the minimum activity. Chimsook (2016) worked on the antioxidant activity of *Dendrobium signatum* leaves where leaves were extracted with ethanol by maceration called M and by sonication-maceration for 30 and 45 min called MS30 and MS45. He reported that MS30 had the stronger free radical scavenging activity than M and MS45 and had moderate radical scavenging ability compared to ascorbic acid which substantiates with the findings of the present study.

In respect to the bulb extract, the n-hexane fraction showed the highest scavenging activity (93.17%) at the concentration of 250 µg/ml and the lowest (63.35%) at the concentration of 100 µg/ml. The DCM fraction exhibited maximum scavenging activity of 91.46% at the concentration of 250 µg/ml and minimum activity of 78.58% at the concentration of 100 µg/ml. Similarly methanolic fraction displayed optimum scavenging activity (92.24%) at the concentration of 50 µg/ml and minimum 81.63% at 200 µg/ml. On the contrary butanol-1 fraction demonstrated the highest value of 93.78% at the concentration of 100 µg/ml and the lowest value (92.08%) at the concentration of 50 µg/ml (Fig. 21.2). Finally, in considering the bulb extract, butanol-1 fraction showed the maximum scavenging activity, and n-hexane fraction showed the minimum scavenging activity. Willams and Saju (2016) worked on antioxidant potential of a wild epiphytic orchid *Acampe praemorsa* (Roxb.) Blatt. of Kanyakumari district, India. DPPH radical scavenging activity of *A. praemorsa (*Roxb.) Blatt. varied from the minimum inhibition of 60.37 ± 0.01% (25 µl) to the maximum inhibition of 69.74 ± 0.01% (100 µl). Aqueous extract varied from the minimum inhibition of 56.00 ± 0.005% (25 µl) to the maximum inhibition of 58.83 ± 0.01% (100 µl). This conclusion is in full agreement with the findings of the present investigation.

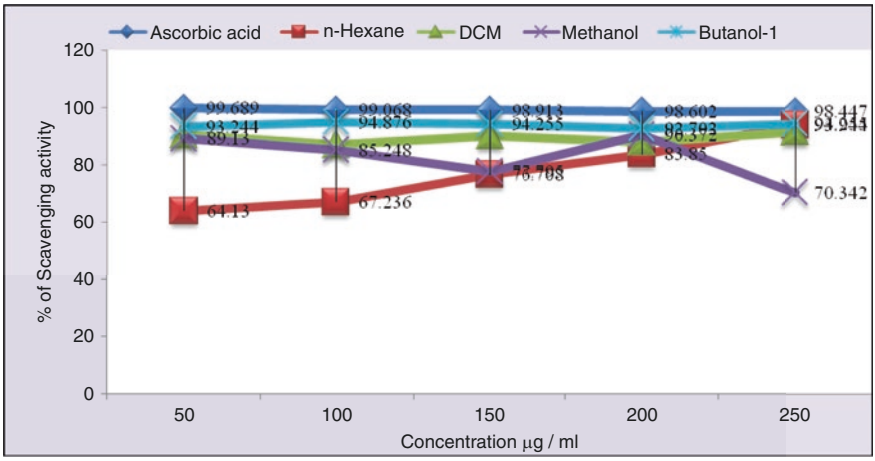

Fig. 21.1 Relative % of scavenging activity or % inhibition of standard antioxidant ascorbic acid and four fractions of bulb of *Eria tomentosa* (Koen.) Hook. f.

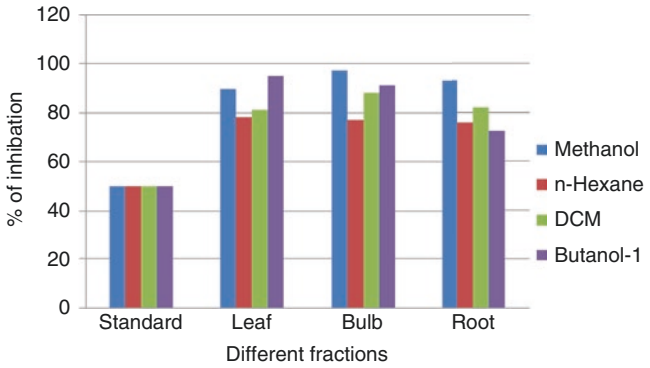

Fig. 21.2 Relative % of scavenging activity or % inhibition of standard antioxidant ascorbic acid and four fractions of root of *Eria tomentosa* (Koen.) Hook. f.

In considering the root extract, n-hexane fraction presented the greatest scavenging activity (84–16%) at the concentration of 200 µg/ml and the lowest (63.66%) at the concentration of 100 µg/ml, while the DCM fraction displayed maximum scavenging activity (93.79%) at the concentration of 250 µg/ml and minimum (84.30%) at 50 µg/ml. Scavenging activity was found to be peaked (91.15%) at the concentration of 100 µg/ml and rounded (89.29%) at the concentration of 200 µg/ml while the methanolic fraction was concerned. Similarly, butanol-1 fraction part confirmed the highest scavenging activity of 90.06% at the concentration of 100 µg/ml and the lowest (85.71%) at the concentration of 50 µg/ml (Fig. 21.3). Nevertheless the root extract of DCM fraction showed the scavenging activity, whereas n-hexane fraction showed the lowest activity. Minh et al. (2016) reported that the antioxidant activity of *Phalaenopsis* sp., a hybrid orchid, evaluated by DPPH (2,2- diphenyl-1-picryl hydrazyl) free radical scavenging assay and β-carotene bleaching method was higher than those of the leaf extracts. It is apparent from the results that butanol-1 fraction was found to be the most effective in showing the highest scavenging activity, and n-hexane fraction was found to be the least effective in showing the optimum scavenging activity in all cases regarding the extracts of leaves, bulbs and roots.

21.6 Anti-inflammatory Property

Among the four different fractions used in the present study, the leaf extract of *E. tomentosa* showed the maximum inhibitory result for the n-hexane fraction (89.35%) and minimum for the butanol-1 fraction (75.95%) following the sequence as n-hexane > DCM > methanol > butanol-1 fraction (Fig. 21.4). In case of bulb extract, n-hexane fraction showed the highest anti-inflammatory activity (97.26%), whereas DCM fraction showed the lowest activity (76.85%) and subsequently maintained the sequence as n-hexane > butanol-1 > methanol > DCM. On the

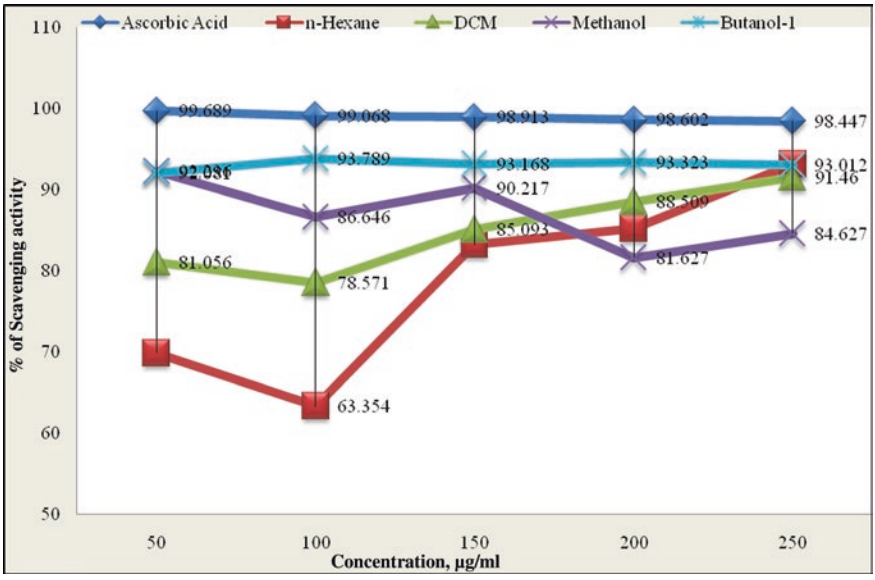

Fig. 21.3 Relative % of scavenging activity or % inhibition of standard antioxidant ascorbic acid and four fractions of leaf of *Eria tomentosa* (Koen.) Hook. f.

contrary, the maximum anti-inflammatory activity was found to be 92.85% for n-hexane fraction, and minimum was found to be 72.45% for butanol-1 fraction showing the succession as n-hexane > methanol > DCM > butanol-1 fraction in relation to root extract.

Abdallah et al. (2014) worked on the anti-inflammatory activity of *Trichodesma trichodesmoides* var. *tomentosum* and found the butanol fraction as the most potent in showing the highest anti-inflammatory activities than their individual fractions. Sukumaran and Yadav (2016) worked on anti-inflammatory potential of *Dendrobium macrostachyum* Lindl. where they observed that the ethanol and water extract was highly effective as albumin denaturation inhibitors (IC_{50}, 114.13 and 135.818 µg/ml, respectively) and proteinase inhibitors (IC_{50}, 72.49 and 129.68 µg/ml, respectively). Chinsamy et al. (2014) studied anti-inflammatory activity of extract of 53 South African medicinal orchids, and significant anti-inflammatory activity was observed in nearly 40% of extracts in the COX-1 assay and 25% of extracts in the COX-2 assay. Overall, the DCM root extract of *Ansellia africana* was the most potent; the DCM tuber extract of orchids *Eulophia hereroensis* was the only extract to significantly inhibit both COX enzymes, while all *Bulbophyllum scaberulum* organic root extracts exhibited COX-2 selective inhibitory activity. These conclusions resemble with the findings of the present experiment.

Based on the results of the present study, it can be concluded that *E. tomentosa* leaf showed maximum positive results for alkaloids, while bulb and root showed the highest result for other secondary metabolites. Butanol-1 fraction was found to be the most effective in showing the highest scavenging activity irrespective of leaf,

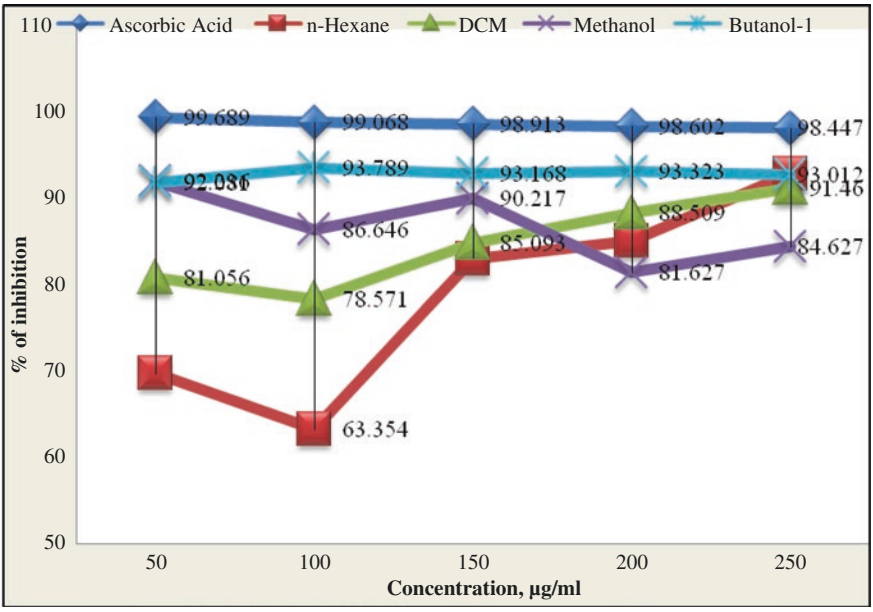

Fig. 21.4 Anti-inflammatory activity of leaf, bulb and root of *Eria tomentosa* (Koen.) Hook. f. at different fractions

bulb and roots. In considering the anti-inflammatory activity, n-hexane fraction of bulb of *E. tomentosa* was regarded as the most efficient in comparison to others. Therefore, the findings of the present study have distinctly focused on the potential medicinal values of this plant and promoted the ongoing research of medicinal orchids in Bangladesh.

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Medicinal Orchids: Production of Bioactive Compounds and Biomass

22

So-Young Park, Thanh-Tam Ho, and Kee-Yoeup Paek

Abstract

Orchids are considered to be the most highly differentiated and horticulturally important plants. Additionally, orchids have been used as traditional medicines in many countries since ancient times. Different organs of orchid plants, such as leaves, stems, and bulbs, contain various biologically active substances such as alkaloids, phenolics, terpenoids, and derivatives thereof. These bioactive compounds are secondary metabolites synthesized from primary metabolites of plants. To improve the utility of orchids, it is important to identify the pharmacological function of these plants. Moreover, the establishment of technologies for the large-scale production of a biomass of orchid plants using field cultivation or biotechnological methods is needed to prevent the overaccumulation of these plants in the natural state, which would ultimately result in these plants being enlisted as endangered species. Among various factors affecting the in vitro culture of medicinal orchids, growth regulators, light, sugar and activated charcoal are the most important. To establish a successful mass production system, it is necessary to determine the optimal concentration at which these factors maximize the production of biomass and bioactive compounds. In this chapter, we provide an overview of medicinal orchids and review recent studies on the in vitro production of biomass and bioactive compounds from these plants.

Keywords

Medicinal orchids · Bioactive compounds · Plant cell and tissue culture · Bioreactor

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22.1 Introduction

Orchidaceae is the largest family of angiosperms comprising several important ornamental plant species that produce a wide variety of distinct flowers with characteristic shapes, size, colors and fragrance (Park et al. 2018). Orchids are used as cut flowers and as potted plants in the horticulture industry (Murthy et al. 2018; Park et al. 2018). In addition to the tremendous horticultural importance of orchid plants, these plants also contain high amounts of bioactive compounds that have been used in various traditional pharmacopeias for centuries (Hossain 2011). Orchids have been used for the treatment of various diseases, such as cancer, tuberculosis, cholera, and eczema, worldwide for a long time (reviewed in Hossain 2011; Schuster et al. 2017).

Anoectochilus formosanus Hayata, commonly known as "jewel orchid," is one of the many medicinal orchids used as traditional medicine for the treatment of hypertension and lung and liver diseases in China, Taiwan, and Vietnam (Ket et al. 2004; Yoon et al. 2007). It is also known as the "King of Medicine" because of its diverse pharmacological effects, such as antioxidant, antiinflammatory, heptoprotective, antitumor, and immunostimulatory activities (Lin et al. 1993; Wang et al. 2002; Yoon et al. 2007). *Gastrodia elata*, a traditional medicinal herb found in Korea and other Asian countries, protects against kainic acid-induced neuronal damage in mouse hippocampus (Kim et al. 2001) and is used for the treatment of depression, epilepsy, obesity, asthma, inflammation, and headaches (Chen et al. 2016; Seok et al. 2018). Root extracts of *Eulophia macrobulbon* exhibit significant antiinflammatory, antioxidant, and anticarcinogenic effects (Schuster et al. 2017). Bhattachryya and Van Staden (2016) reported the potential of *Ansellia Africana*, the "Leopard orchid," for the treatment of Alzheimer's disease. In addition, several members of the genus *Dendrobium*, one of the largest genera in Orchidaceae, exhibit a wealth of medicinal properties (reviewed in Teixeira da Silva and Ng 2017).

22.1.1 Type and Efficacy of Bioactive Compounds

Scientific advances in the field of medicinal orchids have resulted in the discovery of various secondary metabolites, with potential for the treatment of several diseases (Hossain 2011; Bhattacharyya and Van Staden 2016; Singh et al. 2012). A wide variety of chemical compounds, such as alkaloids, terpenoids, polyphenols (e.g., flavonoids), and phenanthrenes, have been isolated from different parts of medicinal orchid plants.

Extracts and metabolites of these medicinal orchids, particularly those isolated from stems and tubers, possess useful pharmacological activities (Gutiérrez 2010). Kinsenoside is one of the most important bioactive compounds present in *A. formosanus* Hayata, with diverse pharmacological effects such as the repression of inflammation and tumor growth and stimulation of the immune system (Du et al. 2000; Yoon et al. 2007). Moscatilin, a phytochemical derived from *Dendrobium* species, has anticarcinogenic effects on different cancer cell lines (Ho and Chen 2003).

Bisbenzylgigantol isolated from *Dendrobium draconis* prevents the development of stem-like phenotypes in human lung cancer cells and adversely affects anchorage-independent growth and tumor cell viability (Bhummaphan and Chanvorachote 2015). A glucomannan isolated from *Dendrobium officinale* stems stimulates splenocytes and RAW 264.7 macrophages (Huang et al. 2016). Vanilla isolated from the orchid *Vanilla planifolia*is is widely used as a flavor-inducing agent in ice cream, bread, and confectionery. It is also used in perfumes and, to some extent, in medicines as a nerve stimulant (Divakaran and Babu 2009).

Gastrodin and its aglycone gastrodigenin (4-hydroxybenzyl alcohol, 4-HBA) are considered as the characteristic and main active constituents of *Gastrodia elata* (Lee et al. 2015; Zhan et al. 2016). Gastrodin, a phenolic glucoside, is used as one of the standards for evaluating the quality of *G. elata.* Phenolics in *G. elata* exhibit prominent neuroprotective, antiinflammatory, and antioxidant activities (Lee et al. 2012; Han et al. 2014). This review summarizes the methods used for the biomass production of medicinal orchids via plant cell and tissue culture.

22.2 Biomass Production of Medicinal Orchids by Micropropagation

Various micropropagation methods have been used for the rapid propagation of plants, depending on plant growth characteristics, for example, somatic embryogenesis in *Rosa rugusa* (Rosaceae) (Jang et al. 2016), callus cultures of *Dorem ammoniacum* (Irvani et al. 2010) and *Bambusa ventricosa* (Wei et al. 2015), and regeneration from mesophyll protoplasts of *Gentiana decumbens* (Gentianaceae) (Tomiczak et al. 2015) and *Arbutus unedo* (Choury et al. 2018). Propagation via axillary buds is an easy and safe method for the production of true-to-type plants in a short time span (Murthy et al. 2014). This method has been used for the propagation of many medicinal plant species, including *Holostemmaada-kodien* (Martin 2002), *Lyscaste* hybrid (Huang and Chung 2011), and *Aegle marmelos* (Puhan and Rath 2012).

In vitro proliferation of the medicinal orchid, *A. formosanus*, was first attempted by Chow et al. (1982). Since then, several techniques have been developed for the micropropagation of *A. formosanus*, such as shoot tip and/or nodal explant culture Ket et al. 2004), symbiotic seed germination (Chou et al. 2004), and seeds from capsules collected from wild plants (Shiau et al. 2002).

22.3 Factors Affecting Micropropagation

During in vitro culture, plant tissues and organs are grown on artificial media as the source of nutrients necessary for growth. Therefore, the success of plant tissue culture is greatly influenced by the media composition. More than a hundred different media formulations have been used for in vitro tissue culture of various plant species. Among these, Murashige and Skoog (MS) medium (Murashige and Skoog

1962) is the most commonly used. The type of media selected for tissue culture depends on the plant species, culture type, and culture stage. Orchids do not require a nutrient-rich medium; instead, a medium low in salt is usually recommended for orchid tissue culture. For example, MS, Knudson's C, Vacin Went, and Lindemann and Mitra media are often used for the micropropagation of *Dendrobium* (Nayak et al. 1997; Roy and Banerjee 2003), terrestrial orchids (Sheelavantmath et al. 2000), *Oncidium* (Chen and Chang 2000), *Cymbidium* (Nayak et al. 1997), *Phalaenopsis* (Chai et al. 2002), *Paphiopedilum* (Adelberg et al 1997; Chen and Chan 2004), *Vanda* (Daehler 2003), and *Doritaenopsi*s (Park et al. 2003). A commercial medium Hyponex, available in many formulations (N-P-K ratios of 20-20-20, 6.5-4.5-19.0, 7-6-19, and 25–5-20), has been frequently used for orchid micropropagation (Ket et al. 2004; Park et al. 2000).

22.3.1 Plant Growth Regulators

Since functions of plant growth regulators, specially auxin and cytokinin, are discovered in higher plants (Miller et al. 1955, 1956), those two main groups of growth regulators have been used for the mass propagation of various medicinal orchids (Bhattacharyya et al. 2016; Naing et al. 2011). Recent studies suggest that the most abundant auxin, indole-3-acetic acid (IAA), is primarily synthesized in a two-step process from the amino acid tryptophan (Zhao 2014) and usually promote root formation. In vitro induction of roots from growing shoots has been achieved using standard media, with or without auxin, depending on the plant genotype (Pout et al. 2000). Nayak et al. (1997) reported root formation in *Cymbidium* and two species of *Dendrobium* on MS medium containing indole-3-butyric acid (IBA), whereas William et al. (2003) reported high rooting rate in the presence of indole-3-acetic acid (IAA). However, marked variation is observed in the rooting potential of different plant species, and systematic trials are often needed to define the conditions required for root induction. William et al. (2003) reported the highest number of shoots in *Vanda spathulata* when grown on media containing a combination of 6-benzylaminopurine (BA) and IAA. Similarly, in *A. formosanus*, germination of shoot tips/nodes and seeds has been reported on media containing cytokinin and auxin (Shiau et al. 2002). Ket et al. (2004) suggest an optimal concentration of thidiazuron (TDZ, 1–2 $mg{\cdot}L^{-1}$) and BA (1 $mg{\cdot}L^{-1}$) for shoot multiplication.

Cytokinin it regulates numerous important processes associated with plant growth and development, such as cell division, chloroplast development, bud differentiation, shoot initiation and growth, and leaf senescence (Brault and Maldiney 1999). Because of these diverse effects, cytokinins are widely used in plant tissue culture. In addition to cytokinins, auxins are incorporated into the culture media to promote the growth of callus, cell suspensions, and organs and to regulate morphogenesis, especially in conjunction with cytokinins. A balance between auxins and cytokinins is necessary for a successful tissue culture. A high cytokinin-to-auxin ratio is generally required for the direct induction of shoots on explants.

Sucrose most explants are heterotrophic during in vitro culture. Therefore, a carbon source, such as sucrose, is required in tissue culture media. In the medium, sucrose is converted to monosaccharides (glucose and fructose) during in vitro culture. This conversion occurs by the action of invertase located in plant cell walls (or extracellular enzymes. Sugars are responsible for maintaining an osmotic potential in the culture medium that is conducive for cell and tissue growth. Rooting in many woody and herbaceous species is improved when the concentration of macro salts is lowered by at least 50% and that of sucrose is increased to 5%.

Activated Charcoal activated charcoal is commonly used in tissue culture media for promoting or inhibiting in vitro growth, depending on the plant species and tissues used. The effects of activated charcoal may be attributed to the establishment of a dark environment (Dumas and Montemmis 1995; Nissen and Sutter 1990) and adsorption of undesirable/inhibitory substances (Linington 1991; Mensuali-Sodi et al. 1993), growth regulators, and other organic compounds (Dumas and Monteuuis 1995; Mensuali-sodi et al. 1993). The addition of activated charcoal seems to improve rooting either by inhibiting light at the shoot base or by adsorbing rooting inhibitors (Dumas and Monteuuis 1995).

Light fluorescence lamps are the most popular source of artificial light used in a tissue culture/growth room. Blue light promotes shoot formation, whereas red light induces rooting in many species (Murashige 1974). In Helianthus tuberosus, red light has a greater stimulatory effect on adventitious root formation than blue light (Economou and Read 1986). Seibert et al. (1975) reported that blue and violet light stimulate adventitious shoot formation in tobacco callus, whereas red light induces adventitious root formation (Economou and Read 1986). White light usually inhibits adventitious root formation but promotes adventitious shoot formation. In nodal cuttings of potato, leaf area, leaf number, stem length, and fresh and dry weight are maximized under white light. These data suggest that the quality of light is an important factor affecting the formation of adventitious shoots and roots.

22.4 Bioreactor Culture

Bioreactor bioreactors are usually described as self-contained, sterile environments that capitalize on liquid nutrient or liquid/air inflow and outflow systems. It is designed for intensive culture and provides maximal opportunity for monitoring and controlling micro-environmental conditions such as agitation, aeration, temperature, dissolved oxygen, and pH (Paek et al. 2001). Bioreactor cultures have many advantages over conventional cultures. Plants in bioreactors grow faster and more vigorously in a shorter time span than in conventional cultures. Moreover, in bioreactors, liquid media are often used for the large-scale growth of various tissues. Since liquid medium is applied to plants, physical environmental conditions, such

as air volume, inoculum density, and gaseous phase, inside the bioreactor can be optimized.

A bioreactor was first used for the micropropagation of *Begonia* (Takayama and Misawa 1981). Since then, bioreactors have been proven suitable for the propagation of various plant species and organs, including shoots, bulbs, microtubers, corms, and somatic embryos (Paek et al. 2001). Automation in bioreactors has been used to reduce the cost associated with micropropagation (Paek and Chakrabarty 2003; Paek et al. 2001; Son et al. 1999). Until the mid-1970s, traditional microbial technology provided the knowledge and equipment for cultivation processes, almost exclusively in the form of stirred tank reactors with flat blade turbines for agitation. At present, a relatively large number and variety of reactor systems are available, thus allowing the rational selection of an appropriate reactor for a given process. Nonetheless, most of the standard equipment designed for microbial cultivation does not meet the requirements for the cultivation of fragile plant cells or cell aggregates (Honda et al. 2001; Paek et al. 2001; Sajc et al. 2000). Paek and Chakrabarty (2003) reviewed different reactor configurations for plant cell suspensions and tissue and organ cultures. Relative advantages and selection criteria for various reactor configurations have been discussed for specific applications. A novel type ebb and flood bioreactor system (a periodic immersion system) has been developed for the mass propagation of several plant species.

22.4.1 Bioreactor Culture for Medicinal Orchids

Several studies have been conducted on bioreactor cultures of cells, tissues, and organs of medicinal orchids such as *Dendrobium candidum* (Cui et al. 2014a, b; Yang et al. 2015; Wang et al. 2016) and *A. formosanus* (Yoon et al. 2007; Wu et al. 2007). The cultivation of medicinal orchids in a bioreactor for biomass production has several advantages over plant production for cultivation purposes. The production of intact plants for cultivation must take into consideration the photosynthetic activity of plants, development of healthy leaves and roots, and successful acclimatization to greenhouse and field conditions. By contrast, biomass production for harvesting bioactive compounds is aimed at maximizing the growth of callus and tissues containing high amounts of bioactive metabolites rather than the development of roots and leaves. Thus, all cultures are optimized by controlling medium composition, medium supply method, air volume, inoculum density, and gas supply.

To increase the content of bioactive substances in tissues, the elicitation method is often used. In the maximized state of biomass, stress signaling substances such as salicylic acid and methyl jasmonate are added to the culture media for increasing the content of useful secondary metabolites. Wang et al. (2016) reported that methyl jasmonate at a concentration of 75 μ M is suitable for the production of alkaloids, polysaccharides, and flavonoids in the culture of *D. candidum* protocorm-like bodies (PLBs).

22.5 Two Case Studies

Anoectochilus formosanus **(jewel orchid)** Fig. 22.1 shows the biomass and plantlet production of *A. formosanus* using the airlift bioreactor system established at the Chungbuk National University. In this system, culture medium, plant growth regulators, and activated charcoal were optimized in a 200–250 ml Erlenmeyer flask for the mass production of multiple shoots for a pharmaceutical purpose. Multiple shoot proliferation was established on Hyponex media supplemented with 1 mgL^{-1} BA or 1–2 mgL^{-1} TDZ. The addition of activated charcoal enhanced multiple shoot formation (Ket et al. 2004). Multiple shoots were then transferred to a 5 L air-lift balloon type bioreactor, and the bioreactor culture system was optimized. Among the three bioreactor systems [continuous immersion, raft (net), and temporary immersion (the ebb and flood system)], the continuous immersion system was the most efficient for shoot proliferation and biomass accumulation (Yoon et al. 2007).

Dendrobium Candidum it is mass multiplied via callus, PLB, or multiple shoots (Cui et al. 2014a, b; Zhao et al. 2008). To ensure the continuous production of biomass and bioactive compounds using a bioreactor, the PLB proliferation method was established on Petri dishes using MS medium supplemented with 0.5 mgL^{-1} α-naphthaleneacetic acid (NAA) and 2.5% (w/v) sucrose (Fig. 22.2b). Bioreactor culture conditions, such as air supply, bioreactor type, culture period, and culture density, were tested in a 3–5 L air-lift bioreactor. Once the bioreactor culture conditions were optimized, proliferated PLBs were transferred to a 20-L bioreactor, and

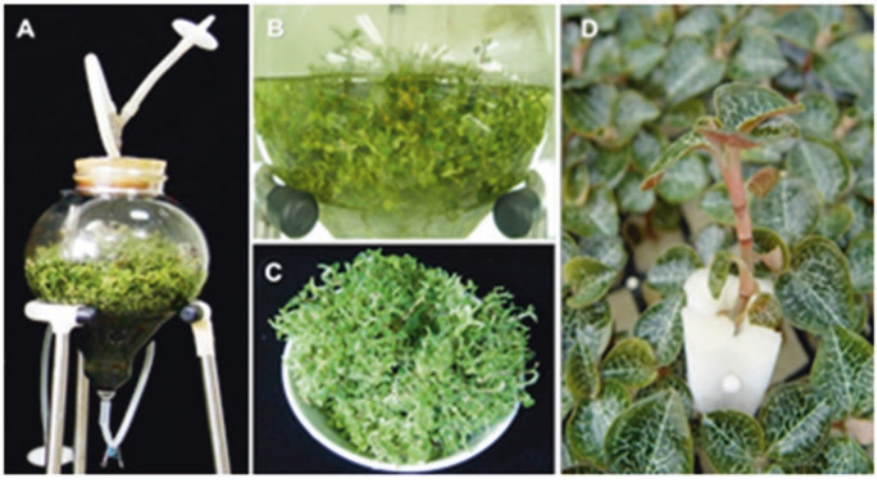

Fig. 22.1 Biomass and plantlet production of *Anoectochilus formosanus* in an air-lift bioreactor. (**a**, **b**) A 5-L air-lift bioreactor culture for the mass production of shoots, (**c**) shoots harvested from the bioreactor culture, (**d**) acclimatized *A. formosanus* plants after 2 months of harvest from the bioreactor

cultures were harvested after 4 weeks (Fig. 22.2c). In a 20-L bioreactor containing 15 L medium, approximately 160.0 g L^{-1} of PLB biomass, on a dry weight (DW) basis, could be obtained; this biomass contained optimum quantities of total polysaccharides (390 mg g^{-1} DW), coumarins (18.0 mg g^{-1} DW), polyphenolics (11.9 mg g^{-1} DW), and flavonoids (4.5 mg g^{-1} DW). Compared with other culture types, PLBs showed the highest antioxidative activity (Fig. 22.3). Analysis of phenolic compounds using high-performance liquid chromatography (HPLC) confirmed that myricetin, gentisic acid, and salicylic acid were present in large amounts (Fig. 22.4).

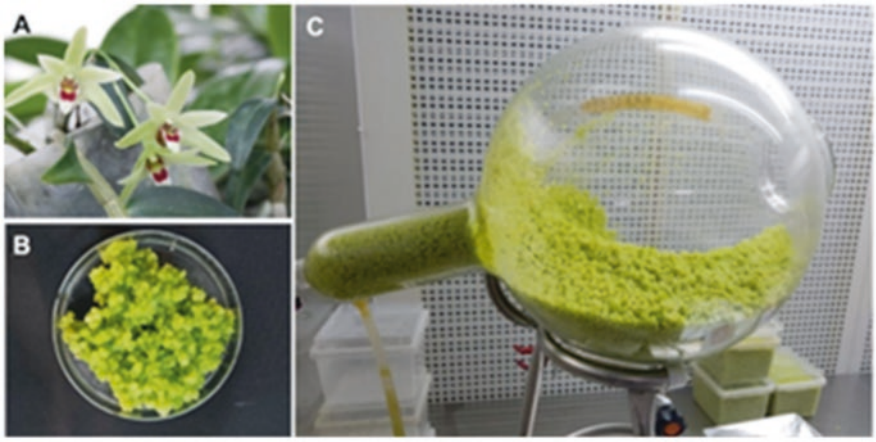

Fig. 22.2 Biomass production of *Dendrobium candidum* protocorm-like bodies (PLBs) in a 20-L air-lift bioreactor. (**a**) *D. candidum* plant with flowers, (**b**) PLBs on a Petri dish, (**c**) PLBs harvested for biomass production

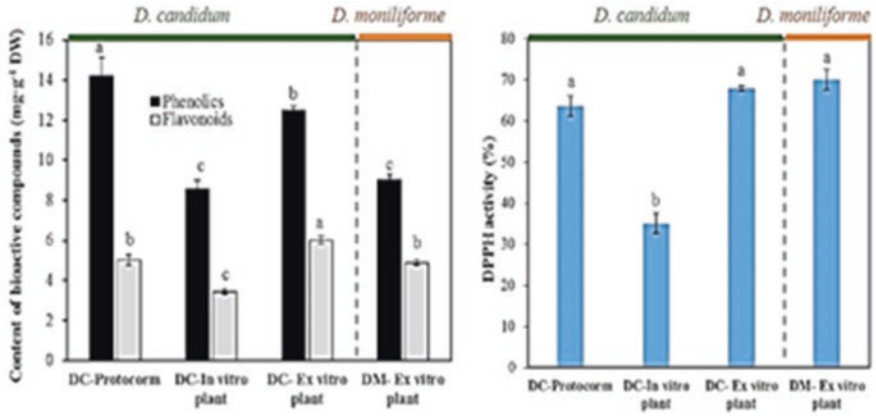

Fig. 22.3 Phenolic and flavonoid content (left) and antioxidant activity (right) of various cultures of *Dendrobium candidum* (DC) and *Dendrobium monifolium* (DM)

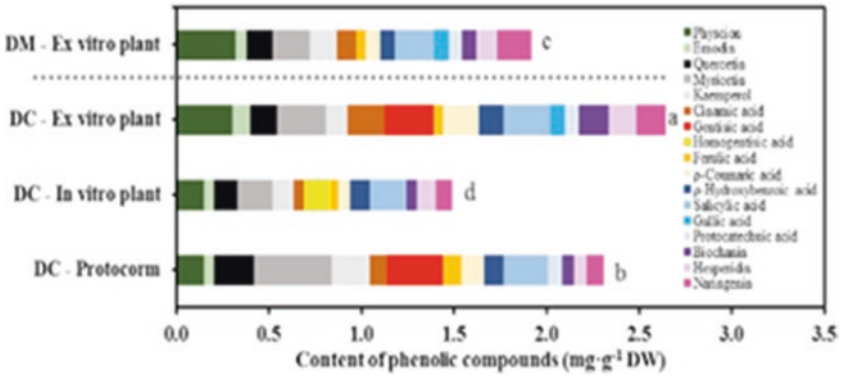

Fig. 22.4 Phenolic profiling of various cultures of *Dendrobium candidum* (DC) and *Dendrobium monifolium* (DM)

22.6 Conclusions and Future Perspectives

Similar to other medicinal plants, orchids contain a variety of bioactive substances and medicinal ingredients. Because of their botanical characteristics, orchids are slower in growth and proliferation than other herbaceous plants and are difficult to cultivate in the field. However, orchids can be mass produced using various in vitro cell and tissue culture methods. In addition, if mass production of orchid plants is needed for industrial purposes such as cosmetics, pharmaceuticals, and medicines, a bioreactor can be used as a powerful production system. Mass production of orchids in bioreactors will increase the availability of medicinal orchids, thus increasing their importance and consequently stimulating further research.

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Ethnomedicinal Aspects of Some Orchids from Darjeeling Himalaya, India

23

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Abstract

The family Orchidaceae is one of the largest flowering plant families with a cosmopolitan distribution. Orchids are profusely growing, particularly in the humid tropics and subtropics. Due to the variety of climatic conditions, India is rich in orchid flora. The Darjeeling Himalaya situated in one of the biodiversity hotspots of the world (Eastern Himalaya) is a virtual goldmine of orchids. The Darjeeling Himalayan region has a rich diversity of medicinal orchids and rich heritage of traditional medicinal practices. Folk medicinal systems like the Nepali Jadi-Buti, the Lepcha system, and the Tibetian medicine are completely based on local plants. To get the data on the traditional uses of ethnomedicinal plants, 20 key informants were interviewed. Quantitative ethnobotanical indices, i.e., fidelity level (Fl) and preference ranking (Pr), were calculated for recorded ethnomedicinal plants. The present ethnobotanical study mainly concerns about 25 species of orchids belonging to 18 genera, including 20 epiphytes and five terrestrials that have been used by the Lepchas of the Darjeeling Himalaya to treat 30 kinds of diseases. Most of the plants have high curing capacity, which could be used in pharmaceutical research in order to achieve adequate revenue. Some of the plants in the study area are facing threats due to anthropogenic activities; hence, sustainable harvesting and conservation initiatives are needed in this region. The conservation and multiplication of important medicinal orchids should go hand

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in hand, and people concerned with the conservation program should get economic incentives. However, no efforts have been made till date for the preservation of the germplasm, their identification, and the estimation of active compounds from medicinal orchids. So the time has come to think globally and act locally.

Keywords
Orchids · Ethnobotany · Darjeeling Himalaya · India

23.1 Introduction

Orchids are considered as the most highly evolved flowering plants. Orchidaceae have about 28,000 currently accepted species, distributed in about 763 genera (Christenhusz and Byng 2016). These nonwoody perennial plants are generally terrestrial or epiphytic herbs. Majority of the species synthesize their own food materials, but some live on dead organic matter (saprophytic) or are aided by a fungus living in their roots to obtain nourishment. There are about 1331 species belonging to 186 genera widely distributed throughout the country (Chowdhery 2009). Out of 1229 species of orchids distributed in the tropical and the alpine regions of India (Chowdhery 1999), 960 species are reported to be from the Indian Himalayan region (Samant and Pant 2006). As many as 545 orchid species are reported from the Eastern Himalayan and 244 from the Trans, North West, and West Himalayan regions (Pangtey et al. 1991; Samant 2002). The East Himalayas and North Eastern India, North West Himalayas, Peninsular India, and Andaman and Nicobar Islands are the major orchid-rich regions in India (Vij 1995).

Orchids are a group of rare plants, and apart from being habitat specific (Linder 1995), they have specialised pollinators (Darwin 1862; Cozzolino and Widmer 2005), as well as specific mycorrhizal associations (Taylor and Bruns 1997; McCormick et al. 2004; Shefferson et al. 2007). Orchid seeds lack endosperm, and the involvement of fungi in seed germination is a well-known fact (Bernard 1909), but the specificity in orchid mycorrhizae has been controversial (Harley and Smith 1983). Some studies show that orchids are specific (Clements 1987; Taylor and Bruns 1997), others show that they are generalist (Hadley 1970; Smreciu and Currah 1989; Masuhara and Katsuya 1989, 1991; Masuhara et al. 1993; Rasmussen 1995) in their mycorrhizal symbioses, whereas some state that specificity is a variable among species (Muir 1989). This phenomenon of specialization makes orchids diverse and rare (Gill 1989; Shefferson et al. 2005; Otero and Flanagan 2006). This specialization may increase the chance of extinction for those orchids that live in endangered or rare habitats (Shefferson et al. 2008). Orchids are valued not only for their attractive flowers, but many of them have been also used for therapeutic purposes (Handa 1986). However, there is some advancement in understanding the orchid family, but threats to the survival of orchids continue to increase day by day. The IUCN red lists of threatened species declared 948 (3.3%) species out of 28,484 orchid species worldwide as threatened (Govaerts et al. 2017).

Darjeeling is a part of the Eastern Himalayan zoo-geographic zone (Negi 1992). The region is well known for its diverse range of vegetation and for being one of the richest in India (Bhujel 1996). The Darjeeling Hill district of West Bengal is situated between 27°13′–28°31′ N latitude and 87°59′–88°53′ E longitude in the Eastern Himalayan region of India. Flora around Darjeeling comprises sal, oak, semi-evergreen, temperate, and alpine forests. Depending on elevation, the climate of the district varies from tropical in the lower hills to subalpine in the high hills. The four major forest types according to altitudinal variation found in Darjeeling Hill areas are tropical moist deciduous forest (300–1000 mts), tropical evergreen lower montane forest (1000–2000 mts), tropical evergreen upper montane forest (2000–3000 mts), temperate forest (3000–3500 mts), and subtemperate forest (above 3500 mts). About 30% of the forest covers found in the lower hills are deciduous. Evergreen forests constitute only about 6% of the total forest coverage.

The Darjeeling Himalayan region is a rich repository of orchid species. There are 109 genera with 392 species reported from the Darjeeling district by many earlier workers (Pradhan and Pradhan 1997; Pearce and Cribb 2002; Yonzone et al. 2012a, b).

The medicinal importance of orchids had been known as early as 250–300 BC by Susruta and Vagbhata in ancient Sanskrit literature. Orchid in Sanskrit is "Vanda," a name adopted for a kind of attractive and monopodial orchids (Deb and Imchen 2008). Orchids were used in ancient Chinese medicines during 2800 BC. The word orchid is derived from the Greek word "orchis" for testicle because of the shape of the root tubers in some species of the genus *Orchis*, and tubers are believed to be useful in treating human virility. *Orchis* gained widespread fame for its unique properties of being an aphrodisiac. Some orchid species are reported to contain alkaloids, flavonoids, phenols, and triterpinoids. Ashtavarga is a group of eight drugs in Ayurvedic formulation, which are used for the preparation of tonics, such as "Chyavanprash," which consists of four orchid species, viz. *Habenaria edgeworthi* Hook. f. (Vriddhi), *H. intermedia* D. Don, (Riddhi), *Malaxis acuminata* D. Don, (Rishbhaka), and *M. muscifera* (Lindl.) Kuntze (Jivaka). Besides these, many orchids are widely used as traditional medicines by people and used in pharmaceutical industries to isolate aeridin, agrostophyllin, anthocyanins, blestrianol, callosmin, cumulatin, gymnopusin, flaccidin, imbricatin, orchinol, stilebnoids, and triterpenoids. India and other parts of the world use many orchid species in their traditional system of herbal medicines.

Plants have been used in traditional medicine for several thousand years. The knowledge of medicinal plants accumulated over the many centuries is based on different medicinal systems, such as Ayurveda, Unani, and Siddha. During the last few decades, there has been an increase in the study of medicinal plants and their traditional use in different parts of the world. Herbal remedies are considered the oldest forms of health care known to mankind on this earth. Prior to the development of modern medicine, the traditional systems of medicine that have evolved over the centuries within various communities are still maintained as a great traditional knowledge base in herbal medicines. Traditionally, this treasure of knowledge has been passed on orally from generation to generation without any written

document and is still retained by various indigenous groups around the world. Documenting indigenous knowledge through ethnobotanical studies is important for the conservation and utilization of biological resources. Ethnobotanical survey has been found to be one of the most reliable approaches to drug discovery. Several active compounds have been discovered from plants on the basis of ethnobotanical information and used directly as patented drugs. As indigenous cultures are closely maintained by tribal and other forest dwellers throughout the world, ethnobotanical investigation is a prerequisite for any developmental planning concerned with the welfare of tribal people and their environment. It is an urgent necessity to record as quickly as possible all information about plants and the role of tribes in conserving the orchid flora. The main focus of the present study is to ascertain detailed information on the use of plants and their therapeutic practices among the Lepcha community of Darjeeling Himalaya (West Bengal) in India.

23.1.1 Ethnobotanical Data Collection

To collect ethnomedicinal information and herbarium specimens, field surveys were carried throughout the forested regions of the Darjeeling Himalaya (Fig. 23.1), including farms and floral nurseries, which were traversed on foot regularly from 2016 to 2018 in different seasons. Efforts were made to study plants in their flowering condition so as to identify them properly. The Lepcha tribal people, including Bonthings and Mun-Bonthings (Lepcha medicine men and Lepcha medicine women, respectively), village chiefs, community leaders, and knowledgeable persons, were interviewed during the course of the study. Information was also gathered in the area from many other elderly people with knowledge of ethnomedicine. To gain rapport with the tribal members, Lepcha social organizations and interpreters belonging to the Lepcha tribe were engaged to help locate and interview informants. Preliminary identification of collected plant material was done with the help

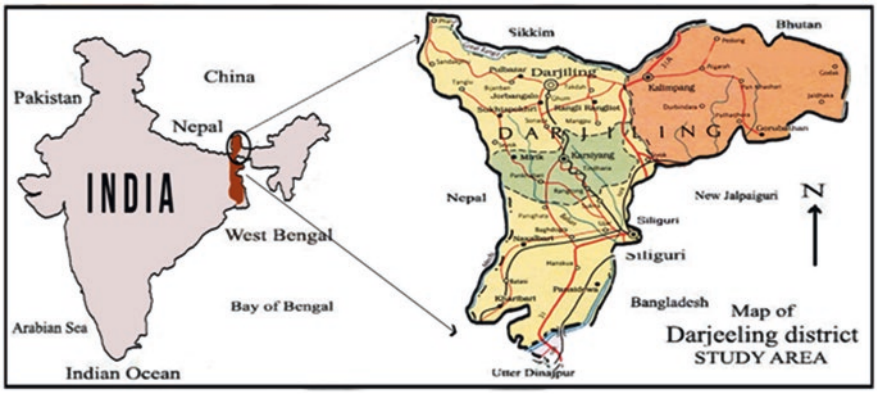

Fig. 23.1 Map of study area (Darjeeling Himalaya)

of these traditional medicine practitioners. Information regarding their use was also recorded with the help of these practitioners and knowledgeable elderly persons. The collected plants were subsequently identified with the help of standard orchid flora (King and Pantling 1898; Pradhan 1976, 1979; Lucksom 2007; Deva and Naithani 1986; Abraham and Vatsala 1981; Bose and Bhattacharjee 1980; Bentham 1881). Standard methods for collection and preservation were used following Jain and Rao (1977). Whole plants and their flowers were preserved in FAA (formalin-acetic-alcohol), and they were mounted on herbarium sheets to study morphological details. Voucher specimens were deposited at the Department of Botany and Microbiology, Acharya Nagarjuna University.

Quantitative Data Collection Ethnobotanical data obtained during the study was summarized using a Microsoft Excel spreadsheet (2007), which was used to calculate proportions like the name of the orchid species, habitat, location, altitude, flowering month, plant parts used, and folklore medicinal uses.

Fidelity Level (Fl) The information obtained from the field, as well as from the informants, was arranged in a proper format, and later analysis was done. Fidelity level was calculated for the most used medicinal plants, which were reported to be used for the treatment of human ailments. This fidelity level value explains the relative healing potential of medicinal plants, and it was calculated by the formula

$$Fl(\%) = Ip / Iu \times 100,$$

where *Ip* is the number of informants who independently indicated the use of species for the same major ailment and *Iu* the total number of informants who gave information about the plant for any major ailment (Alexiades 1996). Before the calculation of *FL*, all human ailments were grouped into major disease categories, using a similar approach employed elsewhere (Heinrich et al. 1998).

Preference Ranking (Pr) This represents one of the simplest analytical tools used by ethnobotanists. In preference ranking, each informant was asked to arrange group of items according to specific criteria (such as personal preference, local economic importance, or species scarcity). Later, each item was assigned a numerical value, with the most important ranking highest (5, in this study), while the least important is assigned a value of 1 (lowest). Then data from a range of informants were summed up to produce overall ranking value. Preference ranking was conducted based on the protocol of Cotton (1996).

23.2 Lepcha Community and Ethnomedicinal Importance of Orchids

Members of this Lepcha tribe are aborigines in Sikkim and the Darjeeling Himalayan region. Famed as born naturalists and known to be a vanishing tribe, the ethnomedicinal traditions of these people are characterized by multiple remedies for a single ailment. India is a treasure house for ethnomedicinal knowledge due to the presence of diverse vegetation, ancient lineage and multi-ethnic groups. All India Coordinated Research Project on Ethnobiology (AICRPE 1992–1998) mentioned that tribes have been using more than 10,000 wild plants for meeting their primary healthcare, food and other material requirements. The study of ethnobotany through a qualitative survey method is a very old history, but the interest in numerical ethnobotany has established progressively in the last couple of decades (Amjad and Arshad 2014). This is the first effort in this region to provide quantitative ethnobotanical data employed by indigenous people. The objectives of the present study were (i) enlisting native ethnomedicinal orchids, (ii) recording the ethnic formulations of this flora along with their mode of preparation, (iii) finding out the correlation between ethnomedicinal uses, and (iv) providing baseline data for pharmaceutical research by the application of quantitative indices. The present study revealed that the Lepchas use 25 species of orchids belonging to 18 genera to treat 30 kinds of diseases.

23.2.1 Demography of Informants

A total of 20 key informants (15 men and 5 women) were interviewed. The age of the informants ranged from 30 to 75 years (Table 23.1). Most of the informants were men compared with the women of this area. The Lepcha community has been professing their original ethnic culture and are also dependent on plant resources for their livelihood. Interestingly, literate persons have less knowledge compared to illiterate persons as they are not interested in this profession and are doing other jobs for their livelihood. Similar observations were recorded elsewhere by other workers (Gedif and Hahn 2003; Uniyal et al. 2006).

23.2.2 Therapeutic Importance of Orchids

The present study revealed that 25 species of orchids belonging to 18 genera were used as medicinal plants by the Lepcha community in the Darjeeling Himalayan region (Table 23.2). The medicinal recipes were prepared by the Lepcha as an extract, decoction, juice, paste, and powder, depending on the plant material. Generally, the parts used from the plants were roots, pseudobulbs, leaves, flowers, fruits, seeds. Based on plant habitat analysis (Fig. 23.2), epiphytes (80%) are frequently used by local people, followed by terrestrial (20%). Some members of the Lepcha community giving ethnomedicinal information are shown in Fig. 23.3a–f.

Table 23.1 The key informants of forest regions of Darjeeling Himalaya

Sl. No.	Name of the tribal physician	Gender	Age (years)	Tribe/race
1	Norchung Lepcha	Male	70	Lepcha
2	Ongdi Lepcha	Male	64	Lepcha
3	Phuty Lepcha	Female	52	Lepcha
4	Palzang Lepcha	Male	46	Lepcha
5	Samchi Lepcha	Male	64	Lepcha
6	Sabittri Lepcha	Female	47	Lepcha
7	Nakbu Lepcha	Male	55	Lepcha
8	Chaman Lepcha	Male	59	Lepcha
9	Sangdub Lepcha	Male	55	Lepcha
10	Sumitra Lepcha	Female	45	Lepcha
11	Thakpa Lepcha	Male	55	Lepcha
12	Lamba bou Lepcha	Male	75	Lepcha
13	Phurba Lepcha	Male	47	Lepcha
14	Machai Lepcha	Male	45	Lepcha
15	Yuden Lepcha	Male	50	Lepcha
16	Samchin Lepcha	Male	45	Lepcha
17	Chunney Lepcha	Female	52	Lepcha
18	Topgay Lepcha	Male	70	Lepcha
19	Sunomit Lepcha	Female	50	Lepcha
20	Chaman Lepcha	Male	30	Lepcha

23.2.3 Mode of Preparation

Traditional practitioners used different parts of the plant to prepare herbal medicine. Depending on the plant part (Fig. 23.4) used for medicinal purposes, the leaf constituents (Fig. 23.4) showed the highest percentage (35%), followed by whole plant (30%), roots (11%), pseudobulbs (8%), flowers (8%), combination of leaf and flower (3%), mixture of root and leaf (3%), and seeds (2%) (Fig. 23.4). The use of leaves in preparation of remedies for various ailments is also very common in other parts of the world (Tabuti et al. 2003; Muthu et al. 2006, Teklehaymanot et al. 2007). Most commonly used methods of plant preparations are paste (33%), followed by juice (31%), decoctions (13%), crushing (9%), other methods (9%), and powder (5%). Methods of preparations were presented in Fig. 23.5. Most of the informants suggested to take the herbal medicines by applying externally (15 remedies) and orally (10 remedies); as well as for eye problems (2 remedies).

23.2.4 Major Disease Categories

About 25 orchids, reported in the present study, have been used in curing 27 different ailments, either singly or in combination. The plants used to treat various diseases were grouped under 14 categories, such as fever (jaundice, cholera, high fever), dysentery (amoebic and blood), skin diseases, bone fractures, liver and

Table 23.2 List of ethnomedicinal orchids in Darjeeling Himalaya used by the Lepcha tribe

S. No	Name of the orchid species	Habitat	Location in Darjeeling Himalaya		Flowering month	Parts used	Folklore medicinal uses	Therapeutic importance
			Location	Altitude (in feet)				
1.	*Acampe papillosa* (Lindl.) Lindl.	Epiphyte	Sukna wild life, Sukna forest, West Bengal	735	June to August	Root	The paste of roots is used in treatment of body pains	Rheumatism
	ANUBH1201					Leaves	The root paste is externally applied on snake bites	Snake bites
							A combination of leaf paste and garlic piece is taken as treatment for stomach disorders, and indigestion	Stomachache
2.	*A. rigida* (Buch. - Ham. ex Sm.) P.F. Hunt	Epiphyte	Near sacred water body, Lamahatta, Tukdah forest, West Bengal	6255	May to June	Whole plant	The decoction of the whole plant produces a bitter tonic used in the treatment of jaundice	Jaundice
	ANUBH1202							
3.	*Aerides multiflorum* Roxb	Epiphyte	Near Latpanchar forests, kurseong, West Bengal	3579	April to May	Whole plant	Root decoction is used in the treatment of body ache	Rheumatism
	ANUBH1203						The juice obtained by the grinding of the whole plant is used to cure cuts and wounds	Cuts/wounds
4.	*Arundina graminifolia* (D.Don) Hochr	Terrestrial	NHPC, NH31A, road side, Kalijhora, West Bengal	745	January to November	Leaves	The paste of the leaves is applied on the body to treat skin infections	Skin infections
	ANUBH1204							

5.	*Bulbophyllum guttulatum* (Hook.f.) N.P. Balakr. ANUBH1205	Epiphyte	Sittong forest, West Bengal	2736	June to August	Pseudobulb	The juice of fresh pseudobulbs is used to treat burn wounds	Burn wounds
6.	*B. leopardinum* (Wall.) Lindl ANUBH1206	Epiphyte	Near road sides of tista bazaar, West Bengal	701	June to August	Whole plant	The whole plant juice is mixed with some other ingredients, and paste applied and tied with a bandage for bone fracture	Bone fractures
7.	*Coelogyne cristata* Lindl. ANUBH1207	Epiphyte	Pankhabari road side, Kurseong, West Bengal	4852	January to April	Pseudobulb	Fresh pseudobulb paste is used as ointment for wounds	Wounds
8.	*Cymbidium aloifolium* (L.) Swartz. ANUBH1208	Epiphyte	11th Tinchuley Lopchu road, Tukdah forest, West Bengal	5006	April to June	Root	Root powder is used to treat mild paralysis	Paralysis
						Leaf	Juice obtained from boiling of fresh leaves is used for the treatment of earache	Earache
						Seeds	The powder of dried seeds is used for healing wounds	Wounds
						Whole plant	The juice of the whole plant is administered orally as a tonic and stimulant	Stimulant

(continued)

Table 23.2 (continued)

S. No	Name of the orchid species	Habitat	Location in Darjeeling Himalaya		Flowering month	Parts used	Folklore medicinal uses	Therapeutic importance
			Location	Altitude (in feet)				
9.	*Dendrobium aphyllum* (Roxb.) C.E.C.Fisch.	Epiphyte	NH 10, Teesta river sides, Rangpo forest, West Bengal	1320	March to May	Leaf	Fresh leaf juice is used to treat skin disorders	Skin infections
	ANUBH1209					Whole plant	The pounded fresh plant is applied to boils	Boils
10.	*D. fimbriatum* Hook.	Epiphyte	Road sides of river Rangeet, West Bengal	1890	March to May	Leaf	The paste of fresh leaves is used to treat boils and pimples	Boils and pimples
	ANUBH1210					Flowers	The juice of flowers and leaves is used for liver and nervous system disorders	Liver and nervous disorders
								Cholera
						Leaf + flower	The decoction of fresh leaves and flowers is taken orally to cure cholera.	
11.	*D. moschatum* (Buch.-Ham.) Sw.	Epiphyte	Rangli forest, West Bengal	1706	May to June	Leaf	The juice obtained from fresh leaves is used for the treatment of earache	Earache
	ANUBH1211						The juice of crushed leaves is used for clotting of blood	Blood clotting
12.	*Eria lasiopetala* (Willd) Ormerod.	Epiphyte	Sukna wild life, Sukna forest, West Bengal	790	March to June	Leaf	The pounded fresh plant is applied to heal boils	Boils
	ANUBH1212					Whole plant	The juice of the whole plant is applied to cuts and wounds	Cuts and wounds

13.	*E. stricta* Lindl.	Epiphyte	Near opposite road side of Mahananda wild life sanctuary, West Bengal	1568	February to May	Whole plant	Plant extract is used to reduce muscular pains in humans.	Muscular pains
	ANUBH1213							
14.	*Panisea uniflora* (Lindl.) Lindl	Epiphyte	NH 10, Birik forest, West Bengal	862	March to June	Leaf	The paste of leaves is used as an external application for pus-forming wounds	Pus-forming wounds
	ANUBH1214							
15.	*Paphiopedilum insigne* (Wall. ex Lindl.) Pfitzer	Terrestrial	Tukdah forest, West Bengal	6003	November to December	Whole plant	The decoction of the whole plant is used against amoebic dysentery	Amoebic dysentery
	ANUBH1215							
16.	*Papilionanthe teres (Roxb.) Schltr.*	Epiphyte	Lish forest, West Bengal	1365	May to June	Root	The decoction of the root is used as an antifertility medicine	Antifertility medicine
	ANUBH1216					Leaf	The paste of fresh leaves is applied during high fever	High fever

(continued)

Table 23.2 (continued)

S. No	Name of the orchid species	Habitat	Location in Darjeeling Himalaya		Flowering month	Parts used	Folklore medicinal uses	Therapeutic importance
			Location	Altitude (in feet)				
17.	*Phaius tankervilleae* (Banks) Blume	Terrestrial	Mazeok Forest, West Bengal	2132	April to May	Whole plant	The paste of whole plant, along with wild ginger is used as medicine in dysentery	Dysentery
	ANUBH1217						The juice extracted from the whole plant is used to treat bone fractures	Bone fractures
18.	*Phalaeonopsis manni* Rchb.f	Epiphyte	Tukdah forest, West Bengal	6009	April to June	Flowers	The paste of flowers in combination with coconut oil is used for hair growth	Hair growth
	ANUBH1218							
19.	*Pholidota articulata* Lindl	Epiphyte	Mangpong forest, West Bengal	940	February to June	Leaves	The juice of leaves is taken orally as a tonic	Tonic
	ANUBH1219							
20.	*Rhynchostylis retusa* (L.) Blume	Epiphyte	Mazeok Forest, West Bengal	2132	March to July	Root	Root paste is used to cure blood dysentery and wounds	Blood dysentery and wounds
	ANUBH1220					Whole plant	The paste of whole plant is applied on the body against various skin diseases	Skin diseases
21.	*Spathoglottis plicata* Blume	Terrestrial	NH 10, Birik forest, West Bengal	843	March to April	Pseudobulb	The paste of pseudobulbs is used for external application to cure rheumatic swelling	Rheumatic
	ANUBH1221							Swelling
						Whole plant	A decoction of the whole plant is used against rheumatism, and the hot fomentation pressed on to as infected part draw out pus relieves pain and encourages blood circulation	Pus remover
								Painkiller
								Blood circulation

22.	*Thunia alba* (Lindl.) Rchb. f.	Terrestrial	Latpanchar forest, West Bengal	1809	May to August	Leaf	The paste obtained from the leaves is applied to treat dislocated bones	Dislocated bones
	ANUBH1222							
23.	*Vanda coerulea* Griff. Ex Lindl.	Epiphyte	Takdah forest, West Bengal	4990	July to December	Flower	Flower juice is used as eye drops against blindness, cataract, and glaucoma	Eye problems
	ANUBH1223							
24.	*V. cristata* wall. Ex Lindl.	Epiphyte	Tukdah forest, West Bengal	5999	May to June.	Leaves	The juice of fresh leaves, along with turmeric, is used in cosmetics for skin care	Skin care and cosmetics
	ANUBH1224							
25.	*V. tessellata* (Roxb.) hook. Ex G.Don	Epiphyte	Near Latpanchar forests, kurseong, West Bengal	3005 ft.	May to July	Root + leaf	Aerial roots and leaves are ground, and the paste is applied for bone fracture	Bone fracture
	ANUBH1225					Leaf	The juice of the leaves is used to cure earache and skin diseases	Earache
								Skin diseases

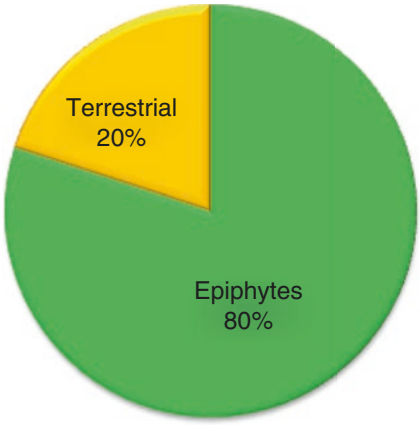

Fig. 23.2 A pie chart showing habitat analysis of orchids

nervous disorders, rheumatism/pain, hair loss/cosmetics-related diseases, aches (stomachache and earache), cuts/wounds/burns/boils/pimples, snake bites, pus, nervous system disorders, eye infections, and fertility problems (Fig. 23.6). The highest number of species (8) has been used in different formulations to treat cuts/wounds/burns/boils/pimples (19%) (Fig. 23.7), while five species were used against skin diseases (12%) (Fig. 23.7).

23.2.5 Fidelity Level of Medicinal Plants

Mesfin et al. (2014) studied the ethnomedicinal plants in Amaro Woreda (Ethiopia) and mentioned that medicinal plants that are known as remedies for a single ailment have 100% FL value as compared to those that used are as remedies for more than one type of ailment. Medicinal plants with high FL values are those that are widely used by the local practitioners. In our study, we found the highest FL value (Table 23.3) for *Bulbophyllum leopardinum*, *Paphiopedilum insigne*, and *Vanda coerulea* (100%) (Fig. 23.8), and lowest value was recorded for *Acampe papillosa* (55.55%) (Table 23.3, Fig. 23.8). Similarly, Ugulu (2012) studied on medicinal plants used for therapeutic Turkish bath by the local people of various areas in Turkey and also reported that *Cucumis sativus* (100%) has the highest FL value and *Thymus vulgaris* (32%) the lowest FL value.

Most of the medicinal plants were freely harvested by users from nature, in which they had been abundantly found. About 90% of the medicinal plants were wild species. Some species, like *Dendrobium moschatum* and *Paphiopedilum insigne*, were reported to be planted by tribes in surrounding environs. Plant collection is a part-time activity for the Lepcha community.

Fig. 23.3 (**a**) Collecting ethnomedicinal information with the help of UC Pradhan, eminent orchidologist; (**b**) collection of plants for herbarium with the help of local people; (**c**) collecting useful plant parts with the help of Chaman Lepcha; (**d, e**) collecting information from key respondents Sabittri Lepcha and Samchi Lepcha; (**f**) some of the key informants showing enthusiasm to give preference ranking of threats

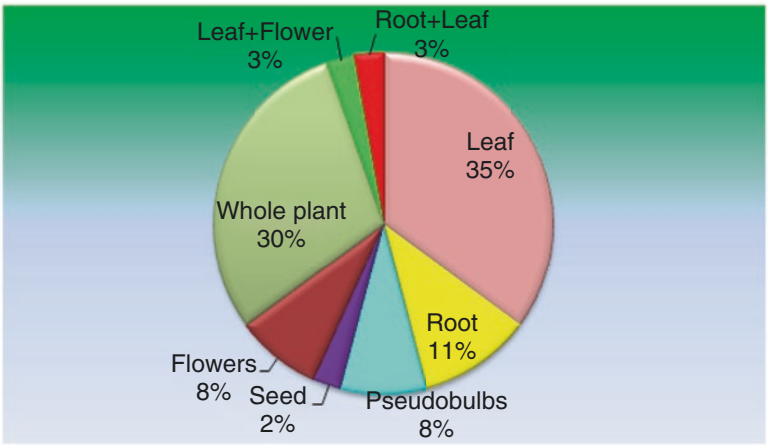

Fig. 23.4 A pie chart showing the different plant parts used by traditional practitioners of the Lepcha community

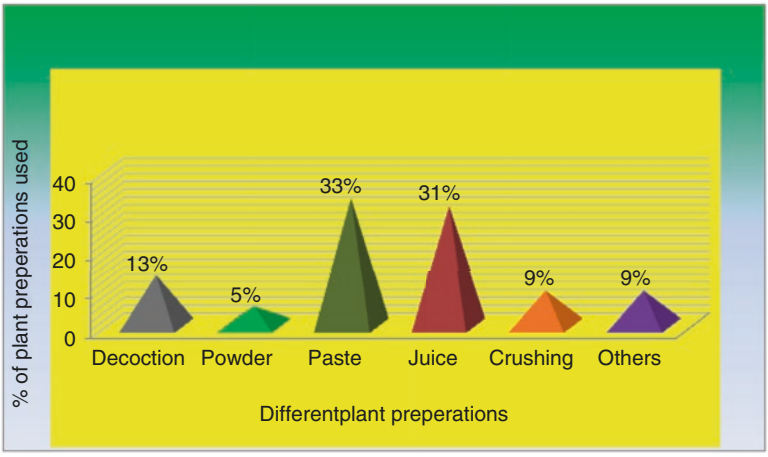

Fig. 23.5 A graph showing the different methods of plant preparations

23.2.6 Preference Ranking (Pr)

In the present study, data from Table 23.4 represent the relative value of six threat factors, as perceived by the five respondents from the study area (Darjeeling Himalaya, West Bengal). The preference ranking based on the degree of threats to ethnomedicinal orchids was conducted with the participation of respondents (Table 23.4). In preference ranking (Fig. 23.9), informants ranked deforestation (Rank 1) as a leading threat, followed by lack of awareness of the public with regard to medicinal plants (Rank 2), climate change and severe weather conditions (Rank

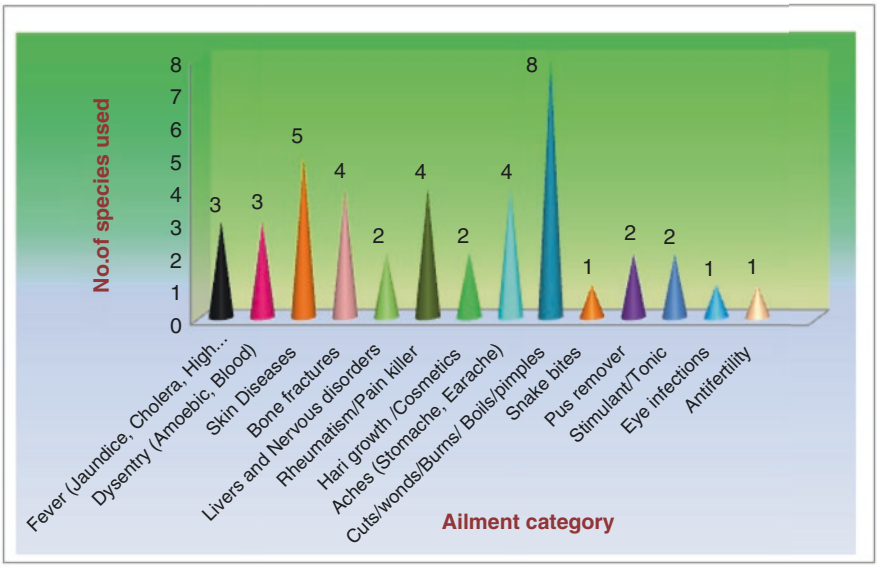

Fig. 23.6 A graph showing the different ailment categories and number of species used for the treatment of different diseases

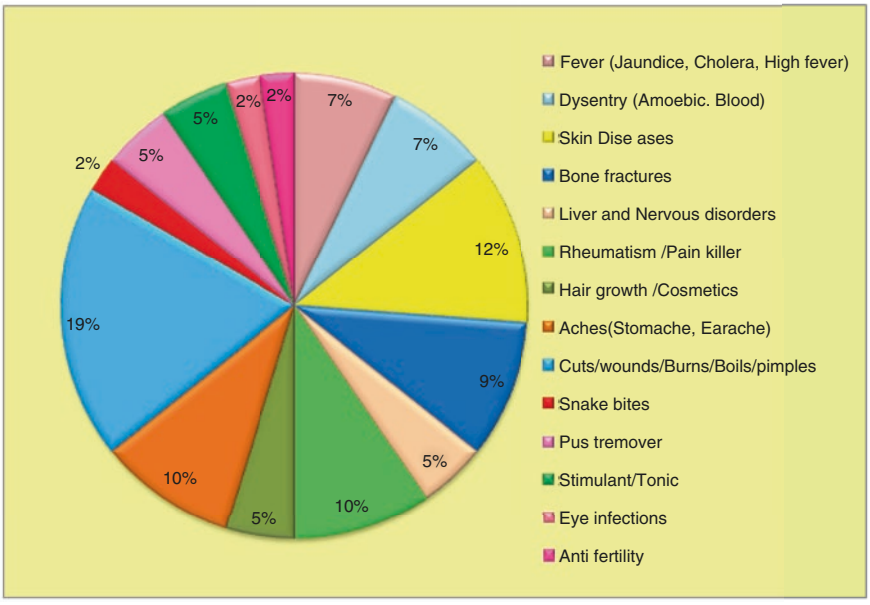

Fig. 23.7 A pie chart showing the percentage of different ailments treated

Table 23.3 Fidelity level of medicinal orchids used by the tribal people of ***Darjeeling Himalaya

S. no.	Name of the orchid species	Therapeutic uses	No. of informants (Ip)	Total no. of informants (Iu)	Fidelity level (Fl %)
1	*Acampe papillosa*	Rheumatism	5	9	55.55%
2	*Arundina graminifolia*	Skin infections	3	4	75%
3	*Bulbophyllum leopardinum*	Bone fractures	10	10	100%
4	*Cymbidium aloifolium*	Paralysis	7	11	63.66%
5	*Dendrobium fimbriatum*	Cholera	5	6	83.33%
6	*D. moschatum*	Earache	5	7	71.43%
7	*Paphiopedilum insigne*	Amoebic dysentery	6	6	100%
8	*Papilionanthe teres*	High fever	6	8	75%
9	*Phalaeonopsis manni*	Hair growth	7	12	58.33%
10	*Thunia alba*	Dislocated bones	9	11	81.81%
11	*Vanda coerulea*	Eye problems	7	7	100%
12	*V. cristata*	Skin care	2	3	66.66%

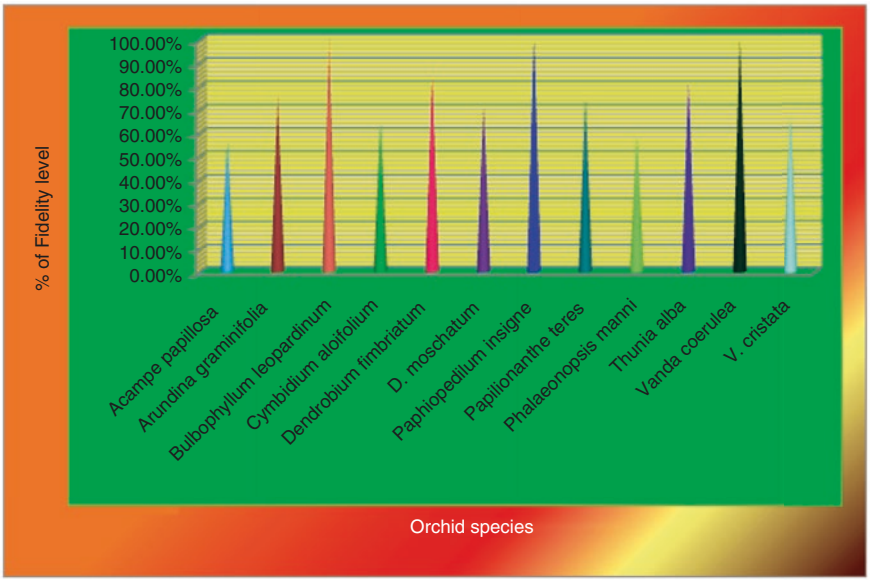

Fig. 23.8 A graph showing the fidelity level (Fl) of different orchids

Table 23.4 Preference ranking (Pr) of factors perceived as threats to ethnomedicinal orchids

Threat factor	Ranking value for each respondent					Total score	Ranking
	A	B	C	D	E	(A + B + C + D + E)	
Human intrusions and disturbance	1	1	1	2	2	7	6th
Overexploitation	3	2	2	4	1	12	4th
Climate change and severe weather	2	3	4	3	2	14	3rd
Lack of awareness of the public with regard to medicinal plants	4	5	3	4	3	19	2nd
Energy production and mining	1	2	1	2	2	8	5th
Deforestation	5	4	5	5	5	24	1st

(A, B C, D, E = respondents from the study area)

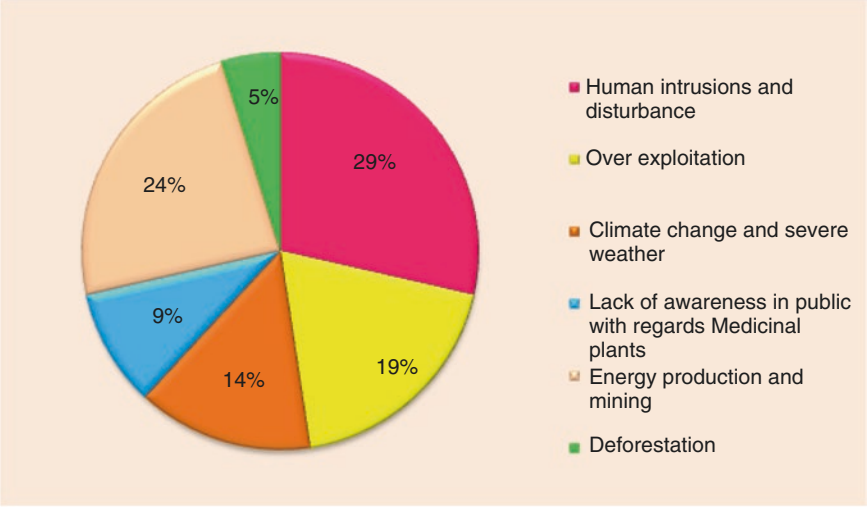

Fig. 23.9 A pie chart showing the preference ranking (Pr) of threats

3), overexploitation (Rank 4), energy production and mining (Rank 5), and human intrusions and disturbance (Rank 6). Similar types of threat factors were also reported by Hussain et al. (2004). They also mentioned that overgrazing, habitat destruction, and overexploitation are increasing day by day, which are the main factors responsible for the extinction of the species. According to Seifu et al. (2006), globally, agricultural encroachment in natural habitat leads to the consequent loss of medicinal plant species.

23.3 Conclusions

Interest in ethnobotany has increased dramatically in recent years. The search for new medicines by the pharmaceutical industry has turned to natural products and ethnobotanical studies as a first step in bioprospecting. These studies are valuable for making a valuable contribution to the cataloguing of biological diversity and hence to the conservation of endangered ecosystems and the human societies, which depend upon them. From the present study, the ethnomedicinal importance of 25 species of orchids belonging to 18 genera from the study area (Darjeeling Himalaya, West Bengal) was recognized. Their usefulness in the treatment of various ailments by the Lepcha community has been analyzed and brought to light. Darjeeling Himalaya is gifted with rich orchid resources; many undocumented orchids with high medicinal properties are still available that need to be properly recognized and explored for their use in therapeutic application. Therefore, there is urgent need to protect the undocumented orchids from this region and to preserve the indigenous knowledge of the local tribes for upcoming generations.

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Evaluation of Phytoconstituents and Antibacterial Activity of *Vanda tessellata* Using In Vitro Model

24

Karabi Biswas and Sankar Narayan Sinha

Abstract

The present work was undertaken to investigate the antimicrobial activity of the leaves and flowers of various solvent (benzene, chloroform, acetone, methanol, ethanol and water) extracts of *Vanda tessellata* of the family Orchidaceae against eight Gram-negative bacteria (*Escherichia coli*, *Vibrio cholerae*, *Shigella dysenteriae*, *Shigella flexneri*, *Serratia marcescens*, *Salmonella typhi*, *Salmonella enterica*) and four Gram-positive bacteria (*Bacillus subtilis*, *Bacillus cereus*, *Micrococcus luteus* and *Staphylococcus aureus*). The fresh plant materials were collected at the end of April 2017 from the Hijli forest located in Ranaghat, West Bengal, India. The antimicrobial activity of the plant extracts was determined by the agar-well diffusion assay. All the extracts showed different degrees of inhibitory potential against all the tested bacteria. Zone of inhibition (ZOI) was measured at 6.9–24.4 mm. Ethanolic extract showed more activity, followed by methanolic, chloroform, acetone and benzene extracts. Water extracts showed less activity due to the absence of secondary metabolites in this extract. This study further imparts the use of whole plant extracts in treating diseases caused by tested microbial organisms.

Keywords

Vanda tessellata · Plant extract · Antibacterial activity

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24.1 Introduction

Research in the field of chemistry of natural products has limitless potential, which is particularly important in countries like India, which has rich biodiversity. The continuous use of synthetic drugs shows unpleasant side effects on one hand, and on the other hand the number of drug-resistant bacteria is increasing. Therefore, plants may be a good source of drugs, and such medicinal plants should be investigated to understand better their characteristics, efficacy, and safety and the major sources of antibacterial drugs (Sofowora 1986; Nascimento et al. 2000). Hence, the screening of plants with folkloric medicinal values is very important to overcome the existing emerging problems (Monica et al. 2013; Bhattacharjee et al. 2015). *Vanda tessellata* (roxb.) is an endangered epiphytic orchid with a leafy stem attaining a height of 30 to 60 cm. This orchid has been used as a source of indigenous medicine, like Ayurveda, and in local traditional medicinal practices (Velanganni et al. 2011). In various countries, this orchid is used largely for the treatment of several diseases, like bronchitis, dyspepsia, piles, inflammations, etc. The route is used in rheumatism and allied diseases, as well as in neurological problems. It is also used in the treatment of otitis and the paste as febrifuge (Basu et al. 1971; Kirtikar and Basu 1999; Ghani 2003).

The plant-derived antibacterial substances are not generally associated with side effects, and they have prospective, therapeutic benefits of curing many infectious diseases (Sibin and Gangaprasad 2012; Bhattacharjee and Islam 2014). In the present study, the experiment was carried out to assess the occurrence of phytochemicals present in *Vanda tessellata* and to evaluate the in vitro antibacterial properties of various solvent extracts of the leaves and flowers of this orchid.

24.1.1 Plant Material and Solvent Extraction

Leaf and flower samples of *Vanda tessellata* were collected from the Hijli Forest, Ranaghat, West Bengal, India.

The plant parts, such as the leaves (Fig. 24.1) and flowers, were washed thoroughly with tap water, shade dried for 14 days, powdered and stored in air tight container. Thirty grams of leaf powder was mixed with 100 ml of its solvent in an Erlenmeyer flask. The solvents used were benzene, chloroform, acetone, methanol, ethanol and water. The mixture was placed undisturbed for three days at room temperature. Each mixture was stirred every 24 h in a rotary shaker for perfect mixing. Extracts were filtered by using Whatmann no.1 filter paper, and filtrate was evaporated under reduced pressure by rotary evaporator to give a semi-solid residue. The flower extract was prepared in the same way as mentioned above and used for antibacterial activity. The percentage yield was obtained using the following formula:

$$W_2 - W_1 / W_0 \times 100,$$

where W_2 is the weight of the extract and the container, W_1 is the weight of the container alone, and W_0 is the weight of the initial dried sample.

Fig. 24.1 Leaves of *V. tessellata*

24.1.2 Bacteria and Antibacterial Activity

For this experiment, some Gram-positive bacteria were used, viz. *Bacillus subtilis* MTCC441, *Micrococcus luteus* MTCC1538, *Staphylococcus aureus* MTCC 3160, *Bacillus cereus* MTCC6629. These bacterial stains were maintained on nutrient agar slant at 4 °C and subcultured for 24 h before use. Gram-negative bacteria exploited here were *Escherichia coli* MTCC443, *Pseudomonas aeruginosa* MTCC2581, *Shigella dysenteriae* (clinically isolated), *Shigella flexneri* MTCC1457, *Vibrio cholerae* MTCC3904, *Salmonella typhi* (clinically isolated), *Salmonella enterica* MTCC3219, and *Serratia marcescens* MTCC97. The pure strains were obtained from IMTECH, Chandigarh.

24.1.3 Phytochemical Tests (Qualitative)

Preliminary phytochemical analysis was conducted using standard methods for the determination of phytoconstituents, viz., alkaloids, tannins, flavonoids, anthocyanins, saponins, carbohydrates, terpenoids, and cardiac glycoside (Sofowora 1993; Harborne 1998; Nuduwake et al. 2007; Rani et al. 2013).

Sterile nutrient broth was inoculated with freshly isolated bacterial culture and incubated for 24 h at 37 °C. The bacterial suspension was found to be approximately 10^7–10^8 cells/ml. After the incubation period, they were used as inocula. About 0.1 ml of suspension containing 10^8 CFU/ml of bacterial strengths was taken into study by agar diffusion method (Estevinho et al. 2008). About 500mg dried plant material was used for 1 ml extract, and the inhibition zones were recorded after 24 h and compared with each solvent, which was negative control.

24.2 Phytochemical Constituents and Antibacterial Activities in *Vanda tessellata*

Successive isolation of bioactive compounds from plant material is dependent largely on the type of solvent used in extraction procedure. The present study showed that the ethanolic plant extract (Fig. 24.2) was provided with a good zone of inhibition, while other extracts had been found to be less active against the tested bacteria (Figs. 24.3 and 24.4). Except ethanolic flower extract, the other extracts of *V. tessellata* showed activity against *Micrococcus luteus.* Water extract was found to have less activity against all the tested bacteria (Table 24.1). Phytochemical screening of this plant parts showed that secondary metabolites, such as alkaloids, terpenoids, flavonoids, phenols, tannins, steroids, and glycosides were present (Table 24.2). Alkaloids, glycosides, tannins, saponins, sitoserols, long-chain aliphatic compounds, and fatty oil have been isolated from other species of *Vanda* (Anwar et al. 2013).

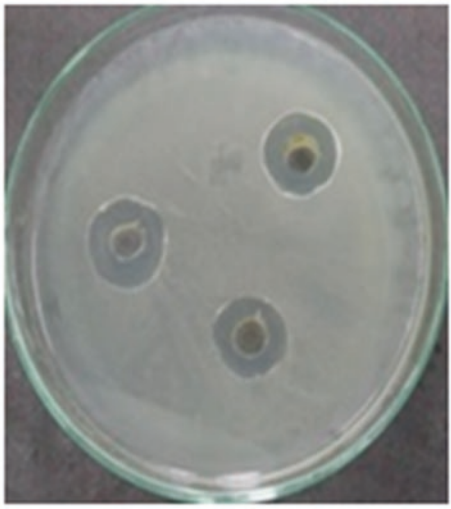

Fig. 24.2 Antibacterial activity of ethanol leaf extract against *M. leuteus*

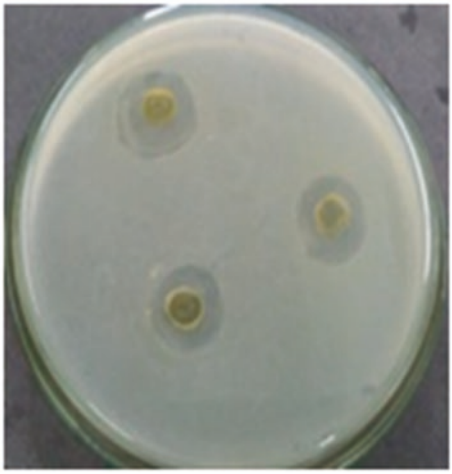

Fig. 24.3 Antibacterial activity of methanol leaf extract against *E. coli*

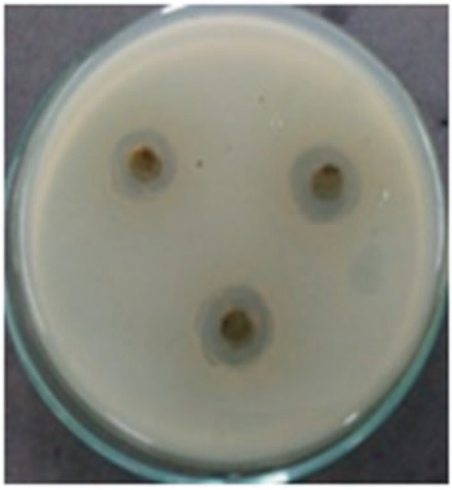

Fig. 24.4 Antibacterial activity of chloroform flower extract against *S. typhi*

Table 24.1 Antibacterial activity of different solvent extracts of *Vanda tessellata* collected from Nadia district, West Bengal

Plant parts	Solvents	Zone of inhibition (mm)											
		Ec	*Vc*	*Sd*	*Sf*	*Sm*	*St*	*Se*	*Pa*	*Bs*	*Bc*	*Ml*	*Sa*
Leaves	Benzene	16	12	11	0	9.3	8.7	10.3	12.8	0	13.4	10.3	10.3
	Chloroform	12	12.9	10	17	11.2	14.4	12.2	14.1	12	15.3	17.2	17.2
	Acetone	14	14.5	13	18.5	10.7	11.5	9.3	0	8	8.7	11.2	11.2
	Methanol	19.6	17.5	13.5	17.5	11.2	12	11.9	13.5	0	3.5	19.5	19.5
	Ethanol	17.5	21.5	14.6	13	12.5	13.7	12.7	12.3	10.7	17.5	24.4	24
	Aqueous	0	12	11	0	8.7	9.3	7.1	11	0	10.4	12.3	12.3
Flower	Benzene	9	0	0	12.3	7.4	8.9	10.3	0	8.4	8.7	0	13.9
	Chloroform	15	12	14	14.9	12.7	13.7	9.4	11.2	12.5	11.2	0	14.7
	Acetone	12	0	0	13.7	9.3	10.5	8.1	8.7	10.2	9.9	0	11.8
	Methanol	16	16.7	0	18.5	18.5	15.2	12.7	10.5	13.9	12.7	0	18.2
	Ethanol	14.5	18	12.5	15.4	16.6	17.8	13.3	14	11.7	13.3	13	16.7
	Aqueous	0	0	0	12	6.9	11.4	0	0	0	10.1	0	13.5

Ec Escherichia coli, *Vc Vibrio cholerae*, *Sd Shigella dysenteriae*, *Sf Shigella flexneri*, *Sm Serratia marcescens*, *St Salmonella typhi*, *Se S. enterica*, *Pa Pseudomonas aeruginosa*, *Bs Bacillus subtilis*, *Bc Bacillus cereus*, *Ml Micrococcus luteus*, *Sa Staphylococcus aureus*

Table 24.2 Qualitative tests for the phytochemicals of different parts of *Vanda tessellate* collected from Nadia district, West Bengal

Phytochemical screening	Leaves	Flowers
Alkaloids	+	+
Amino acids	–	–
Anthocyanins	+	+
Anthraquinones	+	+
Carbohydrates	–	–
Cardiac glycosides	+	+
Coumarins	+	+
Flavonoids	+	+
Flavonones	+	+
Glycosides	+	+
Tannins	+	+
Terpenoids	+	+
Saponins	+	+
Phenols	+	+
Reducing sugar	–	–
Steroids	–	–
Quinones	–	–
Phalobatannin	+	+

+ indicates presence or positive reactions, and –indicates absence or negative reaction

24.3 Conclusion

The present work makes the plant a candidate for bioprospecting for antibiotics. Results also support the folkloric usage of this plant as a therapeutic agent. Further phytochemical studies were required to determine the type of compound responsible for antibacterial activity, which could serve as a useful source for new antibacterial agent.

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Screening of Bioactive Phytochemicals in Some Indigenous Epiphytic Orchids of Bangladesh

25

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Abstract

This investigation portrayed phytochemical screening, antioxidant, anti-inflammatory, antimicrobial, cytotoxic and analgesic activities of three medicinally important epiphytic orchids of Bangladesh, namely *Rhynchostylis retusa* (L.) Bl., *Luisia zeylanica* Lindl. and *Papilionanthe teres* (Roxb.) Schltr. The methanolic extracts of the leaves and roots of *R. retusa* and the leaves and stems of *L. zeylanica* and *P. teres* were used in the present investigation. Quantitative phytochemical screening revealed the presence of a number of bioactive phytochemicals, such as alkaloids, carbohydrates, flavonoids, glycosides, tannins, phenols, xanthoproteics, proteins, saponins, cardiac glycosides, coumarins, quinines, flavonols, steroids, terpenoids, phytosterols, phlobatannins etc. Antioxidant activity of the selected orchids showed significant DPPH free radical scavenging activity compared with that of ascorbic acid at 100 µg/ml. Excellent anti-inflammatory activity was also showed in these orchids, and the highest anti-inflammatory activity was recorded in *P. teres* (88.22%). Total phenol content was measured by using Folin-Ciocalteu reagent gallic acid was used as a reference standard. The maximum total phenol content was 205.33 mg/g GAE in *L. zeylanica*, and the minimum was 36.67 mg/g GAE in the roots of *R. retusa*. Antibacterial activity of methanolic extracts was tested against *Bacillus subtilis*, *Staphylococcus aureus, Salmonella typhi, Pseudomonas aeruginosa* and *Escherichia coli*. The maximum antibacterial activity was observed against *Bacillus subtilis.* The antifungal activity was also studied against *Aspergillus niger* and *Fusarium oxysporum*, and the highest antifungal activity was recorded against *Aspergillus niger* (70%) by *P. teres*. In brine shrimp lethality bioassay, encouraging cytotoxicity was observed after 24 h, and the maximum mortality of nauplii was recorded in *L. zeylanica* (LC50 value 77.175 µg/ml) as compared with anticancer drugs 'vincristine sulphate' (LC50 value 0.25 µg/ml). In analgesic

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assay, remarkable inhibitory effect was observed in acetic-acid-induced writhing test on mice by the extract of *P. teres* (86.19%). The percentage of paw licking inhibition was high in the late phase than in the early phase. The findings of the present investigation will facilitate the designing of new drugs to comate challenging diseases.

Keywords
Phytochemicals · Antioxidant · Cytotoxicity · Antimicrobial · Analgesic · Orchid

25.1 Introduction

Orchids belong to the largest and most evolved family of the flowering plant Orchidaceae, which consist of 25,000–35,000 species fewer than 750–850 genera (Dressler 1993; Singh 2001; Tsavkelova et al. 2001). Orchids are the doyen among ornamentals, attributed outstanding royalty in the world floricultural market considered as one of the most important global cut flower and potted floricultural crop. Apart from their ornamental value, many orchids have high medicinal and glycosidal importance (Hossain 2011). Orchids are a prominent source of bioactive compounds and serve as an important raw material for drug production. It is well known that orchids synthesise and accumulate a number of secondary metabolites generally categorised as alkaloids, flavonoids, carotenoids, anthocyanins, phenols, and sterols, which are present in the leaves, pseudobulbs, roots or flowers of the plant or in the entire plant. Among the different phytochemicals reported from orchids, alkaloids and flavonoids are the most important phytochemicals because of their biological properties.

Orchids have always appeared mysterious and have therefore been the object of intrigue and curiosity of the people for centuries. Ancient cultures believed that magical cures for all sorts of ills could be made from the roots. The use of orchids as health care commodity has a history of thousands year. Most probably the Chinese were the first to use orchids as a source of herbal remedies in 2800 B.C. (Kimura and Migo 1936; Lüning 1974). Since the *Vedic* period (2000 B.C.–600 B.C), some orchids have also been used by the Indians for their curative and aphrodisiac properties (Kaushik 1983). In the Indian *Vedic* scriptures *Sushruta Samhita*, there is mention of orchid under the name *Vanda*. Some parts of Europe, America, Australia and Africa have also been using orchids as herbal cure for a long time.

Incredible diversity, high floricultural and medicinal value, research on orchids is full of potential. However, the fact that orchids could play an important role in herbal medicines is often overlooked (Hossain 2009, 2011). Although a number of orchids are a trustworthy source for bioactive phytochemicals, only a few of them have been investigated for their biological function; others are still unknown. Studies of orchid alkaloids date back to 1892, when E. de Wildeman begun the investigation of orchid alkaloids in some European orchid species. E de Droog analysed 104 species in 78 genera by 1896, and W. Boorsma of the Bogor Botanical

Gardens started exclusive studies on orchid alkaloids in the late 1890s (Ardittii 1992). The first orchid alkaloid isolated in the year 1932 from a Chinese drug 'Chin-Shi-Hu' prepared from *Dendrobium nobile* was named dendrobine (Bhattacharjee 2006). After World War II, Lüning and his associates in Sweden, Lawler and Slaytor in Australia and several scholars in Japan studied the alkaloids in orchids (Lüning 1974; Slaytor 1977). Professor Dodson at the University of Miami was perhaps the first and certainly the most influential scientist who carried out remarkable research on orchid alkaloids (Kong et al. 2003). To date, more than 2000 orchid species have been screened for their alkaloid and/or flavonoid content. In the last three decades, extensive studies on orchids have been carried out, and a number of bioactive compounds were isolated, such as dihydrophenanthrene, ephemeranthoquionone, triterpedoids, bibenzyl derivatives, shihunidine, shihunine, dendrophenol, moscatilin, moscatin, denfigenin, defuscin, amoenumin, cypripedin, crepaditin, rotundatin, cumulatin, gigantol, orchinol, hircinol jibantine, nidemin, loroglossin etc. (Majumder and Sen 1987; Majumder and Chatterjee 1989; Majumder and Pal 1992; Yamaki and Honda 1996; Honda and Yamaki 2000; Krohn et al. 2001; Lo et al. 2004; Zhang et al. 2007; Li et al. 2008). Nearly 100 alkaloids from Orchidaceae have already been isolated, but from chemical considerations, the most important ones are dendrobine, nobiline and nobilonine (Hossain 2011, 2015). Unfortunately, research on the commercial extraction, purification and scientific evaluation of bioactive phytochemicals from orchids is still lacking, except for 'vanilla' from *Vanila planifolia*. Therefore, it is very important to investigate phytochemicals with pharmacological properties and have potential pharmaceutical value to extend the scope of therapies. With the progress of civilisation and the development of human knowledge, orchids have become an important source of medicine for herbal treatment. But being a large group of angiosperms, orchids are ignored in Bangladesh. The screening of bioactive phytochemicals and the study of antimicrobial, antioxidant, anti-inflammatory, analgesic, and cytotoxic activities of medicinally important orchids are very important for new drugs designing to combat diseases. Taking into consideration the above importance, the purpose of the present research program is to investigate the bioactive phytochemical constituents and determine the quantitative value of the methanolic extract of the leaves and roots of *Rhynchostylis retusa* (L.) Bl. and the leaves and stems of *Luisia zeylanica* L. and *Papilionanthe teres* (Roxb.) Schltr. A comprehensive studies were made on antioxidant and anti-inflammatory activities; the determination of antimicrobial (antibacterial and antifungal) activities, and a study of the analgesic and cytotoxic properties of these orchids. The outcome of the present study will help in the discovery and design of new drugs to combat challenging diseases.

Three indigenous orchid species (Fig. 25.1), namely *Rhyncostylis retusa* (L.) Bl., *Luisia zeylanica* L. and *Papilionanthe teres* (Roxb.) Schltr., were collected from the hilly forests of Chittagong in Bangladesh, and the leaves, stems and roots were used for phytochemical investigation.

The plant materials were washed thoroughly under running tap water and oven dried at 60 °C for 72 h. It was then ground into coarse powder by a grinding machine and stored in an airtight container for further study. Twenty-five gm of powder of

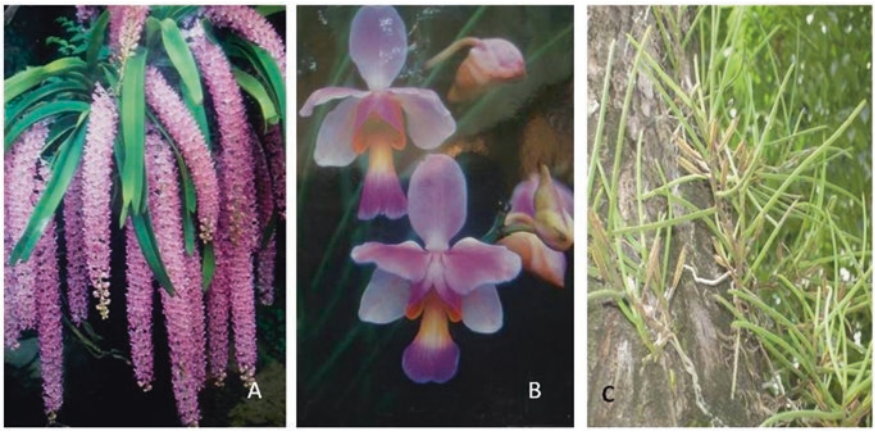


Fig. 25.1 (**a**) *Rhynchostylis retusa* (L.) Bl., (**b**) *Luisia zeylanica* Lindl. and (**c**) *Papilionanthe teres* (Roxb.) Schltr

each plant part was taken in a conical flask with 50 ml methanol, shaken for 30 min and kept overnight, and then shaken again and sonicated for 10 min and filtered through Whatman filter paper no 1. The crude extract was concentrated through rotavaporated under reduced pressure and 40–51 °C temperature by a cyclone vacuum evaporator, dried and used for chemical analysis.

25.2 Quantitative Test for Phytochemicals

For quantitative tests of alkaloids, phlobatannins, flavonoids, saponins, tannins, terpinoids, steroids, glycosides, quinines, coumarins, phytosterols, carbohydrates, cardiac glycosides, flavonones, proteins, xanthoproteics, tannic acids, amino acids and phenols, standard procedures described by Sofowara (1993), Trease and Evans (1989) and Harbrone (1973) were applied. The quantity index of the presence of alkaloid was expressed by '+', '++' and '+++' signs, which mean slight, moderate and significant amount, respectively. No precipitation was indicated by '–' (negative sign).

25.2.1 Test for Alkaloids

For the qualitative testing of alkaloids, the most reliable and rapid testing method described by Aplin and Cannon (1971) was applied with slight modification. Five alkaloid detecting reagents, such as Dragendroff's reagent (D), Hager's reagent (H), Mayer's reagent (M), Wagner's reagent (W) and tannic acid reagent (T), were used. Five grams of fresh and finely pasted plant material was mixed with 10 ml 2% HCl and heated in water bath of 60 °C for one hour. After cooling, the extract was filtered through Whatman no. 1 filter paper. Two drops of concentrated crude extract with one drop of alkaloid detecting reagent were taken on a microscopic groove slide, and relative abundance of precipitation was observed.

25.2.2 Phlobatannin Test

The method for the determination of phlobatannins was followed by Edeoga et al. (2005). Crude extract with 1% aqueous hydrochloric acid was boiled. Deposition of red precipitation was considered evidence of the presence of phlobatannins.

25.2.3 Flavonoid Test

Five grams of powdered plant sample was heated with 10 ml ethyl acetate over a stem bath for 3 min. The mixture was then filtered and added 1–2 ml of dilute ammonia solution and shaken well. A yellow colouration was observed, indicating a positive test for flavonoids (Edeoga et al. 2005).

25.2.4 Saponin Test

Two-gram powdered plant sample with 20 ml of distilled water was boiled in a water bath and filtered. Thereafter, 10 ml of filtrate was mixed with 5 ml of distilled water and shaken vigorously to produce froth. The persistence of foam indicated the presence of saponins (Kapoor et al. 1969).

25.2.5 Quinine Test

One millilitre of concentrated plant extract and 1 ml of concentrated sulphuric acid were mixed togather and allowed some times to develop colour. The development of a red colour indicated the presence of quinine.

25.2.6 Tannin Test

About 5 g of powdered plant sample with 10 ml of distilled water was taken in a test tube, boiled and filtered. A few drops of ferric chloride reagent were added to the filtrate. Blue black precipitate was observed, which indicated the presence of tannins (Harbrone 1973).

25.2.7 Phytosterol Test

Fifty ml concentrated plant extract was dissolved in 2 ml of acetic anhydride and then 1 or 2 drops of conc. Sulphuric acid was added. An array of colour change indicated the presence of phytosterol.

25.2.8 Tannic Acid Test

A few millilitres of plant extract were added in 5 ml 10% of tannic acid. White precipitate indicated the presence of tannic acid.

25.2.9 Terpenoid Test

Exactly 0.5 g of crude powder was dissolved in 5 ml of methanol, and then the extract was treated with 2 ml of chloroform in a test tube. Seven millilitres of concentrated sulphuric acid was added carefully to the mixture to form a layer. An interface with a reddish brown colouration formed, indicating the presence of a terpenoid constituent.

25.2.10 Xanthoproteic Test

A few millilitres of plant extract were added in 1 ml of conc. Nitric acid. White precipitate was observed and boiled and cooled. Then 20% NaoH was added. The presence of orange colour indicated the presence of aromatic amino acids.

25.2.11 Proteins (Biuret Test)

One-two ml of concentrated plant extract, 1 ml of 40% NaOH solution and 2 ml of 1% copper sulphate solution were taken in a test tube. Violet colour developed, which indicated the presence of proteins.

25.2.12 Carbohydrate (Fehling's Test)

About 1 mg sample was dissolved in 2 ml of distilled water. The mixture was heated with 5 ml of equal volume of Fehling's A and B solution. Brick red precipitate indicated the presence of carbohydrates.

25.2.13 Glycosides Test

Crude powder of 0.5 g was dissolved in 5 ml of methanol. Ten millilitres of 50% HCl was added to 2 ml of methanolic extract in a test tube. Then it was heated in a boiling water bath for 30 min. Five millilitres of Fehling's solution was added to the mixture, and the mixture was boiled for 5 min. A brick red precipitate was taken as evidence for the presence of glycosides.

25.2.14 Cardiac Glycoside Test

Two millilitres of plant extract, a few millilitres of glacial acetic acid, ferric chloride and conc. Sulphuric acid were taken in a test tube. Green colour indicated the presence of cardiac glycosides.

25.2.15 Coumarin Test

With 1 ml of extract, 1 ml of 10% NaoH was added and allowed to stand for some time to develop yellow colour, which indicated the presence of coumarin.

25.2.16 Steroid Test

In a test tube 1 ml methanolic plant extract was dissolved in 10 ml chloroform, and equal volume of conc. Sulphuric acid was added. The upper layer turned red, and sulphuric acid layer showed yellow with green fluorescence. This indicated the presence of steroids.

25.2.17 Determination of Phenolic Content

The total concentration of phenolic compound (TPH) in the plant extracts was determined by means of the Folin–Ciocalteu method, as described by Singleton et al. (1999). The Folin–Ciocalteu reagent (FCR) is a mixture of phosphomolybdate and phosphotunstate used for the colorimetric assay of phenolic and polyphenolic antioxidants. The gallic acid (GA) was used as a reference standard. The reaction mixture was prepared by mixing 0.5 ml of methanolic extract, 2.5 ml of 10% FCR and 2.5 ml of 7.5% of $NaHCO_3$, and the control (blank mixture) was prepared with all these chemicals except plant extract. The mixture was then incubated in a thermostat at 45 °C for 45 min. The absorbance of the resulting mixture was measured using a spectrophotometer at 765 nm against blank. Total phenol contents in the plant extract was estimated by the following formula:

$$\text{total phenolic content}, C = \frac{(c \times V)}{m}$$

Here,

C = total phenol content mg/g plant extract in GAE,
c = concentration of gallic acid established from the calibration line,
V = volume of extract,
m = weight of pure plant extract.

25.2.18 Determination of Antioxidant Activity

The free radical scavenging activity of the methanolic extract of the selected orchids and of standard reference solution (ascorbic acid) were investigated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging method described by Kumarasamy et al. (2007). An aliquot of 1.5 ml of 0.05% DPPH solution and 0.1 ml of extract or standard (ascorbic acid) at various concentrations (50, 100, 150, 200 and 250 µg/ml) were mixed. The mixture of DPPH and ascorbic acid or DPPH and extracts was shaken vigorously and incubated for 50 min in the dark at room temperature. DPPH is found as dark-coloured crystalline powder composed of stable free-radical molecules and forms deep violet colour in solution. The scavenging of DPPH free radical (neutralisation) is indicated by the deep violet colour being turned into pale yellow or colourless. The decolourisation of DPPH was determined by measuring absorbance at 517 nm using a spectrophotometer. Ethanol serves as blank. The results were expressed as IC50 value in µg/ml. The percentage inhibition activity was calculated by using the following formula:

$$\%\,\text{DPPH free radical scavenging activity} = \frac{A_c - A_s}{A_s} \times 100$$

Where, A_c = Absorbance of control
A_s = Absorbance of sample or standard

25.2.19 Determination of Anti-inflammatory Activity

Anti-inflammatory activity was assessed through the method of Mizushima and Kobayashi (1968) with slight modification. For the preparation of acetyl salicylic acid solution, 10 mg acetyl salicylic acid (Dispirin CN 100 Reckitt Benckiser, Aspirin 100 mg/tablet were used) was dissolved in 100 ml double-distilled water (DDW). Similarly, 900 mg NaCl was dissolved in 100 ml DDW to prepare iso-saline, and 5 ml of egg albumin was added in 95 ml of iso-saline to prepare 5% egg albumin solution. Two ml of each plant extract and 2 ml 5% egg albumin solution was taken in a separate test tube and used as test groups. Besides 2 ml of 5% egg albumin and 2 ml ethanol was used as negative control while 2 ml acetyl salicylic acid with 2 ml of 5% egg albumin was used as positive standard control. The pH of the reaction mixtures was adjusted at 5.6 ± 0.2 by 1 N HCl. All the reaction mixtures were heated at 57 °C for 20 min. After cooling and filtering, absorbance was measured spectrophotometrically at 660 nm. Anti-inflammatory activity was calculated by using the following formula:

$$\%\,\text{of inhibition} = 1 - \frac{A}{B} \times 100$$

where

A = absorbance of control (5% egg albumin and ethanol),
B = absorbance of test group (5% egg albumin and plant extract), or.
B = absorbance of standard solution (5% egg albumin and acetyl salicylic acid).

25.3 Antimicrobial Tests

25.3.1 Antibacterial Activity Test

Antibacterial sensitivity was carried out by disc diffusion method (Baufr et al. 1966). For this purpose, 2 g of sterilised plant sample was added in 10 ml of pure methanol and kept for 24 h and grounded well by mortar and pestle and filtered through Whatman paper no.1. After settling, the supernatant was collected and used for antimicrobial screening. Four human pathogenic and two other bacteria of both Gm^{+ve} and Gm^{-ve} group, namely *Salmonella typhi* (Schroeter) Warren & Scott. (Str. ATCC 19430), *Salmonella paratyphi* (ex Kauffmann & Edwards) Le Minor & Popoff (ATCC 12176), *Bacillus cereus* (Frankland & Frankland) (ATCC 14579), *Bacillus subtilis* (Ehrenberg) Cohn. (ATCC 19659), *Pseudomonas aeruginosa* and *E. coli* (ATCC25925), were selected for the study. The bacterial cultures were incubated at 35 ± 2 °C for proper growth. The bacterial suspensions were prepared through gradual dilution technique, and inoculum concentration was determined by OD. Ampicillin standard discs were used as positive control, while empty discs soaked only with the solvent were treated as negative control. Sample discs (Whatman filter paper, grade 17, 8 mm diameter) were prepared with dipping in 100 mg/ml, 200 mg/ml and 300 mg/ml concentrated plant extract and air dried. The sample discs, the standard antibiotic discs and the control discs were placed gently on the marked zones in the bacterial plates. The antibacterial activities were expressed in mm of the diameter of inhibition zone surroundings the discs with compared to control.

25.3.2 Antifungal Activity Test

Antifungal activity was determined using the poisoned food technique on potato dextrose agar (PDA) plate at 28 °C by means of the point inoculation method, as reported by Shweta et al. (2015). The preparation of plant extracts was similar to the antimicrobial sensitivity test. Two fungal species, namely *Aspergillus niger* and *Fusarium oxysporum*, were used for this purpose. The test fungi were inoculated at the centre of control (without extract) and poisoned PDA plates (1 ml extract per ml PDA). The plates were left for four days at 24–28 °C, and radial increment of the colonies in both control and poisoned plates was measured. Antifungal activity, in terms of inhibition of the mycelial growth of test fungi using the extracts of selected orchids, was determined based on the following formula:

$$\text{inhibition of mycelial growth } (\%) = \frac{(C - T)}{C} \times 100$$

Where, C = colony diameter on control and T = colony diameter on poisoned plates.

25.3.3 Cytotoxicity Assay

Brine shrimp (*Artemia salina*) lethality assay was conducted for cytotoxicity test followed by Meyer et al. (1982). For the preparation of artificial sea water/brine, 38 g of pure NaCl (iodine free as it is lethal to shrimp nauplii) was dissolved in distilled water to make volume of 1 L. Shrimps were used at small in size, when they are just hatched from the eggs, pale brown or light yellow in colour. Shrimp eggs were added to brine water taken in a 1000 ml beaker, and constant oxygen was supplied by pump machine and incubated at room temperature. After 48–72 h, the eggs hatched.

Five test tubes each conataining 100, 250, 500, 800 and 1000 µl of each plant extract with 5 ml sea water and 10 shrimp nauplii were taken. Vincristine sulphate was taken as standard, and the results were expressed as LC50 in µg/ml. After 24 h, the percentage mortality of brine shrimp nauplii was determined from the number of dead nauplii. LC50 was calculated for selected extracts by treating nauplii with five different concentrations. The percentage of mortality of nauplii was calculated for each concentration by the following equation:

$$\% \text{ mortality} = \left[\frac{\left(N_0 - N_1\right)}{N_0} \right] \times 100$$

Here, N_0 = number of nauplii taken and N_1 = number of nauplii dead.

25.4 Determination of Analgesic Activity

Acetic-acid-induced writhing test was conducted on BALB/c mice of both sexes at 25–30 g weight for analgesic activity assessment according to the method developed by Dubuisson and Dennis (1977). The mice were kept under laboratory condition for 2 weeks for acclimatization providing normal food before starting the experiments and were handled in accordance with the animal study ethical guideline. All the mice were kept away from food for two hours before starting the experiment and were divided into four groups. Group I was injected with normal saline (10 ml/kg) as control, group II received the standard drug 'diclofenac sodium' (20 mg/kg), while the remaining groups III and IV were injected with plant extract 200 and 400 mg/kg weight of mice. After 30 min of injection, the animals were treated with 1% acetic acid. The number of writhing was counted for 10 min after 5 min of acetic acid injection. Antinociceptive activity was expressed as inhibition percent, and the percentages of inhibition were calculated by the following formula:

$$\% \text{inhibition} = \left[\frac{\left(A - B\right)}{A} \right] \times 100$$

where, A = average number of writhing of control group and B = average number of writhing of test groups.

25.5 Phytochemical Screening

The presence of a number of secondary metabolites like alkaloids, flavonoids, phlobatannins, steroids, tannins, saponins, terpenoids, proteins, amino acids, cyanogenic glycosides, xanthoproteics, carbohydrates, tannic acids, flavonols, quinines, coumarins, phytosterols, phlobatannins, phenols and glycosides was confirmed in selected epiphytic orchids of Bangladesh, namely *Rhynchostylis retusa* (L.) Bl., *Papilionanthe teres* (Roxb.) Schltr. and *Luisia zeylanica* Lindl. The methanolic extracts of the selected plants were subjected to qualitative analysis. The presence of different secondary metabolites in the extract of test plants was expressed by a '+' sign, signifying their presence in degrees (from '+' as the minimum to '+++' as the highest quantity). The absence of secondary metabolites was denoted by a '–' sign. Colour intensity was used as analytical responses to these tests. The summary of these tests are given in Table 25.1.

25.5.1 Alkaloids

Alkaloids were assessed qualitatively with five different alkaloid detecting reagents. The methanolic extract of leaves *R. retusa* showed the highest response in Dragendroff's reagent and tannic acid; a moderate response showed in Wagner's reagent, while the lowest response was observed in Hager's reagent and Mayer's reagent. Similarly, the root extract of *R. retusa* showed the highest response in tannic acid and a moderate response in Dragendroff's and Wagner's reagent, while in other reagents it showed the lowest results. The methanolic extracts of leaves and stems of *L. zeylanica* showed the highest results in tannic acid and Wagner's reagent, a moderate result in Mayer's reagent and the lowest results in other reagents. In the case of *P. teres*, the methanolic extracts of leaves and stems showed the highest result in Dragendroff's reagent, Hager's reagent and tannic acid and a moderate result in Mayer's reagent. These findings indicated the degree of presence of alkaloids in different plant parts also.

25.5.2 Steroids, Tannins, Xanthoproteics and Flavonoids

Both the leaves and root extracts of *R. retusa* showed the highest result in steroid, tannin and xanthoproteic tests and a moderate result in cardiac glycoside, glycoside, phlobatannin, flavonoid, carbohydrate and terpenoid tests. In the case of *L. zeylanica*, the highest result was recorded in steroid and xanthoproteic tests and a

Table 25.1 Qualitative chemical estimation of the methanolic extracts of the leaves and roots of *Rhyncostylis retusa* and the leaves and stems of *Luisia zeylanica* and *Papilionanthe teres*

Sl. No.	Name of the test	Specific test name	*R. retusa* (leaf)	*R. retusa* (root)	*L. zeylanica* (leaf & stem)	*P. teres* (leaf & stem)
1.	Alkaloid test	Dregendroff's reagent test	+++	++	+	+++
		Hagger's reagent test	+	+	+	+++
		Mayer's reagent test	+	+	++	++
		Wagner's reagent test	++	++	+++	+++
		Tannin acid test	+++	+++	+++	+++
2.	Carbohydrate test		++	++	+	+
3.	Flavonoid test	Alkali test	++	+	++	+
		Conc. H_2SO_4 test	+	+	+	+
4.	Terpenoid test		++	++	++	++
5.	Steroid test		+++	+++	+++	+++
6.	Saponin test	Foam test	+	+	+	+
7.	Protein test		+	+	+	+
8.	Phenol test		+	+	+	+
9.	Phytosterol test		+	+	++	++
10.	Glycoside test		++	+	+	+
11.	Cardiac glycoside test		++	++	+	+
12.	Flavonol test		+	+	+	+
13.	Coumarin test		+	+	+	+
14.	Quinine test		++	++	+	+
15.	Amino acid test		+	+	+	+
16.	Tannin test	Ammonia test	++	++	++	++
		$K_2Cr_2O_7$ test	+++	+++	+++	+++
17.	Xanthoproteic test		+++	+++	+++	+++
18.	Tannic acid test		+	+	+	++
19.	Phlobatannin		++	+	+	+

['+' represents minimum, '++' represents moderate and '+++' represents the highest degree of presence]

moderate result in phytosterol, flavonoid, tannin, carbohydrate and terpenoid tests. On the other hand, *P. teres* showed the highest result in steroid and xanthoproteic tests and a moderate result in phytosterol, tannin, carbohydrate and terpenoid tests (Table 25.1).

The quantity of the secondary metabolite contents in the extract of test plants and/or their organs varied species to species and their organs. Many of these phytochemicals have potential pharmaceutical value. *L. zeylanica* was first described in

Ayurvedic texts as early as 1500 BC as being used for the preparation of oil to treat fracture (Cooray 1940). *R. retusa* was used for asthma, tuberculosis, cramps, kidney stone, rheumatism and menstrual disorder (Dalgado 1898), while *P. teres* was used for bronchitis and diseases of the nervous system. Although these plants are widely used in folk medicine in Bangladesh and some other parts of the world, their cytotoxicity, phenolic contents and antinociceptive activities have not been investigated, and no precise scientific proof of pharmaceutical properties and therapeutic applications were reported. Recently, a phytochemical investigation by Amin et al. (2011) reported the presence of promising nutritional, pharmaceutical and cosmaceutical bioactive constituents in some indigenous orchids of Bangladesh.

Medicinal plants produce a vast array of therapeutically important secondary metabolites of the classes, alkaloids, terpinoids and phenols (Hess 1975; Goodwin and Mercer 1986; Hopkins 1999). They have strong physiological activities in the animal systems. For this reason, plants containing secondary metabolites are very important to us as a potential ingredient for herbal and many modern drugs. Orchids may play an important role in this sector as it is a prominent source of different bioactive phytochemicals such as alkaloids, flavonoids, tannins and glycosides (Radhika et al. 2013; Shrestha et al. 2015).

Alkaloids are heterocyclic nitrogenous compounds which are basic in nature. These are naturally synthesised in plant body, have antibacterial and antifungal activities are significant for the protection and survival of plant. Some alkaloids have stimulant property as caffeine and nicotine, morphine is an important alkaloid which is used as analgesic and while quinine is used as antimalarial drug.

Glycosides are molecules in which sugar is bound to a non-carbohydrate moiety, usually a small organic molecule. Many glycosides are used as cardiac drugs, laxatives, analgesics or anti-rheumatic, anti-inflammatory and anticancer drugs. Tannins bind to proline-rich proteins and interfere with protein synthesis. They have also been reported to have antiviral effects. Tannins can also be effective in protecting the kidneys. Tannins have been used for the immediate relief of sore throats, diarrhea, dysentery, hemorrhage, fatigue and skin ulcers and as a cicatrizant on gangrenous wounds. Flavonoids are hydroxylated phenolic substance known to synthesise by plants in response to microbial infection. It may be used as an antimicrobial substances against wide array of pathogenic microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. They show anti-allergic, anti-inflammatory, antimicrobial (Cushnie and Lamb 2005) and anticancer activity. Coumarins are also known to act against gram-positive bacterial and fungal infections. Terpenoids are known for anti-inflammatory, lipolytic and anti-cholesteremic activities. It has also been observed and accepted that the medicinal value of plant lies in the bioactive phytocomponents present in the plants. Saponins are glycosides of both triterpenes and steroids, which are characterised by their bitter or astringent taste, foaming property, haemolytic effect on red blood cells and cholesterol binding properties. Saponins have been shown to possess both beneficial (lowering cholesterol) and deleterious (cytotoxic and permeabilisation of intestinal epithelium) properties and to exhibit structure-dependent biological activity. In medicine, it is used to some

extent as an expectorant and as an emulsifying agent (Harbrone 1973). Steroid is a type of organic compound that contains a characteristic arrangement of four cycloalkane rings that are joined to each other. Examples of steroids include the dietary fat cholesterol, the sex hormone estradiol and testosterone and the anti-inflammatory drug dexanethasone. Quinine is a natural white crystalline alkaloid that has antipyretic (fever-reducing), antimalarial, analgesic (pain-killing) and anti-inflammatory properties and a bitter taste.

Maridass et al. (2008) made a qualitative phytochemical analysis of the leaf extracts of 27 genus and 61 species of the Orchidiaceae family, where 37 orchid species showed positive results in reducing sugar and other 21 species showed negative results to it. Among the selected orchids, 48 species contained cyanogenic glycosides, 45 species contained tannin and 14 species showed terepenoid content in their flowers. Thirteen species, such as *Anoectochilus elatus* Lindl., *Bulbophyllum neilgherrense* Wight., *B. tremulum* Wight, *B. xylophyllum* Par & Reichb.f., *Calanthe masuca* (D. Don) Lindl., *Cheirostylis flabellata* Wight, *Cymbidium ensifolium* (L.)., *Dendrobium macrostachyum* Lindl., *Epipogium roseum* (D. Don), *Eria reticosa* Wight, *Liparis atropurpurea* Lindl., *Malleolagracilis* (Lindl.), *Papilionanthe subulata* (Koen.) and *Vanda testacea* (Lindl.), did not show that they were positive in cyanogenic glycoside content. Only eight orchid species contained all types of flavonoids. Chimsook (2016) evaluated the phytochemical screening of *Dendrobium signatum* leaves and reported that carbohydrates, coumarins, alkaloids, flavonoids, phenolics, sterols and glycosides are prominent phytochemicals in orchids.

25.5.3 Total Phenolic Content

The total phenolic content in the methanolic extracts of the selected plant was estimated through the Folin–Ciocalteau method using gallic acid as the chemical standard. The values of total phenol content were expressed as mg GA/g of extracts and shown in Fig. 25.2. The phenolic content varied depending on plant species, as well as plant organs. The highest phenolic content was in the leaf and stem of *L. zeylanica* (205.33 ± 6.34 mgGA/g), followed by the leaf of *R. retusa* (64.67 ± 1.70 mgGA/g), whereas the lowest phenolic content was observed in the root of *R. retusa* (36.67 ± 3.30 mgGA/g).

Phenol is a group of organic compounds whose aromatic ring is bonded to an alcohol group. Phenolic content was investigated in *Dendrobium signatum*, *Vanda cristata* and *Geodorum acutifolius* (Chimsook 2016; Mukesh et al. 2016). This compound constitutes one of the most numerous and ubiquitously distributed groups of secondary plant metabolites, which ranges from simple molecules to highly polymerised compounds. Plants produce phenolic compounds for evolving defense mechanisms to counteract reactive oxygen species (ROS) in order to avoid oxidative damage. Phenolics are secondary plant metabolites ranging from simple structures with one aromatic ring to complex polymers such as tannins and lignins. The interests in phenolic compounds, particularly flavonoids and tannins, have considerably increased in recent years because of their broad spectrum of chemical and diverse

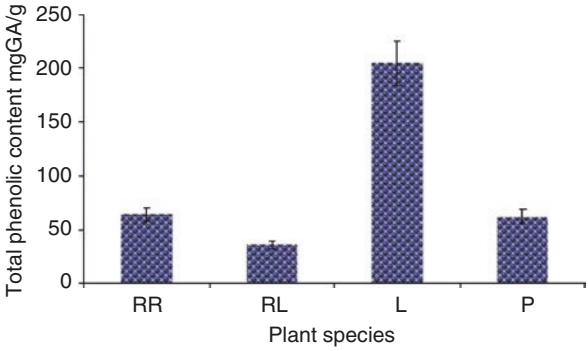


Fig. 25.2 Total phenolic content in *R. retusa*, *L. zeylanica* and *P. teres*

biological properties. In addition to their antioxidant properties, these compounds have been reported to be potential candidates in reducing cardiovascular diseases, anti-carcinogenic activities and anti-allergenic, anti-arthrogenic, anti-inflammatory, antimicrobial and antithrombotic effects. Plant phenolics, in particular phenolic acids, tannins and flavonoids, are known to be potent antioxidants and occur in vegetables, fruits, nuts, seeds, roots and barks. In the case of phenolic compounds, the ability of the phenolics to act as antioxidants depends on the redox potential of their phenolic hydroxyl groups, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers. In addition, they have a metal chelation potential. Extracts of orchids rich in phenolics are increasingly of interest in the food industry because they retard oxidative degradation of lipids and thereby improve the quality and nutritional value of food.

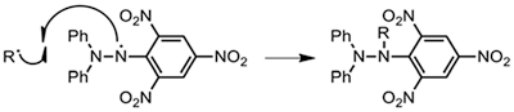
25.6 Antioxidant Activity

DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging activities of the methanolic extracts of the selected orchids were compared with the standard antioxidant ascorbic acid. Excellent scavenging activities were found in different concentrations of all the orchids studied (Table 25.2). Among the five different concentration used, the highest free radical scavenging activity from IC_{50} value was recorded in the leaf and stem extracts of *L. zeylanica* and *P. teres* 92.25% at 150 µg/ml and 82.23% at 100 µg/ml, respectively. Based on observation, it could be concluded that all plant extracts had moderate free radical scavenging activity as compared to ascorbic acid. The antioxidant activity was due to the presence of flavonoids, flavonols and phenols, which may be useful for treating radical-related pathological damage, especially at higher concentration. The methanolic extracts of the selected orchids possess strong DPPH free radical scavenging activity. DPPH acts as an electron acceptor (oxidant or oxidising agent) and causes oxidation of other substances. On the other hand, antioxidants act as an electron donor (reductant or reducing reagent). Antioxidants neutralise DPPH by being oxidised themselves. DPPH is found as dark-coloured crystalline powder composed of stable free-radical

Table 25.2 DPPH free radical scavenging activity of ascorbic acid, *R. retusa*, *L. zeylanica* and *P. teres* at different concentrations

Plant name	Concentration (µg/ml)	Scavenging activity (%)		Average ± SEM
		R_1	R_2	
Ascorbic acid (standard)	50	99.89	99.45	99.67 ± 0.22
	100	99.80	99.88	99.84 ± 0.04
	150	99.34	99.34	99.34 ± 00
	200	99.70	99.64	99.67 ± 0.03
	250	99.20	99.22	99.21 ± 0.01
R. retusa (leaf)	50	67.71	67.81	67.76 ± 0.05
	100	54.32	54.90	54.61 ± 0.29
	150	53.15	53.75	53.45 ± 0.30
	200	57.64	57.01	57.33 ± 0.36
	250	58.13	58.87	58.50 ± 0.37
R. retusa (root)	50	47.74	47.70	47.72 ± 0.02
	100	51.17	51.21	51.19 ± 0.026
	150	45.013	45.03	45.02 ± 0.01
	200	49.52	49.56	49.54 ± 0.02
	250	50.10	50.01	50.20 ± 0.05
L. zeylanica (leaf & stem)	50	87.699	87.743	87.72 ± 0.02
	100	88.89	88.85	88.87 ± 0.02
	150	92.20	92.30	92.25 ± 0.05
	200	87.81	87.77	87.79 ± 0.02
	250	89.25	89.75	89.50 ± 0.25
P. teres (leaf & stem)	50	81.35	81.65	81.50 ± 0.15
	100	82.40	82.06	82.23 ± 0.17
	150	78.10	78.28	78.19 ± 0.09
	200	80.60	80.24	80.42 ± 0.18
	250	81.00	81.02	81.01 ± 0.01

molecules and forms deep violet colour in solution. The scavenging of DPPH free radical (neutralisation) is indicated by the deep violet colour turning into pale yellow or colourless. An antioxidant is a type of compound or molecule that is capable of slowing or preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidising agent. Oxidation reaction can produce free radicals like reactive oxygen species (ROS), e.g. superoxide, hydroxyl, peroxyl and alkoxy radicals, which start chain reactions that damage cells (Matill 1947).



Antioxidants are the most important natural products because they inhibit the initiation of lipid peroxidation, which is related to aging and diseases such as cardiovascular disorder, cancer, inflammation and brain dysfunction. Antioxidants lower the risk of heart diseases and some neurological diseases and cancer. However, many clinical trials do not support the view that antioxidants have a clear effect on the risk of chronic diseases such as cancer and heart diseases (Stanner 2004). There is some evidence that antioxidants might help in preventing other diseases such as muscular degeneration (Bartlett and Eperjesi 2003), suppressed immunity due to poor nutrition (Wintergerst 2006) and neurodegeneration (Wang et al. 2006). Antioxidant activity was assayed by DPPH free radical and found that it is a well-known radical and a trap (scavenger) for other radicals. Therefore, the rate reduction of a chemical upon the addition of DPPH is used as an indicator of the radical nature of that reaction. Because of a strong absorption band centered at about 520 nm, the DPPH radical has a deep violet colour in solution, and it becomes colourless or pale yellow when neutralised.

The free radical scavenging activities of *L. zeylanica*, *Pholidota pallida*, *Dendrobium nutantiflorum* and *Coelogyne breviscapa* were investigated by Rashmi et al. (2015) and reported strong antioxidantal activity of these orchids. Similarly, antioxidant potential was evaluated by Sukumaran and Yadav (2016) in *D. macrostachyum* stem and leaf extracts by in vitro methods, and they reported encouraging free radical scavenging and reducing activity. Haridas et al. (2016) narrated antioxidant activity of *Malaxis rheedii*. The whole plant extracts of *M. rheedii* were screened for various antioxidant assays, viz., DPPH, ABTS• + and ferrous ion chelating assay. A higher level of chelating ability for ferrous ions was observed in the crude methanol and ethyl acetate extracts of *M. rheedii*. Petroleum ether extracts also exhibited remarkable antioxidant activity in DPPH activity and ABTS+ activity assays.

25.7 Anti-inflammatory Activity

The anti-inflammatory activities of the methanolic extracts of the selected orchids were also encouraging (Table 25.3). The highest inhibition of egg albumin denaturation was found in *P. teres* (88.22 ± 0.205), followed by *L. zeylanica* (82.33 ± 0.144), and the lowest inhibition was found in the root of *R. retusa* (71.32 ± 0.151). Moderate anti-inflammatory activity was found in the leaf extract of *R. retusa*.

Significant anti-inflammatory activity was observed in the selected orchids. Inflammation is a process by which the body's white blood cells and substances they produce protect us from infection with foreign organisms, such as bacteria and viruses. Generally, anti-inflammatory drugs have their side effects. Denaturation of proteins is a well-documented cause of inflammation. Inflammation is a part of the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells or irritants. It is characterised by redness, swollen joint that is warm to touch, joint pain, stiffness and loss of joint function. Inflammation is either acute or chronic. Under specific circumstances, it could turn into a chronic state and

Table 25.3 Anti-inflammatory activity of *R. retusa* and *L. zeylanica* and *P. teres*

Plant name	Inhibition (%)			Average
	R_1	R_2	R_3	
Aspirin 100 mg (standard)	49.73	49.78	49.73	49.75 ± 0.024
R. retusa (leaf)	76.56	76.79	76.16	76.50 ± 0.260
R. retusa (root)	71.53	71.25	71.18	71.32 ± 0.151
L. zeylanica (leaf and stem)	82.48	82.14	82.38	82.33 ± 0.144
P. teres (leaf and stem)	88.08	88.51	88.07	88.22 ± 0.205

subsequently become a causative factor in pathogenesis. Inflammation is a self-defense reaction in its first phase, hence regarded as the main therapeutic target and often the best choice to treat the disease and alleviate the symptoms. Anti-inflammatory refers to the property of a substance or treatment that reduces inflammation. Anti-inflammatory drugs make up about half of analgesics, remedying pain by reducing inflammation, as opposed to opioids, which affect the central nervous systems. Perused literatures revealed that orchids have well-known anti-inflammatory activity (Kalaiarasan and Ahmed 2012; Sohag 2015; Sukumaran and Yadav 2016).

25.8 Antibacterial Activity

The antibacterial activity of selected plant extracts was tested against six pathogenic bacteria, namely *Bacillus subtilis*, *B. aureus*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *E. coli*, *Salmonella typhi* and *Staphylococcus aureus*. The methanolic extracts of all the selected orchids showed encouraging antibacterial activities against these bacteria, except *Bacillus aureus* (Fig. 25.3). A particular plant extract was more effective against a particular bacterial strain (Fig. 25.4). The highest antibacterial activity (14 ± 0.31 mm) was observed against *Bacillus subtilis* by the leaf extract of *R. retusa* at 200 µg/ml. In the case of *L. zeylanica*, the highest antibacterial activity (11 ± 0.19 mm) was observed against *Salmonella typhi*, *Pseudomonas aeruginosa* and *E. coli*, while the highest antibacterial activity (12 ± 0.23 mm) was recorded by *P. teres* against *Staphylococcus aureus* in the same concentration. Interestingly, no inhibition zones were observed against *Bacillus aureus* by any of the plant extracts investigated in the present study.

The methanolic extracts of all the selected orchids exhibited notable antibacterial activities against a number of bacteria. An 'antimicrobial' substance is any substance of natural, semi-synthetic or synthetic origin that kills or inhibits the growth of microorganisms but causes little or no damage to the host. Structural modification of the antimicrobials was reported to improve the effectiveness of antimicrobial agents against bacteria, fungi and viruses (Clercq 2001; Poole 2001; Jeu et al. 2003; Zhang et al. 2010). However, of late, research efforts have been put forth to improve the effectiveness of antimicrobial drugs by developing novel and a new class of antimicrobial drugs that can effectively work on multi-targeted sites or organisms

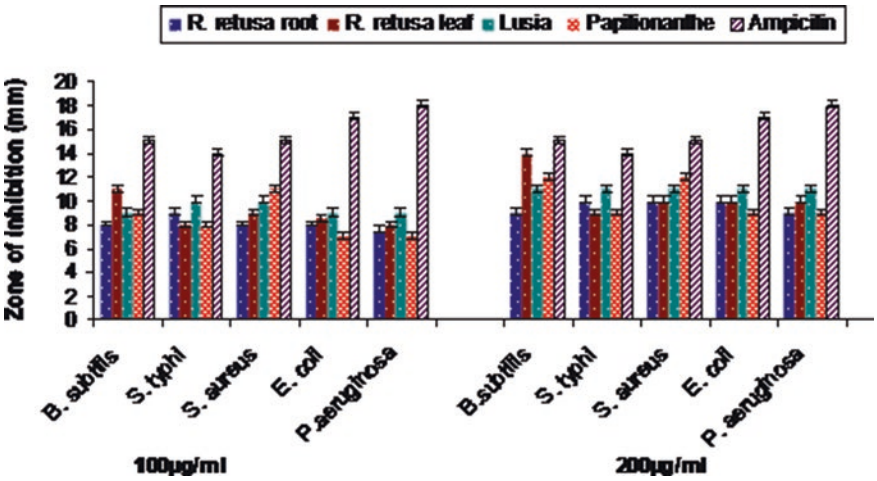


Fig. 25.3 Comparative study of antibacterial activity of methanolic extracts of selected orchids against different bacteria species at different concentrations

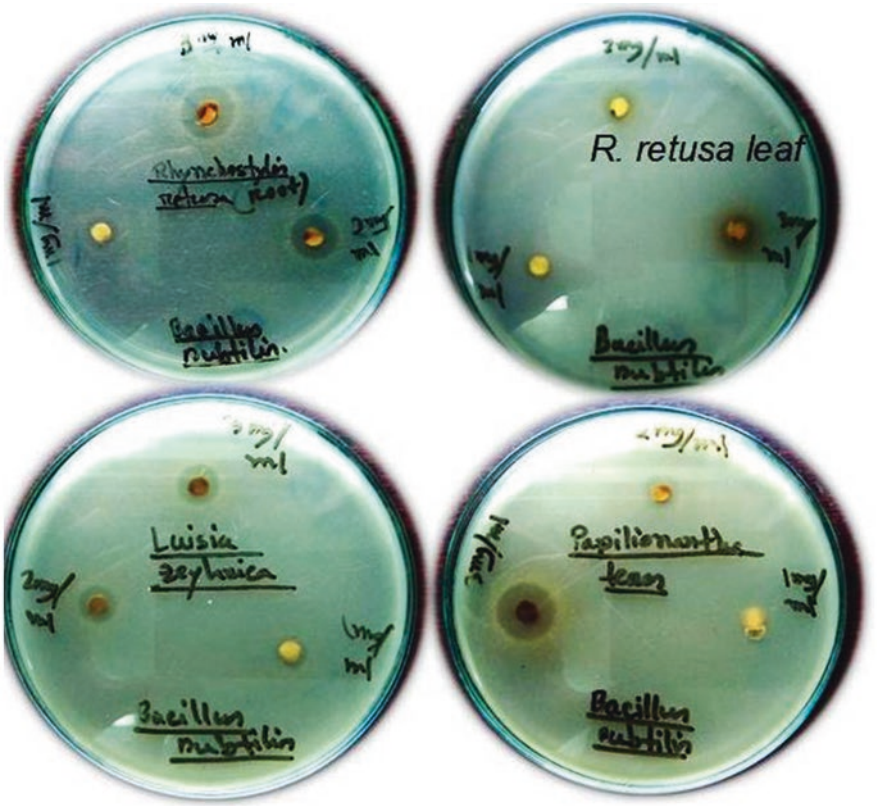


Fig. 25.4 Inhibition against *Bacillus subtilis* by the extracts of *R. retusa* (leaf and root), *L. zeylanica* and *P. teres* at different concentrations

(Esterhuizen et al. 2006; Alka et al. 2010). Antimicrobial properties of orchid plants also play an essential role for preventing traditional disorders. Ethno-pharmacological studies showed that orchids are used in many parts of the world in treating a number of diseases like skin diseases, problems concerning the digestive systems and tumours and for pain relief and reducing fever (Hossain 2011). Antimicrobial activities against a number of bacteria have been reported in many orchids such as *Acampe ochracea*, *Cymbidium aloifilium*, *Coelogyne breviscapa*, *Dendrobium nutantiflorum*, *Luisia zeylanica*, *Pholidota pallida*, *Tragia involucrata* and others (Gutierrez 2010; Panda et al. 2012; Paul et al. 2013; Rashmi et al. 2015; Shubha and Chowdappa 2016).

25.9 Antifungal Activity

The result of antifungal activity of orchid extracts in terms of mycelial growth inhibition of test fungi, i.e. *Aspergillus niger* and *Fusarium oxysporum*, is shown in Figs. 25.5 and 25.6. Poisoning of medium with orchid extracts resulted considerable suppression of mycelial growth of test fungi. In poisoned plates, the fungal colony was comparatively smaller when compared to fungal growth on control plates, and no sporolation occurred. The selected orchid extracts displayed varied antifungal potency.

Noteworthy antifungal activity by the selected orchid extracts in terms of inhibition of mycelial growth and sporolation against *F. oxysporum* and *A. niger* was observed. Antifungal activity have been reported in a number of orchids, such as *A. papillosa*, *C. aloifolium*, *R. retusa L. zeylanica*, *D. nutantiflorum* and *Pholidata pallida* (Radhika et al. 2013; Shweta et al. 2015; Marjoka et al. 2016). Synthetic fungicides are currently used as primary means for the control of plant disease. However, alternative control methods are needed because of the negative public perceptions

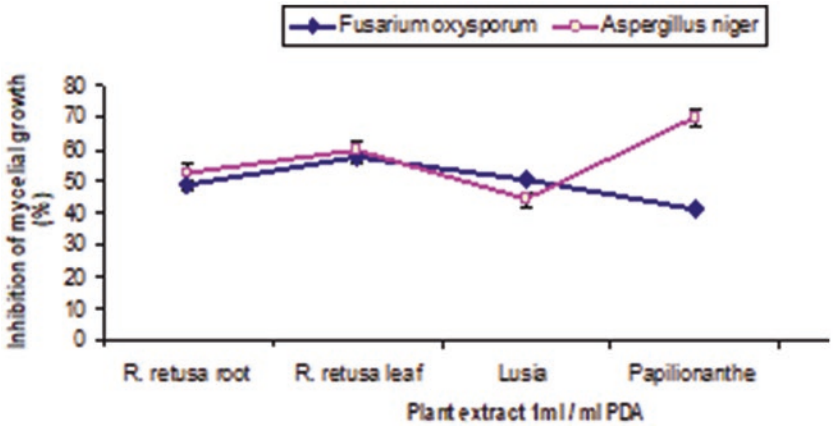


Fig. 25.5 Antifungal activity of methanolic extracts of the selected orchids against *A. niger* and *F. oxysporum*

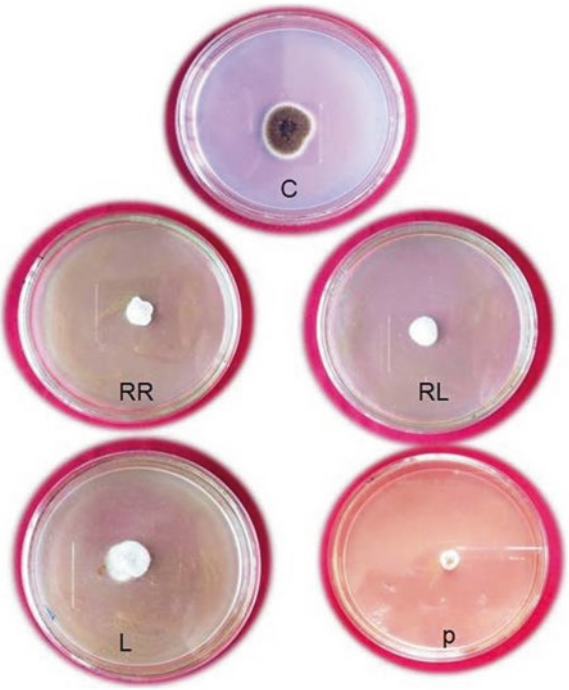


Fig. 25.6 Antifungal activity against *Aspergillus niger* by the extracts of *R. retusa* leaf (RL), *R. retusa* root (RR), *L. zeylanica* (L), *P. teres* (P) and control (C)

about the use of synthetic chemicals, resistance to fungicide among fungal pathogens and the high development cost of new chemicals. The uses of orchids as disease control agents have been studied since they tend to have low mammalian toxicity, less environmental effects and wide public acceptance.

25.9.1 Anticancer Efficacy of Orchids

The brine shrimp lethality bioassay of methanol extract of the selected orchids showed remarkable cytotoxic activity. Among the three epiphytic orchid, the leaf and stem extracts of *L. zeylanica* showed the highest cytoxic activity (LC50 value 77.175 µg/ml), while the anticancer drugs vincristine sulphate showed an LC50 value of 0.25 µg/ml after 24 h of observation. It was also evident that the mortality rate of nauplii increased with the increase of concentration (Table 25.4).

The crude methanolic extracts of the selected orchids demonstrated significant cytotoxicity in brine shrimp lethality bioassay. According to Shrestha et al. (2015) if the LC50 value of the test sample is less than 1000 ppm, the extract is considered to be biologically active. All the extracts in the present study showed higher LC50 value than standard. Orchids are prominent sources of bioactive compounds that have cytotoxicity and are used as a normal source of drug. These drugs are used in herbal medicine to act as an anticancer agent (Radhika et al. 2013; Shrestha et al. 2015; Islam et al. 2016).

Table 25.4 Brine shrimp lethality bioassay for cytotoxic activity of crude methanolic extracts of the selected orchids

Plant extract (μg/ml)	Nauplii mortality (%)			
	R. retusa (root)	*R. retusa* (leaf)	*L. zeylanica*	*P. teres*
Control	0	0	0	0
50	35	25	25	40
100	60	60	60	60
200	90	80	80	80
300	100	100	100	100
400	100	100	100	100
500	100	100	100	100
LC50 (μg/ml)	29.13	16.52	77.175	30.213
LC50 of vincristine sulphate	0.25	0.25	0.25	0.25

Table 25.5 Analysed data of acetic-acid-induced writhing test

Drugs and doses	Number of writhing	% of inhibition
Saline	65.17 ± 2.39	0.00
Diclofenac sodium	20.33 ± 0.80	68.79 ± 1.23
L. zeylanica (200 mg/kg)	17 ± 0.81	73.91 ± 1.25
L. zeylanica (400 mg/kg)	11 ± 0.82	83.12 ± 1.26
P. teres (200 mg/kg)	19.67 ± 1.25	69.82 ± 1.91
P. teres (400 mg/kg)	9 ± 1.63	86.19 ± 2.51

25.9.2 Analgesic Activity

Analgesic assay was conducted with *L. zeylanica* and *P. teres*, and the maximum inhibitory effect was observed on mice by the extract of *P. teres* (86.719%) in acetic-acid-induced writhing test (Table 25.5). Analgesics or painkiller drugs are used to achieve analgesia i.e. relief from pain. Here, 'diclofenac sodium' (20 mg/kg) were used as reference standard, which caused 68.79% reduction of writhing movement in mice. Acetic-acid-induced writhing response is one of the remarkable procedures to evaluate the peripherally acting analgesics. Biphasic analgesic activity, i.e. neurogenic and inflammatory pain, was induced by formalin; concentration-dependent reduction in paw licking was observed in both phases, but reduction was most significant in late phase (Table 25.6). The time spent for licking was greater in 200 mg/kg than 400 mg/kg in all the orchid species.

Encouraging analgesic activity was observed in selected orchids. The word analgesic derives from Greek word *an* + *algose*, meaning without pain. Such drugs were usually known as anodynes before the twenty-ninth century (Encyclopedia Britannica 1878, 1911). An analgesic or painkiller is any member of the group of drugs used to achieve analgesia, relief from pain. Analgesic drugs act in various ways on the peripheral and central nervous systems. They are distinct from anesthetics, which temporarily affect, and in some instances completely eliminate, sensation. Analgesics include paracetamol (known in North America as acetaminophen

Table 25.6 Analysed data for formalin-induced hind paw licking test

Drugs and doses	Time spent for licking		% of inhibition	
	Early phase	Late phase	Early phase	Late phase
Saline	68.16 ± 1.30	52.5 ± 0.92	0.00	0.00
Diclofenac sodium	27.33 ± 2.24	16 ± 1.36	59.90 ± 3.29	69.52 ± 2.60
L. zeylanica (200 mg/kg)	36.67 ± 1.25	30 ± 1.63	46.21 ± 1.83	55.99 ± 2.4
L. zeylanica (400 mg/kg)	22.67 ± 0.47	18 ± 0.82	66.75 ± 0.69	73.59 ± 1.20
P. teres (200 mg/kg)	27 ± 0.82	20.67 ± 1.7	60.39 ± 1.2	69.68 ± 2.49
P. Teres (400 mg/kg)	20 ± 0.82	18 ± 1.63	70.68 ± 1.22	73.59 ± 2.4

or simply APAP), nonsteroidal anti-inflammatory drugs (NSAIDs) such as salicylates and opioid drugs such as morphine and oxycodone. When choosing analgesics, the severity and response to other medication determines the choice of agent; the World Health Organization (WHO) pain ladder specifies mild analgesics as its first step. Analgesic choice is also determined by the type of pain: for neuropathic pain, traditional analgesics are less effective, and there is often benefit from classes of drugs that are not normally considered analgesics, such as tricyclic antidepressants and anticonvulsants.

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GC-MS Analysis of Organic Extracts of *Cymbidium aloifolium* (L.) Sw. (Orchidaceae) Leaves from Eastern Ghats of India

26

Venkatesh Rampilla and S. M. Khasim

Abstract

Phytochemical constituents of various leaf extracts of *Cymbidium aloifolium* (L.) Sw. have been studied using gas chromatography–mass spectrometry (GC-MS) data. The dried leaves powder were extracted with methanol at room temperature by using a Soxhlet extractor. The methanol crude extract of *Cymbidium aloifolium* was again taken with hexane, chloroform and ethyl acetate. The analysis was carried out on an Agilent GC-MS equipped with a HP-5MS fused capillary column; the compounds are separated using helium as a carrier gas at a constant flow of 1 ml/min. A qualitative analysis of various organic extracts showed eight different photochemical compounds, namely n-hexadecanoic acid; 9,12-octadecadienoic acid (Z,Z); 9,12,15-octadecatrienoic acid, (Z,Z,Z); octadecanoic acid; phytol; 2-butyne; 2-cyclopenten-1-one; and 1,4-benzenedicarboxylic acid. Most of the identified compounds are biologically important. This study offers a platform of using *Cymbidium aloifolium* leaves as herbal alternatives for various diseases. The compounds reported in this investigation also have some phylogenetic significance.

Keywords

Cymbidium aloifolium · qualitative analysis · Orchidaceae · GC-MS analysis

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26.1 Introduction

Orchids are scientifically a significant and commercially important group of flowering plants under the family Orchidaceae. It includes about 788 genera (Mabberly 1997) and 25,000–35,000 species (Dressler 1993; Hossain 2011). In India, orchids are grown in high altitude areas of the Himalayas, Western Ghats and Eastern Ghats. Nearly 1129 species and 184 genera (Jalal et al. 2008) were found in India, of which 190 species were recorded in Andhra Pradesh (Reddy et al. 2005). Many orchids having cultural value and are used as herbal medicines and food supplements by tribes in different parts of the world (Khasim and Rao 1999). Phyto-constituents are responsible for the medicinal activity of the plants, and these are classified into primary and secondary metabolites. The screening of active compounds from orchids has led to the invention of novel drugs, and they have efficient protection against various diseases (Dandekar et al. 2015). In recent years, there has been tremendous progress in the study of organic compounds from the medicinal plants and orchids (Keerthiga and Anand 2015; Kalaiarasan and Ahmed John 2011). The combination of gas chromatography (GC) and mass spectrum (MS) is an ideal technique for the qualitative and quantitative analysis of volatile and semi-volatile compounds (Nishaa et al. 2013).

26.2 Ethnobotany and Traditional Use of *Cymbidium Aloifolium*

Traditionally, *Cymbidium aloifolium* has been used in various parts of the world as folk medicine. The tribal community of North East India use the seeds for healing wounds (Medhi and Chakrabarti 2009). In Bangladesh, the plant is used as anti-inflammatories and anticancer agents, while in South India, it is used as emetic and purgative. The leaves of the plant are reported to cure earache, cuts and wounds (Sharief Ahmed Makul et al. 2007). It is an epiphytic orchid distributed widely in Eastern Ghats of Andhra Pradesh. The tribal community of East Godavari district, locally called as Pedda vajanika, are using leaf juice to cure earache. The existing literature indicates that tribal communities of different regions use various phyto-preparations of this plant to cure diseases. No investigation on *Cymbidium aloifolium* has so far been undertaken to provide enough scientific data in favour of reported traditional use. Traditional use varies among local practitioners for boils, earache, vomiting, fever, wounds, paralysis, digestive disorders, sores etc., (Nongdam and Chongtham 2011; Medhi and Chakrabarti 2009; Sharief Ahmed Makul et al. 2007). The various organic extracts of this plant has recently been reported to have antimicrobial and antibacterial activity (Radhika et al. 2013). As a part of the endeavor to search for therapeutic properties of *Cymbidium aloifolium*, we herein presented the GC-MS analysis of various extracts from the leaf.

26.3 Sample Collection and Preparation of Crude Extracts

Leaves of *Cymbidium aloifolium* (L.) Sw. were collected from the Pedda konda sacred grove of the East Godavari district, Andhra Pradesh, India, in January 2015. The specimen was identified with the help of regional floras, and the voucher specimen was deposited at Acharya Nagarjuna University Botany Herbarium (ANUBOTH 11123), Guntur, and Andhra Pradesh, India.

The fresh green leaves of *Cymbidium aloifolium* growing on *Borasus flabellifer* host plant were collected from their natural habitat and packed in polyethylene bags. The leaf samples were washed thoroughly in running tap water to remove soil particles. The plant samples were shade dried and ground into fine powder and stored in air-tight polythene bags until use. The dried leaves powder (150 g) was extracted in methanol at room temperature by using a Soxhlet extractor for 12–18 h. Crude extracts were prepared according (Amzad Hossain 2011). The crude methanol extracts were evaporated by a vacuum rotary evaporator (Buchi Labortech Ag, model l, R-215) under reduced pressure. The crude extract was diluted with water and extracted successively with n-hexane, ethyl acetate and chloroform. The extracts were filtered using Whatman no. 41 filter paper to obtain particle-free extract. The residue was re-extracted twice with solvents (E-Merk) used to obtain extracts. The 2 µl of each sample was injected into the GC-MS instrument for phytochemical analysis.

26.4 GC-MS Equipment

The GC-MS analysis of various crude extracts from leaves was performed using an Agilent GC-MS (Model-5975c inert MSD with Triple-Axis Detector, USA) equipped with an HP-5MS fused capillary column (30 m × 0.25 mm i.d., film thickness 0.25 µm). Inert helium gas was used as a carrier gas at a constant flow rate of 1ml/1min. An aliquot of 2 µl of various extract solution of the sample was injected into the column with injector temperature of 250 °C. Mass transfer line and injector temperature were set at 220 °C and 300 °C, respectively. The oven temperature was programmed from 50 to 150 °C at 3 °C/min, then held isothermal for 10 min and finally raised to 250 °C at 10 °C/min. In gas chromatography–mass spectroscopic detection, an electron ionisation system with ionisation energy of 70 ev was used, and the detector was operated in scan mode from 40 to 500 amu (atomic mass unit). The total running time was 55.3 min.

Interpretation of GC-MS data was carried out using the National Institute of Standard and Technology (NIST) database library 2.0 version, which has more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known component stored in NIST library. The name, molecular weight and structure of the components of the test materials were ascertained.

26.5 GC-MS Analysis of Organic Extracts

Leaves of *C. aloifolium* are linear-oblong, obtuse, 50 cm long and 3 cm broad. Inflorescence arises from the base of the stem tuber, and raceme is 50 cm long. The various plant extracts analysed using GC-MS had led to the identification of eight different organic compounds, and their retention time (RT) and concentration (peak area %) were shown in Table 26.1. The typical gas chromatograms of chemical constituents of ethyl acetate extract (Fig. 26.1), chloroform extract (Fig. 26.2) and hexane extracts (Fig. 26.3) have been shown in repective figures. Molecular formula, molecular weight, the nature of the compounds, and biological activity are presented in Table 26.2. A total of six chemical compounds were identified in ethyl acetate and chloroform extract each; five were traced in hexane extract. The compounds like n-hexadecanoic acid (Fig. 26.4c); octadecanoic acid (Fig. 26.5b); 9,12-octadecadienoic acid (Z,Z) (Fig. 26.4d), 9,12,15-octadecatrienoic acid, (Z,Z,Z) (Fig. 26.5a) and phytol (Fig. 26.5c) were found in all extracts. However, phytol was not detected in ethyl acetate extract. 2-butyne (Fig. 26.4a) and 2-cyclopenten-1-one (Fig. 26.4b) are found in ethyl acetate extract only, while 1,4-benzenedicarboxylic acid, bis(2-hydroxyethyl) ester (Fig. 26.5d) is detected in chloroform extract only.

Table 26.1 Chemical composition of different extracts of *Cymbidium aloifolium*

Extracts	Name of the compound	Retention time (min)	Peak area%
	n-hexadecanoic acid	50.409	22.6956
	Phytol	52.538	4.5813
n-hexane extract	9,12-octadecadienoic acid (Z,Z)	52.803	28.5815
	9,12,15-octadecatrienoic acid, (Z,Z,Z)	52.872	38.3769
	Octadecanoic acid	53.11	5.7647
	2-butyne	13.623	4.5256
	2-cyclopenten-1-one	17.907	6.1662
Ethyl-acetate extract	n-hexadecanoic acid	50.399	20.4864
	9,12-octadecadienoic acid (Z,Z)	52.792	24.1308
	9,12,15-octadecatrienoic acid, (Z,Z,Z)	52.861	37.3557
	Octadecanoic acid	53.105	7.3353
	n-hexadecanoic acid	50.388	21.0246
	Phytol	52.533	3.5791
	9,12-octadecadienoic acid (Z,Z)	52.776	24.754
Chloroform extract	9,12,15-octadecatrien-1-ol, (Z,Z,Z)	52.845	34.2954
	Octadecanoic acid	53.099	6.6246
	1,4-benzenedicarboxylic acid, bis(2-hydroxyethyl) ester	53.428	9.7223

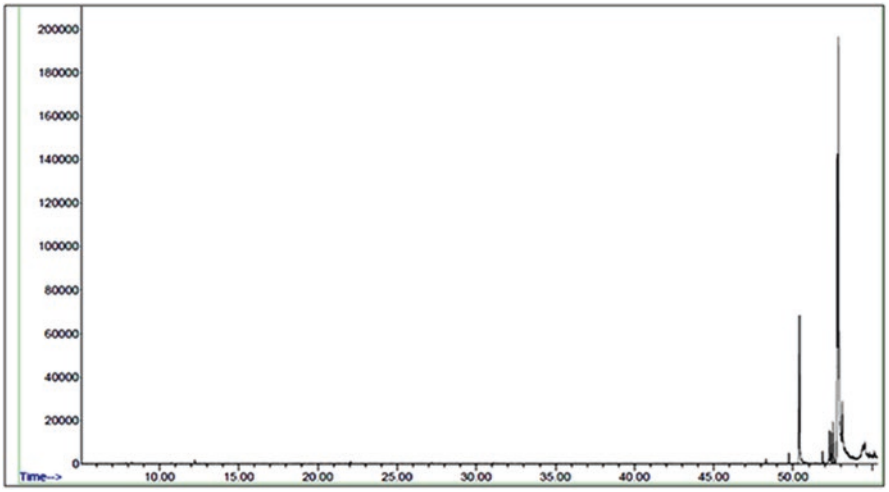

Fig. 26.1 A typical gas chromatogram of the chemical constituents of ethyl acetate extract

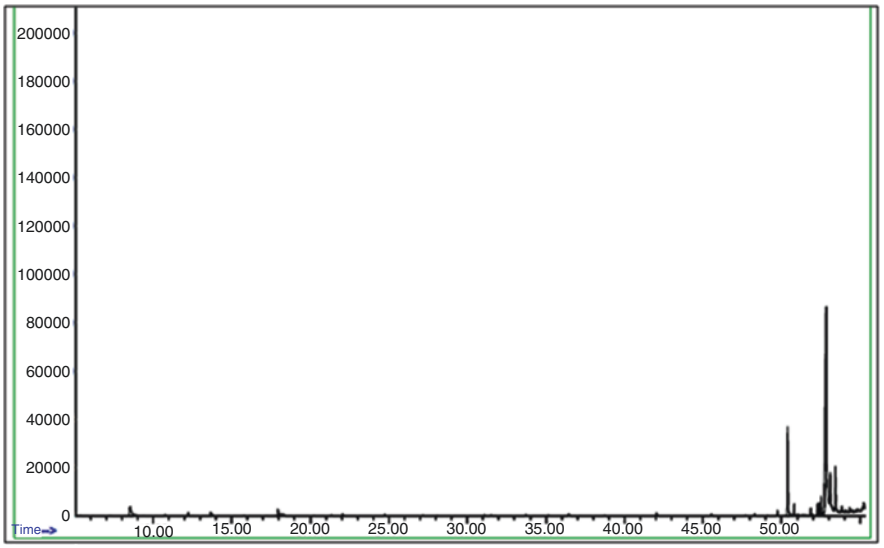

Fig. 26.2 A typical gas chromatogram of the chemical constituents of chloroform extract

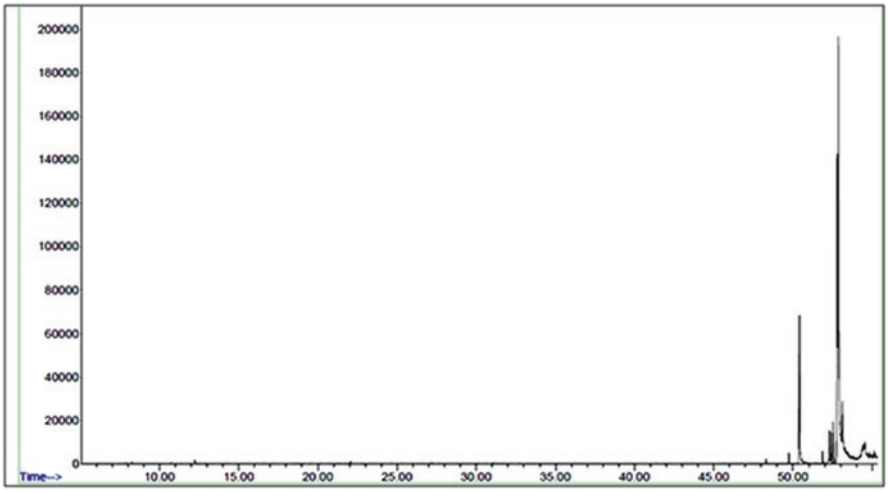

Fig. 26.3 A typical gas chromatogram of the chemical constituents of hexane extract

26.6 Orchid Chemicals and Their Importance

In the present study, the GC-MS analysis of the various organic extracts of *Cymbidium aloifolium* showed the presence of eight compounds. In terms of percentage amounts 9, 12, 15-octadecatrienoic acid (Z, Z, Z); 9, 12-octadecadienoic acid (Z, Z); and n-hexadecanoic acid were predominant in all three extracts. Compounds such as 9,12-octadecadienoic acid (Z,Z) and 9,12,15-octadecatrienoic acid, (Z,Z,Z) are polyunsaturated fatty acid (PUFA) compounds. PUFAs play a key role in cellular and tissue metabolism and electron and oxygen transport and also reduce the risk for coronary heart disease (Funk 2001; Mozaffarian et al. 2005). The 2-cyclopenten-1 was only ketone identified in *C. aloifolium*. It acts as an inducer for heat shock protein that has antiviral activity (Rossi et al. 1996a, b).

In recent times, there is a growing awareness in correlating photochemical components and their biological activities (Fernie et al. 2004; Summer et al. 2003). The 9,12,15-octadecatrienoic acid, (Z,Z,Z) has anti-inflammatory, hypercholesterolemia, cancer preventive, hepatoprotective, nematicide and antiarthritic activities (http://www.ars- http://www.ars-grin.gov/duke/chem-activities.html). Octadecanoic acid is a saturated fatty acid, and it might act as a cholesterol-reducing agent (Hunter et al. 2009). Phytol is an acyclic diterpene, and it is also a precursor for vitamins E and K1. Phytol is a promising novel class of pharmaceuticals used for the treatment of antiarthritis and other chronic inflammatory diseases (Ogunlesi et al. 2009).

C. aloifolium belongs to the subtribe Crytopodiinae, tribe Cymbidieae, subfamily Epidenchoideae of Orchidaceae (Dressler 1993). The compound n-hexandecanoic acid reported in the present study was also recorded in *Bulbophyllum kaitense* (Kalaiarasan and Ahmed john 2011). This chemical data would indicate that the tribe Cymbidieae has close affinity with genus *Bulbophyllum*.

Table 26.2 GC-MS analysis showed the phytochemical compounds, their nature, molecular formula, molecular weight and biological activities of *Cymbidium aloifolium*

Sl. no	Name of the compound	Nature of the compound	Molecular formula	M.W	Biological activity	Reference No.
1	n-hexadecanoic acid	Palmitic acid(saturated fatty acid)	$C_{16}H_{32}O_2$	256	Antioxidant, hypocholesteromic, nematicide, hemolytic, 5-alpha reductase inhibitor, antipsychotic	Vijisaral and Subramanian (2014), Sermakkani and Thangapandian (2012), Akpuaka et al. (2013)
2	Phytol	Acyclic, diterpene	$C_{20}H_{40}O$	296	Antimicrobial, anti-cancer, anti-inflammatory, hypocholesteromic, nematicide, anti-arthritic, anticoronary, anti-androgenic, diuretic	http://www.ars- http://www.ars-grin.gov/duke/chem-activities.html, Ogunlesi et al. (2009)
3	9,12-octadecadienoicacid(Z,Z)-	Linoleic acid	$C_{18}H_{32}O_2$	280	Hypercholesterolemic, nematicide, 5-alpha reductase inhibitor, antihistaminic, insectifuge, antieczemic	http://www.ars- http://www.ars-grin.gov/duke/chem-activities.html
4	9,12,15-octadecatrienoic acid, (Z,Z,Z)-	Linolenic acid ester	$C_{18}H_{30}O_2$	278	Anti-inflammatory, hypercholesterolemic, cancer preventive, hepatoprotective, nematicide, antiarthritic,	http://www.ars- http://www.ars-grin.gov/duke/chem-activities.html
5	Octadecanoic acid	Stearic acid	$C_{18}H_{36}O_2$	284	Antifungal, antitumour, antibacterial, cholesterol-reducing agent	Vijisaral and Subramanian (2014), Sermakkani and Thangapandian (2012), Akpuaka et al. (2013), Hunter et al. (2009)
6	2-butyne	Alkyne	C_4H_6	54	A simple asphyxiant	CRC Handbook of Chemistry and Physics (2013)
7	2-cyclopenten-1-one	Ketone	C_5H_6O	82	Inducer of Hock protect Shock 70 with antiviral activity	Antonio Rossi et al. (1996a, b)
8	1,4-benzenedicarboxylic acid, bis(2-hydroxyethyl) ester	Ester	$C_{12}H_{14}O_6$	254	Antitumour	Da Hong Wang and Wen Yi Tao (2009)

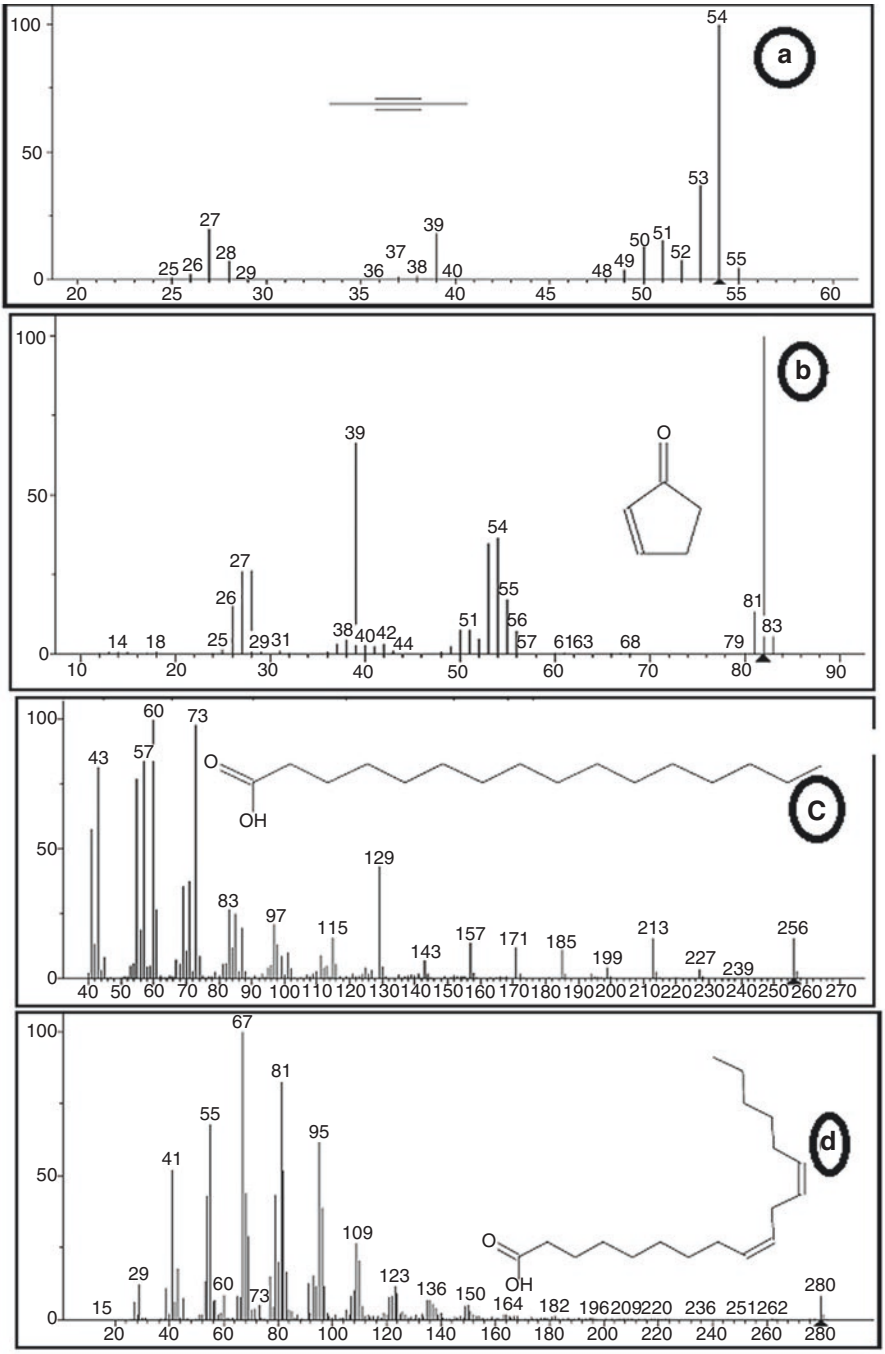

Fig. 26.4 **a**. 2–Butyne, **b**. 2-Cyclopenten-1-one, **c**. n-Hexadecanoic acid, **d**. 9, 12-Octadecadienoic acid (Z,Z)

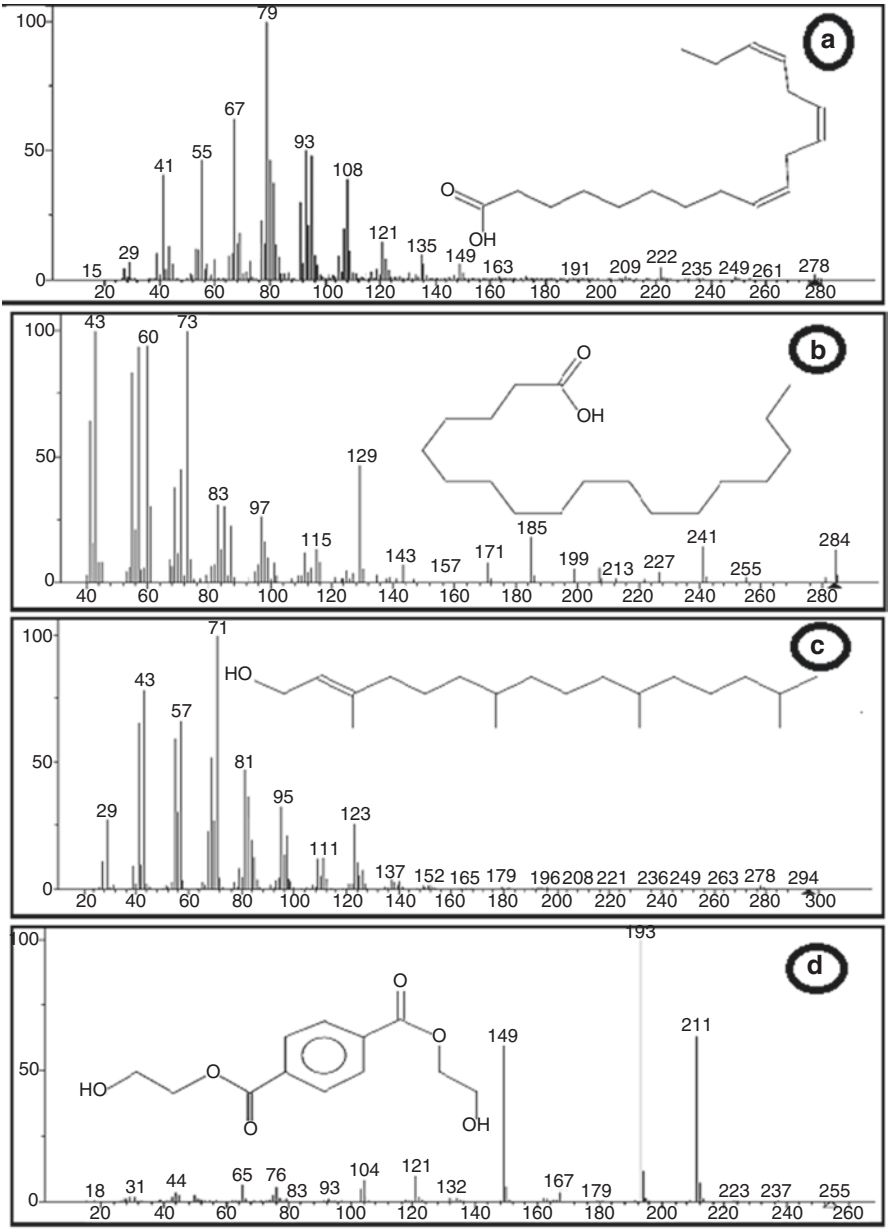

Fig. 26.5 **a.** 9, 12, 15-Octadecatrienoic acid, (Z, Z, Z), **b**. Octadecanoic acid, **c.** Phytol, **d.** 1, 4-Benzenedicarboxylic acid, bis (2-hydroxyethyl) ester

On the other hand, Veerraju et al. (1989) opined that Cymbidieae would have some affinity with the Bletiinae based on chemical data. However, based on recent studies on seed and embryo, it did not share any character with any other genera of Bletiinae.

26.7 Conclusion

The present study characterised the phytochemical profile of the various organic extracts of the *Cymbidium aloifolium* leaves. The compound 1,4-benzenedicarboxylic acid bis(2-hydroxy ethyl) ester was reported from chloroform extract only. Similarly, 2-butyne and 2-cyclopentene-1-one were identified from ethyl acetate extract only. The identified various bioactive compounds have therapeutic properties that can be useful for the treatment of various diseases. These compounds reported from this investigation have some phylogenetic significance.

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Anticancer Property in *Acampe praemorsa and Aerides odorata* (Orchidaceae), an *In Vitro* Approach

27

K. Jhansi and S. M. Khasim

Abstract

The present study aims at the *in vitro* cytotoxic effect of epiphytic orchids, viz., *Acampe praemorsa* and *Aerides odorata.* Plants were collected from the Eastern Ghats of the Visakhapatnam district, Andhra Pradesh, India. Leaf materials were shade dried; ethyl acetate and methanolic leaf extracts were prepared for this investigation. The leaf extracts were tested for their inhibitory effect on HeLa and MCF-7 cell lines, which were evaluated by the MTT assay; these plants showed good anticancer activity and did not show any adverse effect to normal cells. Both extracts showed good anticancer activity on the MCF-7 cell line than on the HeLa cell line. The methanolic leaf extract of *Aerides odorata* has significant cytotoxicity effect on the MCF-7 with concentration ranging from 5 to 100 µg/ml, with an IC 50 (µg/ml) value 26.211. The findings from this study showed that the methanolic extract of the *A. odorata* leaf possesses vast potential as a medicinal drug in breast cancer treatment.

Keywords

In vitro cytotoxicity · Methanol · Ethyl acetate · HeLa cell line · MCF-7 cell line · MTT assay

27.1 Introduction

The Orchidaceae is one of the largest families with more than 30,000 species spread over to 750 genera distributed throughout the world from the tropics to the Alpine states (White and Sharma 2000). They are the most diverse among the flowering

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plant families with over 184 genera and 1331 species in India (Kumar and Manilal 1994). The usage of orchids in medicine has a long history, as indicated in ancient records stating that these plant parts have been utilised to treat various diseases by the Chinese, Indians, Sumerians and Egyptians (Bulpitt 2005). In many countries like China and in some parts of Europe, America, Australia and Africa, orchids have been used as traditional drugs since times immemorial (Dash et al. 2008). The earliest Middle East report of plant remedies is in a 4000-year-old Sumerian clay tablet, which included some orchids (Kong et al. 2003). They are also one of the ingredients in ancient Indian systems of medicine called Ayurveda (Bijaya pant 2013).

The present study deals with the *in vitro* cytotoxic effect of two orchid species, viz., *Acampe praemorsa* and *Aerides odorata*, against HeLa and MCF-7 cancer cell lines. In Andhra Pradesh (India), the Koya community uses the pulverised plant *A. praemorsa*, mixed with egg white and calcium, to produce a paste for application on fractured limbs to promote healing (Akarsh 2004). Ten drops of warm butter extracted from cow milk taken on the leaf of this plant are bandaged to the legs of kids to cure tetanus (Behera et al. 2013). The leaf juice is applied over the nipple for stomach ache, and it is also used for earache and for controlling body temperature (Shanavaskan et al. 2012). The forest dwellers in Araku valley showed that leaf paste, along with a piece of garlic, taken seven days is effective for the relief of chest pain and stomach disorder caused by hyperacidity (Padal et al. 2013). The leaf juice of this plant has been used to control mild tuberculosis (Dash et al. 2008); the leaf paste of this plant is used to treat cuts and wounds (Bijaya Pant 2013) and also to cure boils in the ear and nose (Hossain 2009). The whole plant and leaves have been used to treat pneumonia, dyspepsia, epilepsy, paralysis, inflammation, waist ache and fractures (Akhtar et al. 2017). The leaves of *A. praemorsa* and *Luisia zeylanica* and the aerial roots of *Cymbidium aloifolium* are used to fix human bone fractures (Behera et al. 2013). A wide range of chemical compounds have been isolated from various parts of orchids. These compounds are believed to be effective in reducing fevers, increasing WBC count, curing eye infection, treating fatigue and headache and, most importantly, functioning as an anticancer agent (Bulpitt 2005). Indian *Vanda* orchids have antiproliferative effects against various types of cancer (Ho and Chen 2003).

The leaves of *Acampe praemorsa* and *Aerides odorata* were collected from Paderu, Visakhapatnam, Eastern Ghats of Andhra Pradesh, India, and were washed and shade dried; the dried material was made into a coarse powder. The dried powdered leaf material was extracted in ethyl acetate and methanol solvents; the resulting extracts were filtered and then concentrated.

27.1.1 Human Cell Lines

The HeLa (cervical cancer) and MCF-7 (breast cancer) cell lines were obtained from NCCS, Pune (India), and the cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and

antibiotics such as penicillin/streptomycin (0.5 mL^{-1}), in 5% CO_2/95% air atmosphere at 37 °C temperature.

27.1.2 Preparation of Crude Leaf Extract and MTT Assay

For MTT [3-(4, 5- dimethylthiazol-2-yl)-2, 5- diphenyl tetrazolium bromide] assay, each leaf extract was weighed separately and dissolved in dimethyl sulphoxide. With the media, make up the final concentration to 1 mg/ml and the cells were treated with series of concentrations from 10 to 100 µg/ ml. MTT assay is a colorimetric assay that measures the reduction of yellow MTT by mitochondrial succinate dehydrogenase. The assay depends both on the number of cells present and on the assumption that dead cells or their products do not reduce tetrazolium. The MTT enters the cells and passes into the mitochondria, where it is reduced to an insoluble, dark-purple-coloured formazan crystals. The cells are then solubilised with a DMSO, and the released, solubilised formazan reagent is measured spectrophotometrically at 570 nm.

Cell viability was evaluated by the MTT assay with three independent experiments with six concentrations of crude leaf extract in triplicates. HeLa and MCF-7cell lines were trypsinised, and the Tryphan Blue assay was performed to identify viable cells in cell suspension. Cells were counted by the haemocytometer and seeded at a density of 5.0×10^3 cells/well in 100 µl media in 96 well plate culture media and incubated overnight at 37 °C. After incubation, the old medium was taken off, and the 100 µl fresh media with different concentrations of crude leaf extract in represented wells in 96 plates was added. After 48 h, the crude leaf extract was discarded, the fresh media with MTT solution (0.5 mg/mL^{-1}) was added to each well, and plates were incubated at 37 °C for 3 h. At the end of incubation time, precipitates were formed as a result of the reduction of the MTT salt to chromophore formazan crystals by the cells with metabolically active mitochondria. The optical density of solubilised crystals in DMSO was measured at 570 nm on a microplate reader. Percentage growth inhibition was calculated using the following formula, and the concentration of test drug needed to inhibit cell growth by 50% in value is generated from the dose-response curves for each cell line using with ORIGIN software:

$$\% \text{ Inhibition} = \frac{\left(\text{Control} - \text{Treatment}\right)}{\text{Control}} \times 100$$

27.2 *In Vitro* Cytotoxic Effect Against Cancer Cell Lines

MTT assay for the *in vitro* cytotoxicity assessment of the ethyl acetate and methanolic leaf extracts of *A. praemorsa* and *A. odorata* was carried out at six different concentrations of 5, 10, 25, 50, 75 and 100 µg/ml on two different cell lines, MCF-7

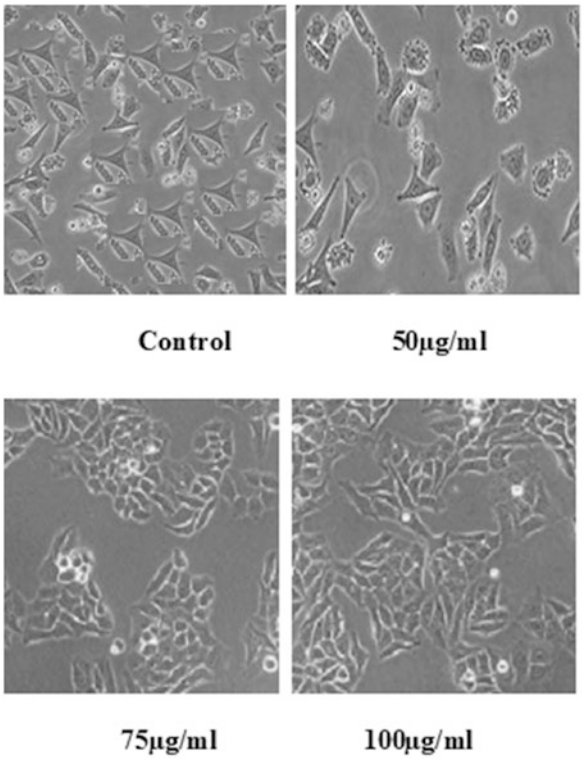

Fig. 27.1 Highest anticancer activity of the methanolic extract of *A. odorata* on the MCF-7 cell line

(breast cancer) and HeLa (cervical cancer) (Figs. 27.1 and 27.2). At 100 µg/ml concentration of the crude leaf extract, the inhibition rate of cancer cell lines is high, which means that the percentage of viable cancer cells is less (Tables 27.1 and 27.2). The percentage of viable cancer cells decreases with the increase in the concentration of the crude leaf extract from 5 to 100 µg/ml (Figs. 27.3 and 27.4). IC_{50} values were recorded for all orchid leaf extracts. In vitro cytotoxic activity is measured in terms of IC_{50} value, which is half maximal inhibitory concentration that measures the potency of crude leaf extract (drug) in inhibiting the cancer cell line.

27.2.1 *Acampe praemorsa*

At 100% concentration of the ethyl acetate leaf extract of *Acampe praemorsa*, 61.128% of MCF-7 cells were inhibited (38.872% cells are viable) with an IC_{50} value of 49.276 µg/ml; 57.44% of HeLa cells were inhibited (42.56% of cells are viable) with an IC_{50} value of 61.68 1 µg/ml. Similarly, at 100% concentration of methanolic extract, 58.795% of MCF-7 cells were inhibited (41.205% of cells are viable) with an IC_{50} value of 55.904 µg/ml; 53.984% of HeLa cells were inhibited (46.016% of cells are viable) with an IC_{50} value of 76.94 µg/ml (Table 27.1). Here,

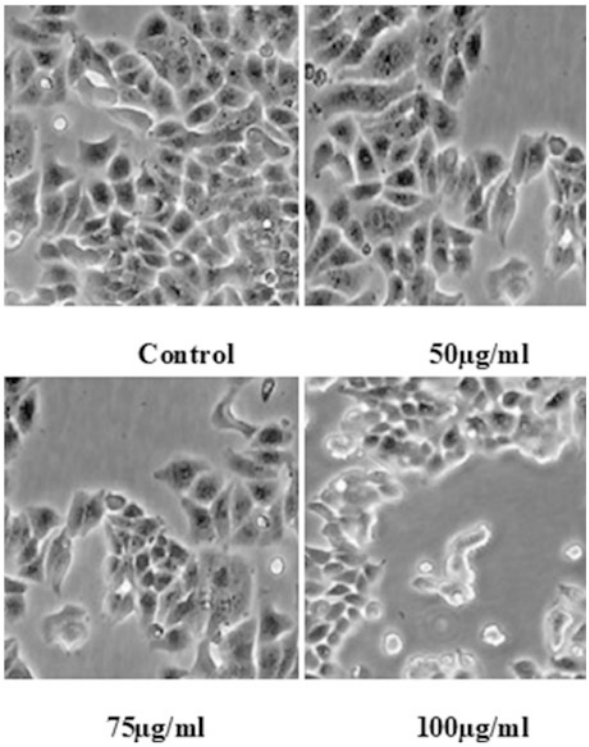

Fig. 27.2 Poor anticancer activity of the methanolic extract of *A. praemorsa* on the HeLa cell line

MCF-7 cell lines were more prone to death to ethyl acetate extract. Hence, the highest *in vitro* cytotoxicity was seen in the ethyl acetate extract against the MCF-7 cell line (Fig. 27.1) with the least IC_{50} value of 49.276 µg/ml (Table 27.1), which was enough to kill 50% of MCF-7 cancer cells. In other cases, poor *in vitro* cytotoxicity was seen in methanolic extracts against the HeLa cell line.

27.2.2 *Aerides odorata*

At 100% concentration of the ethyl acetate leaf extract of *Aerides odorata*, 61.759% of MCF-7 cells were inhibited (38.241% of cells are viable) with an IC_{50} value of 41.094 µg/ml; 58.072% of HeLa cells were inhibited (41.928% of cells are viable) with an IC_{50} value of 59.061 µg/ml. In the case of 100% concentration of methanolic extract, 60.69% of MCF-7 cells were inhibited (39.31% of cells are viable) with an IC_{50} value of 26.211 µg/ml; 58.70% of HeLa cells were inhibited (41.3% of cells are viable) with an IC_{50} value of 52.167 µg/ml (Table 27.2). Here also, MCF-7 cells were more prone to death to methanolic extract when compared to HeLa cell lines. Hence, *in vitro* cytotoxicity of methanolic extract was highest against the MCF-7

Table 27.1 Cytotoxic properties of ethyl acetate extract of orchids on MCF-7 and HeLa cell lines

Cell line	Species	Concentration (ug/ml)		Absorbance at 570 nm		Average	Average−blank	% of growth inhibition	IC_{50} (ug/ml)
MCF-7	*A. praemorsa*	100	0.805	0.807	0.809	0.807	0.8	61.128	49.276
		75	0.898	0.9	0.902	0.9	0.893	56.60	
		50	1.052	1.054	1.056	1.054	1.047	49.12	
		25	1.161	1.163	1.164	1.162	1.155	43.87	
		10	1.195	1.197	1.198	1.196	1.189	42.22	
		5	1.235	1.237	1.239	1.237	1.23	40.23	
		Control	2.065	2.066	2.065	2.065	2.058	0	
		Blank	0.007	0.008	0.007	0.007	0		
	A. odarata	100	0.792	0.794	0.796	0.794	0.787	61.759	41.094
		75	0.889	0.891	0.893	0.891	0.884	57.046	
		50	0.993	0.995	0.997	0.995	0.988	51.993	
		25	1.105	1.107	1.109	1.107	1.1	46.55	
		10	1.161	1.163	1.165	1.163	1.156	43.829	
		5	1.185	1.187	1.188	1.186	1.179	42.712	
		Control	2.065	2.066	2.065	2.065	2.058	0	
		Blank	0.007	0.008	0.007	0.007	0		

HeLa	*A. praemorsa*	100	0.815	0.817	0.819	0.817	0.812	57.44	61.681
		75	0.899	0.901	0.903	0.901	0.896	53.04	
		50	0.967	0.969	0.971	0.969	0.964	49.47	
		25	1.091	1.093	1.095	1.093	1.088	42.97	
		10	1.178	1.18	1.182	1.18	1.175	38.42	
		5	1.243	1.245	1.247	1.245	1.24	35.02	
		Control	1.913	1.914	1.913	1.913	1.908	0	
		Blank	0.005	0.006	0.005	0.005	0		
	A. odarata	100	0.803	0.805	0.807	0.805	0.8	58.072	59.061
		75	0.891	0.893	0.895	0.893	0.888	53.46	
		50	0.975	0.977	0.978	0.976	0.971	49.019	
		25	1.08	1.082	1.084	1.082	1.077	43.554	
		10	1.162	1.164	1.165	1.163	1.158	39.309	
		5	1.196	1.197	1.199	1.197	1.192	37.527	
		Control	1.913	1.914	1.913	1.913	1.908	0	
		Blank	0.005	0.006	0.005	0.005	0		

Table 27.2 Cytotoxic properties of methanolic leaf extracts on MCF -7 and HeLa cell lines

Cell line	Orchid extract	Concentration (ug/ml)	Absorbance at 570 nm			Average	Average−blank	% of growth inhibition	IC_{50} (ug/ml)
MCF-7	*A. praemorsa*	100	0.854	0.856	0.857	0.855	0.848	58.795	55.904
		75	0.933	0.935	0.937	0.935	0.928	54.908	
		50	1.065	1.067	1.068	1.066	1.059	8.543	
		25	1.174	1.176	1.178	1.176	1.169	43.198	
		10	1.205	1.207	1.209	1.207	1.2	41.691	
		5	1.278	1.279	1.281	1.279	1.272	38.193	
		Control	2.065	2.066	2.065	2.065	2.058	0	
		Blank	0.007	0.008	0.007	0.007	0		
	A. odorata	100	0.814	0.816	0.818	0.816	0.809	60.69	26.211
		75	0.871	0.873	0.875	0.873	0.866	57.93	
		50	0.922	0.924	0.925	0.923	0.916	55.49	
		25	0.995	0.997	0.998	0.996	0.989	51.94	
		10	1.068	1.07	1.072	1.07	1.063	48.35	
		5	1.176	1.178	1.179	1.177	1.17	43.14	
		Control	2.065	2.066	2.065	2.065	2.058	0	
		Blank	0.007	0.008	0.007	0.007	0		

HeLa	*A. praemorsa*	100	0.881	0.883	0.885	0.883	0.878	53.984	76.94
		75	0.956	0.958	0.959	0.957	0.952	50.105	
		50	1.045	1.047	1.049	1.047	1.042	45.388	
		25	1.158	1.159	1.161	1.159	1.154	39.518	
		10	1.215	1.217	1.219	1.217	1.212	36.478	
		5	1.295	1.297	1.299	1.297	1.292	32.286	
		Control	1.913	1.914	1.913	1.913	1.908	0	
		Blank	0.005	0.006	0.005	0.005	0		
	A. odorata	100	0.791	0.793	0.795	0.793	0.788	58.70	52.167
		75	0.85	0.852	0.854	0.852	0.847	55.60	
		50	0.963	0.965	0.967	0.965	0.96	49.68	
		25	1.036	1.038	1.039	1.037	1.032	54.08	
		10	1.105	1.107	1.109	1.107	1.102	42.24	
		5	1.181	1.183	1.185	1.183	1.178	38.26	
		Control	1.913	1.914	1.913	1.913	1.908	0	
		Blank	0.005	0.006	0.005	0.005	0		

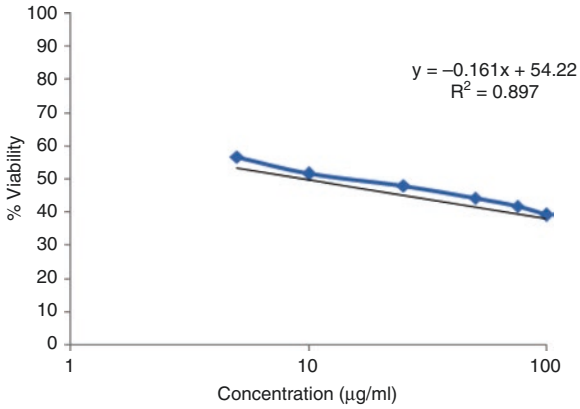

Fig. 27.3 Highest cytotoxic effect of the methanolic extract of *A. odorata* on the MCF-7 cell line

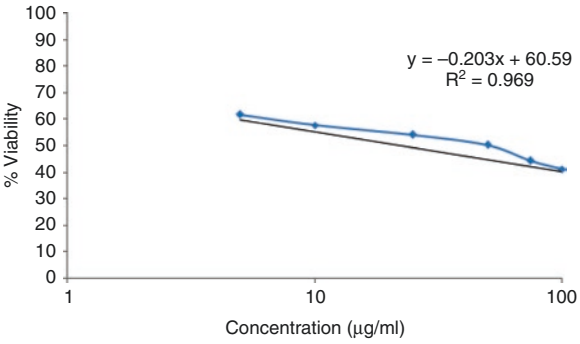

Fig. 27.4 Poor cytotoxic effect of the methanolic extract of *A. praemorsa* on the HeLa cell line

cell line with the least IC_{50} value of 26.211 µg/ml (Table 27.1), which was enough to kill 50% of MCF-7 cancer cells (Fig. 27.2). Poor *in vitro* cytotoxicity was seen in the ethyl acetate extract against the HeLa cell line with an IC_{50} value of 59.061 µg/ml.

In the two orchids studied here, the higher *in vitro* cytotoxicity was observed against the MCF-7 cell line through the methanolic extract of *A. odorata*; poor *in vitro* cytotoxicity was seen against the HeLa cell line through the methanolic extract of *A. praemorsa*.

27.2.3 Orchid Chemicals as Anticancer Agents

Cancer is associated with abnormal, uncontrolled cell growth. It is a group of diseases caused by the loss of cell cycle control. In the contemporary world, breast cancer is the most commonly occurring cancer among women around the world, and the current available therapies are not safe as they are toxic to normal cells, along with cancerous cells. The plant extracts' preparation shows a potential

anticancer effect for the treatment of different types of cancer (Sivaraj et al. 2014). Experimental works have been conducted for many years across the world, and various chemical compounds were isolated from these plant extracts, which possess pharmacological activities. A wide variety of orchid chemicals, such as alkaloids, flavonoids, terpenoids, tannins, steroids, phenols and glycosides, have been isolated (Mari Suji and Christudas 2016). Denbinobin, a naturally occurring phenanthroquinone isolated from the genus *Dendrobium*, is known to have antioxidant activity against lung carcinoma, human ovary adenocarcinoma and human promyelocytic leukemia cell lines (You et al. 1995). Phytochemical analysis of *A. praemorsa* was done earlier by Maridas et al. (2008), and they found that cyanogenic glycosides and flavonoids were present in *A. praemorsa*. Further, *A. praemorsa* has been reported to contain a phenanthropyran derivative, i.e. praemorsin (1, 7-dihydroxy-3-methoxy-9, 10-dihydrophenanthropyran) (Anuradha and Prakash 1994).

A bibenzyl derivative, moscatilin, isolated from *Dendrobium loddigesii*, has anticancer properties (Ho and Chen 2003). This compound was originally purified from the Indian orchid *D. moschatum* (Majumder and Sen 1987). Another compound, kinsonoside isolated from *Anoectochilus formosanus*, has got diverse pharmacological effects, including the repression of tumour growth (Du et al. 2000, Yoon et al. 2007). Similarly, bibenzylgigantol extracted and purified from *D. draconis*, prevents the development of stem-like phenotypes in human lung cancer cells and adversely affects tumour cell viability (Bhummaphan and Chanvorachote 2015). Further, terpenoid and phenolic groups of compounds isolated from *D. lasianthera* and *Arachnis flas-aeris* have cytotoxic efficacy against T47D breast cancer cells. Prasad and Koch (2014) studied the antitumour properties of the ethanolic extract of *D. formosum* and suggested an alternative in the treatment of cancer. Prasad et al. (2017) observed that the ethanolic extract of *D. chrysanthum* showed dose-dependent cytotoxic effect against the HeLa cell line. In the present study, the methanolic extract of *Aerides odorata* showed the highest cytotoxic effect on the MCF-7 cell line, whereas poor cytotoxicity was recorded in regard to the methanolic leaf extract of *Acampe praemorsa* against the HeLa cell line.

27.3 Conclusion and Future Perspectives

The results of our study evidently demonstrated the cytotoxic activity of the ethyl acetate and methanolic leaf extracts of *A. praemorsa* and *A. odorata.* Thus, both the extracts seem to possess profound cytotoxic activity against cancer cell lines. The methanolic extract of *A. odorata* against MCF-7 cell lines reveals that the plant had the highest anticancer activity, whereas the methanolic extract of *A. praemorsa* against HeLa cell lines had shown poor anticancer activity. Further research is to be carried out to fractionate and purify the extract to find the molecules responsible for the anticancer activity. More effort is needed to explore drugs that attack cancerous cells without causing damage to normal cells and to save humans.

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Phytochemical Screening and Evaluation of Antimicrobial Potential of *Dendrobium fimbriatum* Hook

28

Sankar Narayan Sinha and Karabi Biswas

Abstract

Research on medicinal plants is important to Indian subcontinent because most of its rural population relies on it as a mode of medicine. Orchids have tremendous potential in horticulture and pharmacognosy; five *Dendrobium* species are included in the Chinese pharmacopoeia. They are assumed to be effective in some diseases or syndromes related to the deficiency of yin in the kidney, lung and stomach. They are also used as remedies for fever, red tongue, atrophic gastritis and diabetes, but this potential has remained largely untapped in India. Many orchids were used in Chinese traditional medicine as a remedy for a number of treatments. Medicinal plants, such as the orchid *Dendrobium fimbriatum*, were collected from the Manipur University campus. The present study was undertaken to find the antimicrobial activity and phytochemical profile in different extraction media. The percentage yield from the orchid was highest in warm ethanol extraction with 12.6%, followed by ethyl acetate and lowest in cold ethanol.

Plant extract showed the presence of antioxidants like alkaloids, terpenoids, flavonoids, tannins and glycosides. The antibacterial activity of the ethanol extract of *D. fimbriatum* showed significant bioactivity by inhibiting the growth of microbial species selected for the test. The zone of inhibition shown by the extracts was comparable to the standard antibiotics. Therefore, the present study indicated that *D. fimbriatum* possesses good antibacterial activities. Further work is needed to explore the active principle from the plant extracts for further pharmaceutical studies.

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Keywords
Dendrobium fimbriatum · Phytochemical screening · Antimicrobial potential

28.1 Introduction

Orchids are the most extravagant group of flowering plants in nature distributed throughout the world from the tropics to high alpine. They exhibit an incredible range of diversity in the size, shape and color of their flowers. Though orchids are grown primarily as ornamentals, many are used as herbal medicines and food, and others have cultural value among different cultures and tribes in different parts of the world.

Orchids have been used in many parts of the world in traditional healing systems, as well as in the treatment of a number of diseases since the ancient time. Though Orchidaceae is regarded as the largest family of plant kingdom, a few studies have been done regarding their medicinal properties.

Dendrobium fimbriatum is a large-sized, warm-to-cold growing epiphyte, lithophyte or terrestrial orchid with long, erect, arching or pendulous, light-yellow green with aged long stems that are thickest in the middle and many oblong to lanceolate, acute or acuminate, deciduous leaves. Flowering occurs in March through May but can bloom at most any time on a pendant, axillary, many [6–15] flowered raceme Flower arises from the nodes near the apex of leafless and mature canes and has sour smelling flowers.

D. fimbriatum Hooker. is an important orchid traditional medicine used for resetting fractured bones and possesses antioxidant activity. It has been reported to possess constituents like rhein, fimbriatone etc. However, there is a dearth of scientific data on its phytochemical and pharmacological evaluation. Thus, on the basis of published reports, the recent study was aimed to evaluate the phytochemical profiles and antibacterial potentials of the finger-lipped orchid *D. fimbriatum.*

28.1.1 Plant Materials and Preparation of Solvent Extracts

The leaves and flowers of *Dendrobium fimbriatum* were collected in April 2015 from the Manipur University campus, Imphal, Manipu, India (Table 28.1) and was identified and authenticated by Prof. S. K. Mukherjee, Plant Taxonomist, Department of Botany, University of Kalyani, Kalyani, India. A voucher specimen (Voucher no. sns/bot/15) was deposited in the herbarium of the university. Since the same species in various ecological locations may not have the same antibiotic or antimicrobial activity, information regarding the time and place of collection, their state of maturity, habitat and altitude are recorded.

Before extraction, the plant materials were freed off adhering soil particles and washed in running tap water, followed by distilled water. The plant (leaves and

Table 28.1 Collection details of plant specimen

Name of the plant with family	Site of collection	Altitude (msl)	Time of collection	Temperature on the day of collection (°C)	Topography	Soil type
Dendrobium fimbriatum (Orchidaceae)	Manipur University campus, Manipur, India (24°48′50.2812″N and 93° 57′1.0044″ E)	784 m	April 2015	22	Hilly area	Rocky soil

flowers) was shade dried at ambient temperature (30 °C) and was powdered using an electronic blender. Solvent extracts were prepared by dipping the powdered material in 600 ml of each of the solvents, viz., ethanol (warm and cold) and ethyl acetate in a soxhlet apparatus for 72 h at 30 °C until complete extraction.

The extract was examined for sterility after Milipore filtration by passing 2 ml of the sterile extract mixed with 10 ml of sterile nutrient broth. This was incubated at 37 °C for 24 h. A sterile extract was confirmed by the absence of turbidity or the transparency of the broth medium after the incubation period (Ronald 1995).

The extraction yield (mg of dry extract per g of lyophilised sample) was determined. A portion of each ethanolic extract was evaporated (Buchi R-215) under vacuum at 40 °C and then dried in an air oven at 105 °C for 3 h. The final dry weight was used to calculate extraction yield.

$$\text{Extraction yield} = \text{Wt of extracted material} / \text{wt of raw material} \times 100$$

28.1.2 Bacterial Strains and Antibacterial Activity

Three Gram-positive (*Bacillus subtilis*, *Staphylococcus aureus* and *Micrococcus luteus*) and five Gram-negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*, *Vibrio cholerae*, *Shigella dysenteriae* and *Shigella flexneri*) were used as test organisms in this study. All the bacterial cultures were procured from ID and BG Hospital, Kolkata. The bacterial strains were maintained on nutrient agar slant at 4 °C and were subcultured before use.

To evaluate the antibacterial activity of various solvent extracts of different parts of the *Dendrobium fimbriatum*, standard agar well diffusion assay was carried out. The plates were incubated at 37 °C for 24 h for antibacterial activity by the appearance of a zone of inhibition surrounding the well. Each test was conducted three times, and antibacterial potential was expressed as the mean of diameter of the inhibition zones(mm) produced by different extracts when compared to controls.

28.1.3 Phytochemical Tests

The phytochemical screening of the secondary metabolites alkaloids, terpenoids, flavonoids, tannins, glycosides, saponins, phenols, steroids and anthraquinones was done for the leaf and floral extracts using the prescribed methods (Harborne 1983; Trease and Evans 1989). For the alkaloid test, 2 ml of the extract was measured in a test tube, to which picric acid solution was added. The formation of orange colouration indicated the presence of alkaloids. For the terpenoid test, 0.2 g extract was taken, to which 2 ml chloroform and 3 ml of concentrated H_2SO_4 was added carefully to form a layer. A reddish brown colouration formed at the interface indicates the presence of terpenoids. For the flavonoid test, 5 g of the sample was soaked and completely extracted with acetone. The residue was extracted in warm water after evaporating acetone on a water bath. The mixture was filtered and the filtrate was used for the test. Exactly 5 ml of 10% sodium hydroxide was added to an equal volume of the extract. The appearance of a yellow solution indicated the presence of flavonoids. For tannins, 3 g of the sample was boiled in 50 ml distilled water for 30 min on a hot plate. The mixture was filtered, and a portion of the filtrate was diluted with sterile water in a ratio of 1:4, and three drops of 10% ferric chloride solution were added. A blue or green colour indicated the presence of tannins. For the glycoside test, 25 ml of 1% sulphuric acid was added to 5 ml of the extract in a test tube and boiled for 15 min, cooled and neutralised with 10% sodium hydroxide, and then 5 ml of Fehling solution A and B was added. A brick red precipitate of reducing sugars indicated the presence of glycosides. For the saponin test 1 g of the sample containing 10 ml of sterile distilled water in a conical flask was boiled for 5 min. Following filtration, 2.5 ml of the filtrate was added to 10 ml of sterilised distilled water in a test tube. The test tube was stopped and then shaken vigorously for about 30 s. After 30 min, honey comb froth appeared, which indicated the presence of saponins. For the phenol test, 25 ml of extract was added to 2 ml of ferric chloride solution; the formation of a deep bluish green solution indicates the presence of phenol. To test the steroids, 2 ml of acetic anhydride was added to 0.5 g of the extract, along with 2 ml of H_2SO_4. The colour changes from violet to blue, indicating the presence of steroids. Finally, for anthraquinones, 0.5 g of the plant extract was shaken with 10 ml of aqueous H_2SO_4 and then filtered while hot, then 5 ml of benzene was added to the filtrate and shaken; the benzene layer was separated and half of its volume—5 ml of 10% ammonia solution—was then added. The presence of violet, pink or red colouration in the ammonical (lower) phase indicates the presence of anthraquinones.

28.2 Phytochemical Screening and Antimicrobial Efficacy of *Dendrobium fimbriatum*

The plant was collected from the hilly region of Manipur in April 2015. This medicinal orchid is a very good source of phytochemicals, which are responsible for various medicinal properties. Plant details, along with their medicinal value and active constituents, are depicted in Table 28.2.

The results of the present study showed that different solvents had significant effect on the extractable solid yields of different parts. Highest yields of extractable solid residue was found in warm ethanol extract (12.6%) of leaf of *D. fimbriataum* followed by ethyl acetate and cold ethanol extract (11.5% and 10.7%). Flower of this orchid using warm ethanol extract showed highest solid yields (8.8%) followed by ethyl acetate and cold ethanol extract (8.01%, and 6.3%). Table 28.3 shows the presence of phytochemicals in this orchid extracts. It contained alkaloids, terpenoids, flavonoids, tannins, glycosides, saponins, phenols, and anthraquinone. These metabolites are present in different parts are known to have varied pharmacological action in human and animals (Ndukwe et al. 2007).

The results of the antibacterial activity screening of the orchid extracts are shown in Tables 28.4, 28.5 and 28.6. The antibacterial activity of the ethanol and ethyl acetate extracts of the leaves and flowers of *Dendrobium fimbriatum* was noted against eight human pathogenic bacteria, such as *Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Vibrio cholera*e, *Shigella*, *dysenteriae* and *Shigella flexneri*. However, maximum activity was exhibited by the ethanolic extracts, particularly the warm ethanolic extract of the leaves and flowers of *D. fimbriatum*.

In the present investigation, the ethanolic extract showed better antimicrobial activity, which might be due to the easy release of the bioactive components in this solvent that are found to be potent antibacterial agent. The inhibition of all Gram-positive and Gram-negative bacteria by the extraction indicated the presence of various broad spectrum antimicrobial substances. Usually, Gram-negative bacteria are more resistant than Gram-positive bacteria (Tortora et al. 2001; Yao and Moellering 1995), but in the present investigation, all the tested Gram-negative bacteria were found to be inhibited significantly by the crude solvent extracts of the plants, which indicated that these extracts might be used to treat human pathogen. Present studies showed that these extracts might be able to penetrate the outer membrane of Gram-negative pathogenic bacteria.

Table 28.2 Plant details for the present study

Name of the plant	Family	Brief description	Important chemical constituents	Medicinal properties
Dendrobium fimbriatum	Orchidaceae	It is medium to large-sized, warm-to-cold growing epiphyte, lithophyte or terrestrial found in north East India, from the Western Himalayan range to the Malaysian peninsula. This species is deciduous in nature and remains dormant during winter and blooms in early spring on the upper nodes of its leafless canes; each raceme can have up to 20 fragrant flowers; epiphytes: Stems are as long as 2–4 ft, tapering towards the apex, sub-erect; leaves are oblong/lanceolate, pointed at its apex and narrowed towards the base, 4–7 cm long and 1–2 cm in width at its mid portion.	Fimbriatone, confusarin, crepidatin, physcion, rhein, ayapin, scopolin, methylether, n-octacostyl ferulate	The leaves of the plants may be either boiled or soaked in warm water, which can be consumed as tonic as it promotes the production of body fluid. It can also be applied on fractured areas to set cracked bones.
		Racemes appear laterally from the nodes of the leafy stems, pendulous with 5–9 flowers; the peduncle has many sheathing bracts at its base; flowers are 3–4 cm across; sepals and petals have bright yellow lip of the same colour with a large orbicular reddish-brown patch around their middle portion; sepals are oblong, bluntly spreading; petals are wider than the sepals, oblong, lip orbicular with undulate and fringed margins.		It is also used as remedies for fever, red tongue, atrophic gastritis and diabetes.

Table 28.3 Phytochemical analysis of *D. fimbriatum*

	Cold ethanolic extract		Warm ethanolic extract		Ethyl acetate extract	
Phytochemical	Leaves	Flowers	Leaves	Flowers	Leaves	Flowers
Alkaloids	+	+	+	+	+	+
Terpenoids	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+
Tannins	+	+	+	+	+	+
Glycosides	+	+	+	+	+	+
Saponins	+	+	+	+	+	+
Phenols	+	+	+	+	+	+
Steroids	−	−	−	−	−	−
Anthraquinones	+	+	+	+	+	+

+ indicates presence or positive reactions; − indicates absence or negative reactions

Table 28.4 Antibacterial activities of warm ethanolic extracts of *Dendrobium fimbriatum*

	Zone of inhibition (mm)							
Plant parts	Bs	Ml	Sa	Pa	Ec	Vc	Sd	Sf
Leaves	22	18	17	14	15	12	11	10
Flowers	20	17	17	13	14	11	10	8

Bs Bacillus subtilis, *Ml Micrococcus luteus*, *Sa Staphylococcus aureus*, *Pa Pseudomonas aeruginosa*, *Ec Escherichia coli*, *Vc Vibrio cholera*e, *Sd Shigella dysenteriae*, *Sf Shigella flexneri*

Table 28.5 Antibacterial activities of the ethyl acetate extracts of *D. fimbriatum*

	Zone of inhibition (mm)							
Plant parts	Bs	Ml	Sa	Pa	Ec	Vc	Sd	Sf
Leaves	16	14	13	12	13	10	10	9
Flowers	14	12	11	11	11	9	9	8

Bs Bacillus subtilis, *Ml Micrococcus luteus*, *Sa Staphylococcus aureus*, *Pa Pseudomonas aeruginosa*, *Ec Escherichia coli*, *Vc Vibrio cholera*e, *Sd Shigella dysenteriae*, *Sf Shigella flexneri*

Table 28.6 Antibacterial activities of the cold ethanolic extracts of *D. fimbriatum*

	Zone of inhibition (mm)							
Plant parts	Bs	Ml	Sa	Pa	Ec	Vc	Sd	Sf
Leaves	15	14	12	11	12	9	9	8
Flowers	12	11	10	10	9	8	8	8

Bs Bacillus subtilis, *Ml Micrococcus luteus*, *Sa Staphylococcus aureus*, *Pa Pseudomonas aeruginosa*, *Ec Escherichia coli*, *Vc Vibrio cholera*e, *Sd Shigella dysenteriae*, *Sf Shigella flexneri*

The results of the study also showed that the flower extracts of *Dendrobium fimbriatum* are potentially good antibacterial agents and support the traditional application of medicinal plant. More research is needed to isolate bioactive substances from this orchid.

28.3 Conclusion

The antibacterial activity of the ethanolic and ethyl acetate extracts of the leaves and flowers of *Dedrobium fimbriatum* used in traditional medicine was tested against eight species of bacteria, such as *Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Vibrio cholera*e, *Shigella dysenteriae* and *Shigella flexneri*. All the solvent extracts showed significant antibacterial activity against most of the bacteria tested.

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