Pragya Tiwari
Jen-Tsung Chen *Editors*

Advances in Orchid Biology, Biotechnology and Omics

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Editors
Pragya Tiwari
Department of Biotechnology
Yeungnam University
Gyeongbuk, Korea (Republic of)

Jen-Tsung Chen
Department of Life Sciences
National University of Kaohsiung
Kaohsiung, Taiwan

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Preface

Orchids comprise the most exotic and multi-colored group of flowering plants, classified in the family Orchidaceae. The multi-faceted attributes and promising socio-economic applications in the present era have commercialized orchid cultivation and global trade, substantially improved via the advances in high-throughput technologies, omics biology, and metabolic engineering approaches. Widely cultivated for ornamental purposes as the cut flower and artificially propagated varieties, the present decade has witnessed the popularity of orchids on a global level, with researchers investigating the multi-faceted attributes and applications of orchids in the food sector, healthcare, and industries. Novel and high-value varieties of orchids are being developed via substantial contributions in advanced plant tissue culture techniques, plant breeding, and more recently, the genetic manipulation studies in orchids for plant trait improvement and value addition.

Orchids include approximately 30,000–35,000 species, which are found in diverse habitats across the world. The very first report suggested that the Chinese were the pioneers in the cultivation and description of orchids, with the description of *Bletilla striata* and *Dendrobium* species in the book, *Materia Medica* of the twenty-eighth-century BC by a Chinese legend. In addition, traditional medicine systems, like Ayurveda also reports the extensive usage of orchid species for therapeutic purposes. Some distinct characteristics of the orchid—adaptive mechanisms, mycorrhiza-dependent germination, perennial nature, and absence of woody structures and the flowers include bilateral symmetry (zygomorphism), resupinate flowers, fused stamen and carpels, and highly modified petals (labellum). Furthermore, orchids exhibit monopodial (stem grows from a single bud, with the growth of new leaves on the apex every season) and sympodial growth (adjacent shoots are produced, grow to a certain size, bloom, and then replaced), growing laterally and following the surface for support. The orchids usually flower in the spring season and some of the key species that are grown as ornamentals include *Renanthera*, *Paphiopedilum, Cattleya, Phragmipedium, Dendrobium,* and *Vanilla* sp.

The cultivation and demand of exotic orchid varieties have witnessed a tremendous upsurge, attributed to the improved understanding and knowledge in areas of

orchid biology, classification, phytochemistry, and cultivation strategies, among other areas. Plant tissue culture and traditional plant breeding approaches form the basis of orchid cultivation, contributing immensely to the cultivation of exotic orchid varieties, however, multiple challenges including slow growth, complex orchid genomes, and poor efficiency of transformation are major limitations. The classical plant breeding approaches comprising crossbreeding and mutational breeding, molecular marker-assisted breeding, in vitro orchid propagation, and cryopreservation have addressed these challenges to a considerable extent. These traditional approaches also provided a sound platform for introducing genetic manipulation of novel orchid varieties for trait improvement. The last decade has witnessed the extensive application of plant tissue culture techniques for the propagation and conservation of orchids, *via* utilizing different approaches and explants, namely shoot nodes, stems, flower stalks, root tips, etc. facilitating the translational success of several varieties. Conventional breeding approaches in orchid propagation and conservation have witnessed key translational success in the development of novel varieties as well as conservation of the species with novel attributes.

In this direction, efforts were also made to understand the molecular mechanisms of orchid mycorrhizal symbiosis for elucidating genetic information. While the plant–fungal interactions are key to orchid development, the association of fungal endophytes and their prospects in the production of antimicrobials highlight key prospects in the discovery and development of novel antimicrobials. Another interesting contribution aims to discuss the societal impact of some medicinal orchids, providing valuable insights into the history and ethnomedicinal uses and the prospects of socio-economic applications in healthcare. *Catasetum* genus consists of showy epiphytic orchids, defines novel attributes, and is highly prioritized in horticulture; however, most of the species are difficult to cultivate without a greenhouse. The conservation of members in the *Catasetum* genus, therefore, requires immediate attention and conservation via biotechnological strategies, as discussed in a key literature contribution.

In recent times, genetic engineering approaches have focused on trait improvement by creating novel hybrids of genera, for example, *Oncidium*, *Vanda*, *Phalaenopsis*, *Cymbidium*, and *Dendrobium*, among others. *Agrobacterium*-mediated transformation of orchids has been the most successful technique to date creating novel transgenics in orchid genera like *Oncidium*, *Vanda*, *Dendrobium*, and *Phalaenopsis.* In addition, to overexpression of key genes in heterologous systems for desired traits, gene silencing studies have also been attempted in orchids like *Oncidium* and *Dendrobium* species. The biotechnological interventions in different orchid varieties have focused on the alteration of flower fragrance, color, disease resistance, and shelf-life, aiming for improved plant traits and varieties. A few key examples of transgenic orchid varieties include RNAi-based gene silencing in *Phalaenopsis equestris* for flower color, gene overexpression in *Dendrobium Sonia* for altering orchid morphology, and organogenesis and *in vitro* development *via* permanent magnetic fields in *Phalaenopsis* species, among others. The scientific approaches have made remarkable contributions to the development of exotic

varieties displaying multi-faceted attributes, namely novel plant traits, different color patterns, and disease resistance, among others.

In the present era, orchid cultivation has witnessed a tremendous upsurge attributed to their recognition as food ingredients, floriculture, and/in healthcare. Moreover, omics and computational approaches have significantly improved our understanding of different concepts in orchid biology via better insights into the metabolic pathways and their roles in the biosynthesis of diverse metabolites and physiological mechanisms in orchid biology. While proteome analysis of orchid species focused on flower development and micropropagation methods, while the omics approaches have identified the developmental stages in orchid biology and improved orchid breeding, conservation, and commercialization of novel varieties. With the emerging importance and multi-faceted role of orchids in floriculture, the food sector, and healthcare, the respective book aims to discuss the recent advances/developments in orchid biology, biotechnology, and omics approaches. The book provides further insights into the progress and the prospects in orchid breeding, the importance of key medicinal orchids and their societal impact, and how the association of the fungal endophytes with members of Orchidaceae defines key prospects as antimicrobials in drug discovery, an interesting yet less-explored area of investigation in orchids. Some prospective chapters discuss specific examples in detail including ethnomedicinal, phytochemistry, and biotechnological strategies for the conservation of Orchids in the *Catasetum* genus, and some terrestrial orchids. The book provides valuable insights and contributions from renowned experts in orchid biology and biotechnology from all over the world, with 9 chapters discussing different sub-themes of wider significance and applications in orchid biology.

This book provides comprehensive insights into the existing and emerging trends in orchid biology and discusses the advances/contribution of omics, plant breeding, and biotechnological approaches in this interesting field. In addition, it aims to bridge the gaps in knowledge deficiencies and provide a combined platform discussing multi-faceted areas of orchid biology and biotechnology in a single book. With the development of high-throughput approaches and omics interventions, orchids have gained enormous popularity in socio-economic applications and witnessed a global demand for exotic varieties. Therefore, the respective book will play a key role in providing an excellent basis for graduate, and post-graduate students, Ph.D. scholars, and researchers, to improve and widen their scientific knowledge in the field of orchid biology, updates on biotechnological/omics approaches in orchid cultivation and how these developments project to remarkably impact orchid industry and commercialization on a global platform. With this aim, the book brings together high-quality chapters from eminent researchers/experts across the world and hopes to serve as a platform of literature for future initiatives in orchid biology. Finally, the editors would like to thank the effort of all authors for organizing their chapters and the assistance and instructions from the editorial office of the publisher are much appreciated.

Gyeongbuk, Republic of Korea — Pragya Tiwari
Kaohsiung, Taiwan — Jen-Tsung Chen

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Editors and Contributors

About the Editors

Pragya Tiwari is working as Research Professor in the Department of Biotechnology, Yeungnam University, Republic of Korea. She possesses research/teaching experience of more than 8 years in national/international institutions in the field of plant sciences, with research experience in areas of phytomolecules from medicinal plants, plant-associated endophytes, plant-microbe interactions, and computational biology in drug designing. She has been a recipient of various research fellowships/recognitions from CSIR and ICAR, Govt. of India, and gained scientific recognitions for her work in inter-disciplinary areas of plant sciences. She has been an active participant/presenter in more than 30 symposiums/conferences on international/national platforms and holds life memberships of scientific societies including Korean Society of Biotechnology and Bioengineering, Indian Science Congress Association, and The Society of Biological Chemist, India. Her research work has gained broad interest through highly cited publications, book chapters, and invited lectures. Her area of research comprises plant-endophyte interactions in *Panax ginseng* and the significance of bioactive metabolite (ginsenoside) in promoting plant growth and as bio-stimulants in sustainable agriculture.

Jen-Tsung Chen is currently a professor at the National University of Kaohsiung in Taiwan. He teaches cell biology, genomics, proteomics, medicinal plant biotechnology, and plant tissue culture. His research interests include bioactive compounds, chromatography techniques, in vitro culture, medicinal plants, phytochemicals, plant physiology, and plant biotechnology. He has published over 100 scientific papers and serves as an editorial board member for *Plant Methods* and *Plant Nano Biology*.

Contributors

Muhammad Adil Pharmacology and Toxicology Section, University of Veterinary and Animal Sciences, Lahore, Jhang Campus, Jhang, Punjab, Pakistan

Kolsum Ahmadzadeh Department of Horticultural Sciences and Engineering, Faculty of Agriculture, University of Kurdistan, Sanandaj, Iran
Medicinal Plants Breeding and Development Research Institute, University of Kurdistan, Sanandaj, Iran

Angel Josabad Alonso-Castro División de Ciencias Naturales y Exactas, Departamento de Farmacia, Universidad de Guanajuato, Guanajuato, Mexico

Nora E. Anghelescu Faculty of Horticulture, University of Agronomic Sciences and Veterinary Medicine of Bucharest, Bucharest, Romania

Raffaella Balestrini Consiglio Nazionale delle Ricerche-Istituto per la Protezione Sostenibile delle Piante, Torino, Italy

Khosro Balilashaki Department of Horticultural Science, Faculty of Agricultural Science, University of Guilan, Rasht, Iran

Suparna Bhattacharjee Agrotechnology and Rural Development Division, CSIR-North East Institute of Science and Technology, Jorhat, Assam, India

Papori Borah Agrotechnology and Rural Development Division, CSIR-North East Institute of Science and Technology, Jorhat, Assam, India

Jean Carlos Cardoso Department of Biotechnology, Plant and Animal Production, Lab of Plant Physiology and Tissue Culture, Center of Agricultural Sciences, Federal University of Sao Carlos (DBPVA, CCA/UFSCar), Araras, Sao Paulo, Brazil
Graduate Program of Plant Production and Associated Bioprocesses, CCA/UFSCar, Araras, Sao Paulo, Brazil

Candy Carranza-Álvarez Programa Multidisciplinario de Posgrado en Ciencias Ambientales, Universidad Autónoma de San Luis Potosí, San Luis Potosí, San Luis Potosí, Mexico
Facultad de Ciencias Químicas, Universidad Autónoma de San Luis Potosí, San Luis Potosí, San Luis Potosí, Mexico
Facultad de Estudios Profesionales Zona Huasteca, Universidad Autónoma de San Luis Potosí, Ciudad Valles, San Luis Potosí, Mexico

Luis J. Castillo-Pérez Programa Multidisciplinario de Posgrado en Ciencias Ambientales, Universidad Autónoma de San Luis Potosí, San Luis Potosí, San Luis Potosí, Mexico

Jen-Tsung Chen Department of Life Sciences, National University of Kaohsiung, Kaohsiung, Taiwan

Zahra Dehghanian Department of Biotechnology, Faculty of Agriculture, Azarbaijan Shahid Madani University, Tabriz, Iran

Silvia De Rose Dipartimento di Scienze della Vita e Biologia dei Sistemi, Università di Torino, Torino, Italy

Fatemeh Feizi Department of Horticultural Science, Faculty of Agricultural Science, University of Guilan, Rasht, Iran

Javier Fortanelli-Martínez Instituto de Investigación de Zonas Desérticas, Universidad Autónoma de San Luis Potosí, San Luis Potosí, San Luis Potosí, Mexico

Dipti Gorh Agrotechnology and Rural Development Division, CSIR-North East Institute of Science and Technology, Jorhat, Assam, India

Vahideh Gougerdchi Department of Plant Breeding and Biotechnology, Faculty of Agriculture, University of Tabriz, Tabriz, Iran

Tanmita Gupta Agrotechnology and Rural Development Division, CSIR-North East Institute of Science and Technology, Jorhat, Assam, India

Mohammad Musharof Hossain Department of Botany, University of Chittagong, Chittagong, Bangladesh

Carla Midori Iiyama Department of Biotechnology, Plant and Animal Production, Lab of Plant Physiology and Tissue Culture, Center of Agricultural Sciences, Federal University of Sao Carlos (DBPVA, CCA/UFSCar), Araras, Sao Paulo, Brazil
Graduate Program of Plant Production and Associated Bioprocesses, CCA/UFSCar, Araras, Sao Paulo, Brazil

Hironori Kaminaka Faculty of Agriculture, Tottori University, Tottori, Japan

Shamsa Kanwal Microbiology Section, University of Veterinary and Animal Sciences, Lahore, Jhang Campus, Jhang, Punjab, Pakistan

Arshpreet Kaur Department of Botany, Panjab University, Chandigarh, India

Elaheh Kavusi Department of Plant Breeding and Biotechnology, Faculty of Agriculture, University of Tabriz, Tabriz, Iran

Rabia Naeem Khan Microbiology Section, University of Veterinary and Animal Sciences, Lahore, Jhang Campus, Jhang, Punjab, Pakistan

Chihiro Miura Faculty of Agriculture, Tottori University, Tottori, Japan

Kalpataru Dutta Mudoi Agrotechnology and Rural Development Division, CSIR-North East Institute of Science and Technology, Jorhat, Assam, India

Sandip V. Pawar University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh, India

Silvia Perotto Dipartimento di Scienze della Vita e Biologia dei Sistemi, Università di Torino, Torino, Italy

Galih Chersy Pujasatria The United Graduate School of Agricultural Science, Tottori University, Tottori, Japan

Hugo Magdaleno Ramírez-Tobias Facultad de Agronomía y Veterinaria, Universidad Autónoma de San Luis Potosí, San Luis Potosí, San Luis Potosí, Mexico

Priyanka Roy Centre for Infectious Disease, CSIR-North East Institute of Science and Technology, Jorhat, Assam, India

Siddhartha Proteem Saikia Agrotechnology and Rural Development Division, CSIR-North East Institute of Science and Technology, Jorhat, Assam, India

Prasanna Sarmah Agrotechnology and Rural Development Division, CSIR-North East Institute of Science and Technology, Jorhat, Assam, India

Jaspreet K. Sembi Department of Botany, Panjab University, Chandigarh, India

Fabiano Sillo Consiglio Nazionale delle Ricerche-Istituto per la Protezione Sostenibile delle Piante, Torino, Italy

Xiaoyun Tang College of Art Colleges of Landscape Architecture, Fujian Agriculture and Forestry University, Fuzhou, China

Pragya Tiwari Department of Biotechnology, Yeungnam University, Gyeongsan, Gyeongsangbuk-do, Republic of Korea

Daniel Torres-Rico Facultad de Ciencias Químicas, Universidad Autónoma de San Luis Potosí, San Luis Potosí, San Luis Potosí, Mexico

Yavar Vafaee Department of Horticultural Sciences and Engineering, Faculty of Agriculture, University of Kurdistan, Sanandaj, Iran
Medicinal Plants Breeding and Development Research Institute, University of Kurdistan, Sanandaj, Iran

Maryam Vahedi Department of Horticultural Science, Faculty of Agricultural Sciences and Engineering, College of Agriculture and Natural Resources, University of Tehran, Tehran, Iran

Jagdeep Verma Department of Botany, Sardar Patel University, Mandi, Himachal Pradesh, India

Joe Abdul Vilcherrez-Atoche Graduate Program of Plant Production and Associated Bioprocesses, CCA/UFSCar, Araras, Sao Paulo, Brazil
Graduate Program of Plant Genetics and Breeding, Escola Superior de Agricultura Luiz de Queiroz, University of São Paulo, Piracicaba, Sao Paulo, Brazil

Vikramaditya G. Yadav Department of Chemical and Biological Engineering, University of British Columbia, Vancouver, BC, Canada
School of Biomedical Engineering, University of British Columbia, Vancouver, BC, Canada

Understanding the Molecular Mechanisms of Orchid Mycorrhizal Symbiosis from Genetic Information

Chihiro Miura, Galih Chersy Pujasatria, and Hironori Kaminaka

1 Introduction

Mycorrhiza, the oldest plant–microbe holobiont ever described, is an intricate plant–fungus relationship (Frank 2005; Selosse et al. 2017). The fungus enters the plant's root system and forms specialized structures depending on the mycorrhizal types. The earliest-to-evolve type, arbuscular mycorrhiza, is found in almost all flowering plants (Delaux et al. 2013) and is mainly characterized by the formation of tree-like hyphal structures (arbuscules), although other structures, such as vesicles, are also formed. The second type is ectomycorrhiza (ECM), which is found in several tree species, such as Pinaceae, Fagaceae, and Betulaceae (Smith and Read 2008). The third type, which is the main topic of this chapter, is orchid mycorrhiza (OM). Orchid mycorrhizal fungi penetrate orchid seeds or roots through the suspensor (Peterson and Currah 1990; Richardson et al. 1992; Rasmussen and Rasmussen 2009) or epidermal hairs (Williamson and Hadley 1970) and then form dense mycelium coils known as pelotons. Although other mycorrhizal symbioses exhibit mutualism, OM symbiosis is known as parasitism: Other mycorrhizal plants obtain minerals from fungi instead of supplying photosynthetic products to the fungi, whereas orchids depend on carbon, nitrogen, and phosphorus sources provided by OM fungi (Cameron et al. 2006, 2007; Kuga et al. 2014), at least during their germination—a characteristic classified as initial mycoheterotrophy (Merckx 2013). Most orchids indicate the dual (photosynthetic and mycoheterotrophic) carbon acquisition strategy for growth and development—a phenomenon known as partial mycoheterotrophy (Gebauer and Meyer 2003; Merckx 2013)—whereas

C. Miura · H. Kaminaka (✉)
Faculty of Agriculture, Tottori University, Tottori, Japan
e-mail: cmiura@tottori-u.ac.jp; kaminaka@tottori-u.ac.jp

G. C. Pujasatria
The United Graduate School of Agricultural Science, Tottori University, Tottori, Japan

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others have even evolved to be fully mycoheterotrophic and rely completely on mycorrhizal fungi. Such orchids are commonly leafless or achlorophyllous (Lallemand et al. 2019; Li et al. 2022).

OM fungi are mainly represented by filamentous basal orders of Agaricomycotina: Sebacinales and Cantharellales (Weiß et al. 2016; Miyauchi et al. 2020). Some of the members of these orders resemble *Rhizoctonia*, a famous plant pathogen, necessitating the name "*Rhizoctonia*-like fungi." Regardless of the taxonomical disputes, the members of this group are *Ceratobasidium*, *Sebacina*, *Serendipita*, and *Tulasnella.* However, some orchids—especially fully mycoheterotrophic ones—evolve to associate with ECM fungi or even ascomycetous fungi (Taylor and Bruns 1997; Sisti et al. 2019). They can also indirectly obtain carbon from dead wood, in which their mycorrhizal fungi grow, or simply form a mycorrhizal network with nearby living trees (Suetsugu et al. 2020). Interestingly, some orchids can even switch their mycorrhizal fungi across development stages (Umata et al. 2013; Chen et al. 2019), and OM fungi may turn parasitic against orchid seeds (Adamo et al. 2020). Thus, OM symbiosis indicates a remarkable physiological diversity among all kinds of mycorrhiza to date.

Along with traditional studies, molecular studies of OM have been advancing in recent decades, ranging from mycorrhizal diversity to physiological omics, such as transcriptomics, proteomics, and genomics. Their use is advantageous because they can reveal even the innermost physiological phenomena that are easily overlooked when using in vivo assays. However, guidelines for OM symbiosis analysis using these omics techniques are unavailable. In this chapter, the tentative methods of orchids' whole-genome sequencing (WGS) and transcriptome analysis will be introduced as well as their applications and prospects.

2 Methodology of the Genomics and Transcriptomics of Orchids

2.1 Sample Preparation, Sequencing, and Bioinformatics for Orchid Genome Sequencing

Whole-genome sequencing generally involves five steps: DNA extraction and isolation, genomic DNA library construction, sequencing, de novo assembly, and annotation (Fig. 1). Because some choices or options exist in these steps, researchers need to select suitable methods for their samples. Here, we introduce the methodologies used by researchers for orchid WGS.

(i) DNA extraction and isolation

The two main methods for genomic DNA extraction include solvent extraction, such as a modified cetyltrimethylammonium bromide protocol (Murray and Thompson 1980; Inglis et al. 2018; Hu et al. 2019), or column extraction, such as DNeasy Plant Mini Kit protocol (Qiagen) and DNAsecure Plant Kit

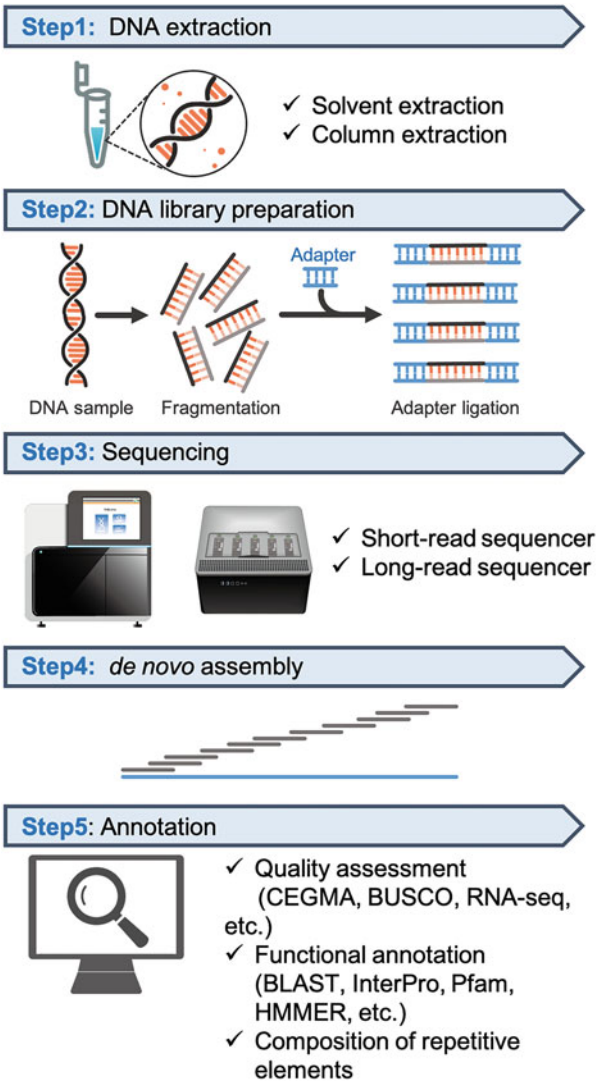

Fig. 1 Schematic overview of a whole-genome sequence analysis. The illustrations were modified and/or created with images from TogoTV (©2016 DBCLS TogoTV/CC-BY-4.0)

(TIANGEN). In any case, high-purity genomic DNA above a certain amount is necessary for obtaining high-quality sequence data. Leaves, shoots, and flowers tend to be used for DNA extraction, whereas roots, rhizomes, or bulbs are not used because these parts potentially include symbionts, except for an aseptic culture.

(ii) Genomic DNA library construction and sequencing

The two main ways to obtain WGS are short- and long-read sequencing (Goodwin et al. 2016). Regarding orchid WGS performed to date, the first method is Illumina sequence technology, and the latter is the PacBio sequel

system or Oxford Nanopore Technologies. Orchid WGS is often assembled using both short and long reads. Combining short- and long-read data improves genome assemblies of orchids whose genomes reveal a high content of repetitive elements that encompass ~82% (Li et al. 2022). How is sequencing depth achieved using these sequence technologies to produce high-quality assembled genomes? Notably, some short and long reads frequently contain sequence errors (Sims et al. 2014), which can be overcome by increasing the number of sequencing reads. A high-quality assembly of a eukaryote genome can generally be achieved based on more than ×70 sequence depths from hybrid approaches that combine short- and long-read sequencing technologies (Faino and Thomma 2014). For orchids, many studies have a coverage depth of approximately 240-fold, with at least 54-fold sequence coverage generating high-quality reference genomes (Table 1). The sequence coverage is calculated based on the estimated genome size. Although several methods exist for measuring genome size, two have mainly been conducted in the orchid WGS: flow cytometric and *k*-mer analyses. In the former analysis, the content of relative DNA extracted from leaves and stained with a fluorescent dye is compared between query and reference samples using flow cytometry (Sliwinska 2018). In the latter analysis, the genome size is estimated based on sequence data using the *k*-mer method (Simpson 2014). In this method, the read sequences are fragmented by approximately 17–31 base pieces in this manner, and the same sequence fragments are counted. The genome size is estimated based on the count distribution of these fragments (see details in Simpson (2014)). Genome size data are important for evaluating the assembled sequence quality, ploidy, and heterozygosity levels.

(iii) Assembly and annotation

De novo genome assembly tools include velvet (Zerbino and Birney 2008), SOAPdenovo (Luo et al. 2012), Abyss (Simpson et al. 2009; Jackman et al. 2017), Platanus (Kajitani et al. 2014), ALLPATHS-LG (Gnerre et al. 2011), and MaSuRCA (Zimin et al. 2013). Collected reads from orchids can be assembled using three main software tools: velvet, SOAPdenovo, and Platanus. Recently developed software, such as Canu, can enable long-read assembly, contributing to WGS accuracy (Koren et al. 2017). Repetitive element accumulation could make orchid genomic assembly challenging. Whole-genome sequencing analysis showed that repetitive elements generally occupy approximately 68% of orchid genomes or even 82% of the *Platanthera guangdongenesis* genome (Li et al. 2022). Some software tools for the analysis of repetitive elements, such as RepeatModeler/RepeatMasker (https://www.repeatmasker.org/), RepeatScout (https://github.com/mmcco/RepeatScout), and LTR_FINDER (Xu and Wang 2007), are beneficial. To improve sequencing accuracy, researchers need to select better tools according to the sequencing method and genome features.

Table 1 Summary of the published whole-genome sequencing data of orchids

Subfamily	Species	DNA ext. protocol	Sequencer	Genome assembler	Chromosomes ($2n=2x$)	Assembled genome size (Gb)	Sequence coverage (fold change)	Protein-coding genes	Repetitive elements	Reference
Apostasioideae	*Apostasia ramifera*	CTAB	Illumina HiSeq2000	SOAPdenovo2	Draft	0.36559	156	22841	44.99%	Zhang et al. (2021)
	Apostasia shenzhenica	modified CTAB	Illumina HiSeq2000, PacBio, 10X Genomics Linked-Reads	ALLPATHS-LG	68 ($n=34$)	0.349	229	21841	42.05%	Zhang et al. (2017)
Epidendroidae	*Bletilla striata*	Genomic DNA Kit (Qiagen)	PacBio Sequel II, Illumina	LACHESIS	32 ($n=16$)	2.37/2.43[a]	85.4	26673/ 26891[a]		Jiang et al. (2022)
	Cymbidium sinense	modified CTAB	GridION, Illumina?	NextDenovo	40 ($n=20$)	3.45	258	29638	77.78%	Yang et al. (2021)
	Dendrobium catenatum Lindl.	modified CTAB	Illumina HiSeq2000	SOAPdenovo2, Platanus	38 ($n=19$)	1.01	220	28910	78.10%	Zhang et al. (2016)
	Dendrobium chrysotoxum	modified CTAB	MGI-SEQ2000, PacBio, NovaSeq	Canu	38 ($n=19$)	1.37	290	30044	62.81%	Zhang et al. (2021)
	Dendrobium huoshanense	CTAB	PacBio, Illumina HiSeqX-Ten	SMARTdebovo, Pilon	38 ($n=19$)	1.285	352	21070	79.38%	Han et al. (2020)
	Dendrobium nobile	modified CTAB	MGISEQ-2000, PacBio Sequel II, MGISEQ-2000	Canu, Pilon	38 ($n=19$)	1.19	110	29476	61.07%	Xu et al. (2022)
	Dendrobium officinale	modified CTAB, DNeasy Plant Mini Kit (Qiagen)	Illumina HiSeq2000, PacBio	SOAPdenovo	Draft	1.35	125	35567	63.33%	Yan et al. (2015)
	Dendrobium officinale	modified CTAB	PacBio, Illumina Hiseq4000, HiSeq2500	Mecat2	38 ($n=19$)	1.23	208	27631	76.77%	Niu et al. (2021)

(continued)

Table 1 (continued)

Subfamily	Species	DNA ext. protocol	Sequencer	Genome assembler	Chromosomes ($2n=2x$)	Assembled genome size (Gb)	Sequence coverage (fold change)	Protein-coding genes	Repetitive elements	Reference
	Gastrodia elata	DNeasy Plant Mini Kit (Qiagen)	Illumina HiSeq2500	ALLPATHS-LG	Draft	1.06	169	18969	66.18%	Yuan et al. (2018)
	Gastrodia elata	VAHTS	PacBio Sequel II, MGI-SEQ2000	CANU	36 ($n=18$)	1.043	107	21115	66.36%	Xu et al. (2021)
	Gastrodia elata	modified CTAB and DNeasy Plant Mini Kit (Qiagen)	Illumina NovaSeq6000, PacBio	FALCON Unzip assembler v0.4	36 ($n=18$)	1.045	242	18844	74.92%	Bae et al. (2022)
	Gastrodia elata (Achlorophyllous)	DNeasy Plant Mini Kit (Qiagen)	Illumina HiSeq2000	SOAPdenovo	Draft	1.12	351	24484	68.34%	Chen et al. (2020a, b)
	Gastrodia menghaiensis	DNAsequre Plant Kit	Illumina HiSeqX-Ten, Illumina HiSeq2500, PacBio	FALCON	36 ($n=18$)	0.863	408	17948	62.57%	Jiang et al. (2022)
	Papilionanthe Miss Joaquim 'Anges'	Nanobind Plant Nuclei Big DNA Kit (Circulomics Inc)	Illumina NovaSeq 6000, GridION	Flye	38 ($n=19$)	2.5	35	31529	78.00%	Lim et al. (2022)
	Phalaenopsis aphrodite	CTAB, DNeasy Plant Mini Kit (Qiagen)	Illumina HiSeq2000/2500	ALLPATHS-LG	38 ($n=19$)	1.025	469	28902	60.30%	Chao et al. (2018)
	Phalaenopsis equestris	modified CTAB	Illumina HiSeq2000	SOAPdenovo	Draft	1.086	110	29431	62%	Cai et al. (2015)
	Phalaenopsis KHM190 cultivar	CTAB	Illumina HiSeq2000	Velvet	Draft	3.1	97	41153	59.74%	Huang et al. (2016)
Orchidoideae	*Platanthera guangdongensis*	modified CTAB	PacBio	Canu, Pilon	42 ($n=21$)	4.2	99	22559	82.18%	Li et al. (2022)
	Platanthera zijinensis	modified CTAB	PacBio	Canu, Pilon	42 ($n=21$)	4.19	99	24513	77.38%	Li et al. (2022)

Vanilloideae	*Vanilla planifolia*	modified CTAB	Illumina HiSeq4000	SOAPdenovo2, Mnia	Draft	2.2	92.4			Hu et al., (2019)
	Vanilla planifolia	KeyGene	Illumina HiSeq4000, GridION, PromethION	Miniasm	28 (n=14)	736.8/ 744.2[a]	54	29167/ 29180[a]	44.30%	Hasing et al. (2020)

[a] Haplotype A/B

2.2 *Sample Preparation, Sequencing, and Bioinformatics for Transcriptome Analysis of OM Symbiosis*

RNA-seq-based transcriptome analyses generally involve four steps: RNA extraction and purification, cDNA library preparation, RNA sequencing, and data analysis (Fig. 2). In this section, we introduce the methodologies where some choices exist for transcriptome analysis of OM associations.

(i) RNA extraction and purification

RNA is often extracted using a column method, such as the RNeasy Plant Mini Kit (Qiagen), or an organic solvent method, such as TRIzol reagent (Invitrogen). In any case, RNA-seq requires a sufficient amount of high-quality RNA. Because RNA is more unstable than DNA and environmental conditions can easily affect expression patterns, sampling methods should effectively be

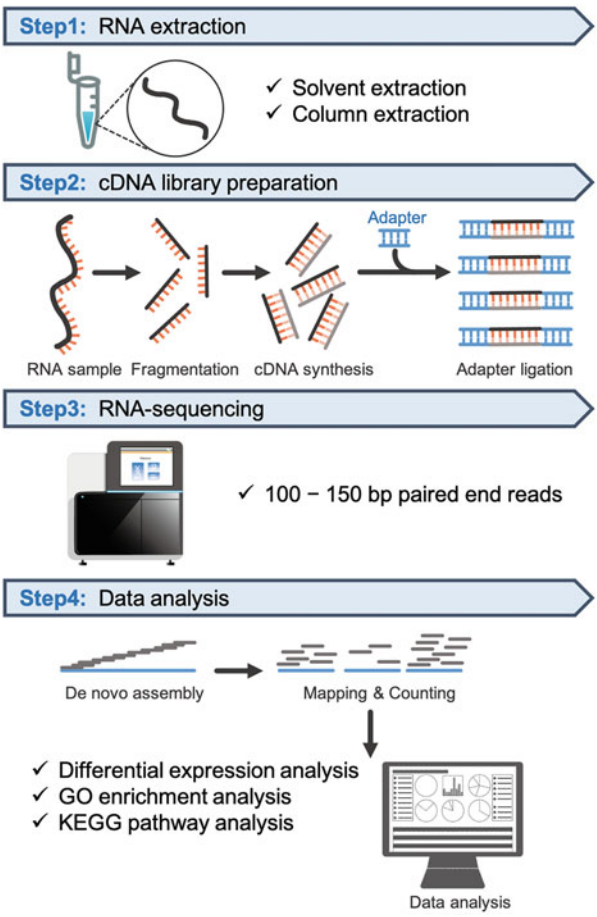

Fig. 2 Schematic overview of an RNA-sequencing analysis. The illustrations were modified and/or created with images from TogoTV (©2016 DBCLS TogoTV/CC-BY-4.0)

considered when collecting samples in situ. For example, naturally collected tissue samples should be soaked in an RNA preservation solution, such as RNAlater (Qiagen), and processed for RNA extraction as soon as possible.

(ii) cDNA library preparation and sequencing

Transcriptome analyses of OM roots or protocorms have mainly been performed using Illumina short-read sequencing platforms (Yeh et al. 2019). The cDNA libraries are prepared using commercially available kits according to the objectives of analysis: The various types of library prep kits are available, for example, the kits for strand-specific RNA-seq, for removing ribosomal RNA, and for small RNA-seq. Our primary concerns in RNA-seq experiments are the number of biological replicates and the sequencing depth required for each sample. Unfortunately, there is no clear answer to this issue (Sims et al. 2014). Lamarre et al. (2018) recommended at least four biological replicates per condition and 20-M reads per sample to be almost sure of obtaining approximately 1000 differentially expressed genes (DEGs) if they exist, according to the meta-analysis with 16 RNA-seq projects involving the tomato fruit model (*Solanum lycopersicum*). One may reason that a higher number of biological replications and sequence reads are more accurate and more sensitive to detecting DEGs, but this is often difficult to achieve, especially in the analysis of orchids in nature. Although only a few RNA-seq studies exist for mycorrhizal symbiosis using wild orchids, Suetsugu et al. (2017) and Valadares et al. (2020) performed RNA-seq analysis with three biological replicates of *Epipactis helleborine* and *Oeceoclades maculata*, respectively.

(iii) Data analysis

The bioinformatics pipelines vary depending on the available reference genome sequence. When reference genome sequences are available, data analysis is divided into the following parts: mapping and counting of reads and downstream analyses, such as differential expression, clustering, and pathway analyses. In addition to these steps, the pooled reads need to be aligned themselves to generate a de novo reference assembly when reference genome information is unavailable. The extracted RNA from symbiotic roots or protocorms contains plants and fungal RNAs. How to analyze multispecies transcriptome analysis remains controversial. Because most aligners are optimized for a single organism rather than multispecies datasets (Chung et al. 2021), the de novo assembled sequences are preferably divided into single species. Previous studies have often applied BLAST searches of the de novo assembly data against the NCBI nonredundant protein (nr) database to predict the origins of the contigs (Perotto et al. 2014; Suetsugu et al. 2017; Valadares et al. 2020, 2021). Perotto et al. (2014) examined the transcriptome of *Serapias vomeracea* protocorms inoculated with *Tulasnella calospora*. The de novo assembled transcriptomes were either compared with the NCBI-nr database using the BLSTX algorithm on the Blast2Go program (Conesa et al. 2005) with a cutoff *E* value $<1.0e{-}10$ or analyzed with an EST3 classifier, which determines the origin of sequences in mixed sequence sets by codon frequencies (Emmersen et al. 2007). Although the *T. calospora* genome has been

sequenced (Kohler et al. 2015) as a part of a DOE JGI Community Sequencing Program coordinated by F. Martin (INRA, Nancy, France), only 79 sequences (0.84%) matched *T. calospora* genes with an *E* value <1.0e−10 in Perotto's study (2014). This result reflected an extremely high degree of variability in the ribosomal DNA sequences of *Tulasnella* (Moncalvo et al. 2006; Suárez et al. 2006; Taylor and McCormick 2008; Cruz et al. 2011; Fuji et al. 2020). The transcriptome study of symbiotic *Bletilla striata* protocorms by Miura et al. (2018) utilized the assembled genome scaffolds provided from pure cultures of *Tulasnella* sp. The plant-derived sequences were confirmed by subtracting the result of a BLAST search of the assembled *Tulasnella* genome from the de novo reference assembly of the transcriptome of symbiotic protocorms. Several issues are being discussed, such as how to define an *E* value threshold for the BLAST search and how to handle unannotated sequences other than plant and fungi.

3 New Insights into the Molecular Mechanisms of OM Symbiosis

3.1 Orchid Genome Summary

The whole-genome sequences of orchids have been deposited in the NCBI (https://www.ncbi.nlm.nih.gov/data-hub/genome/?taxon=4747) or the Chinese National Genomics Data Center Genome Sequence Archive (https://ngdc.cncb.ac.cn/gsa/) for 12 species at the chromosome level and 11 species of draft genomes. These analyses estimate that the haploid genomes are 0.35–4.3 Gb, which contain approximately 25,000 protein-coding genes (Table 1). The assembled average genome size of 1.7 Gb is 4.5 and 14.2 times larger than that of rice (*Oryza sativa* cv. Nipponbare) and *Arabidopsis* (*Arabidopsis thaliana* col-0 ecotype), respectively, and approximately the same as that of tree cotton of 1.7 Gb (*Gossypium arboreum*) (Fig. 3). The orchid genomes contain a large number of repetitive sequences; that of *Platanthera guangdongensis* comprises 82% repetitive elements (Li et al. 2022), making it the most significant proportion of the orchid genome to date. The ratio is similar to *Zea mays* (approximately 85%). Although the biological function of repetitive DNA sequences remains largely unknown, these sequences are important in the regulation of mammalian gene expression (Faulkner et al. 2009). In plant species, transposable elements are important for epigenome alterations under stress (Ragupathy et al. 2013). According to RNA-seq analysis by Vangelisti et al. (2019), AM fungi induce the expression of specific retrotransposons in sunflower roots (*Helianthus annuus* L.), implying a function for retrotransposons during symbiotic interaction. Thus, a large number of repetitive sequences in orchid genomes may be involved in regulating symbiosis.

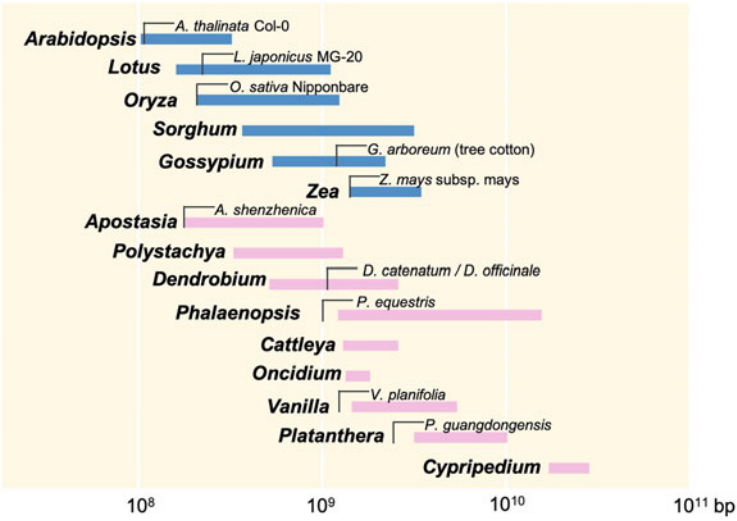

Fig. 3 Overview of plant genome sizes. The genome size ranges were estimated using the Kew Garden C-value database (https://cvalues.science.kew.org/). Flow cytometry was selected as the estimation method. The genome sizes of each plant species were based on the assembled genome size by whole-genome sequencing

Most studies of orchid WGS detected at least two whole-genome duplications (WGDs) events in Orchidaceae (Zhang et al. 2017; Xu et al. 2021, 2022; Jiang et al. 2022). Most monocots are likely to share older WGD, and younger WGD might represent an independent event specific to the Orchidaceae lineage (Zhang et al. 2017). One may infer that WGD events have driven gene family extension, thereby expanding the evolutionary potential for functional diversification. For example, a comparative genome analysis of the Venus flytrap (*Dionaea muscipula*) and its close relatives revealed that a common WGD is the source of gene recruitment to carnivory-related functions of carnivorous plants (Palfalvi et al. 2020). Orchidaceae is one of the most diverse groups of flowering plants, comprising approximately 25,000 species (Dressler 1993; Cribb et al. 2003; Chase et al. 2015). Unlike most plants, almost all orchid species are heterotrophic in their early life stages (Leake 1994). Future studies should determine whether WGD events contribute to the evolution characterizing the orchid species, such as mycoheterotrophy. However, orchids have lost some gene families, such as photosynthesis-related genes and a part of the MADS-box genes from their genomes. According to WGS analysis of leafless orchid *Gastrodia elata* and *P. guangdongensis*, the number of missing gene families was higher in the fully mycoheterotrophic orchids than in most photosynthetic plants, and many of the lost genes were involved in photosynthesis, corroborating their inability to perform photosynthesis (Yuan et al. 2018; Li et al. 2022). Most orchids lack the type I M-beta MADS-box genes involved in endosperm development initiation (Masiero et al. 2011). Almost all orchids are initially mycoheterotrophic: They produce tiny, endosperm-free seeds dependent on

mycorrhizal fungi for nutrient uptake during seed germination. The absence of M-beta genes is thought to be related to endosperm deficiency (Zhang et al. 2017). However, some orchid species undergo double fertilization and form a rudimentary endosperm (Pace 1907; Sood and Mohana Rao 1988), and the loss of M-beta may not be directly related to the loss of endosperm formation in orchids (Qiu and Köhler 2022).

3.2 Nutritional Mode or Nutrition Transport

Almost all orchids depend on carbon and other nutrients provided by mycorrhizal fungi during seed germination and subsequent early growth, which is classified as initial mycoheterotrophy. Some orchids completely depend on fungal carbon during their entire life cycle ("full mycoheterotrophy") or combine autotrophy and mycoheterotrophy at maturity ("partial mycoheterotrophy" or "mixotrophy"). The orchid genome architecture reflects their lifestyle. Fully mycoheterotrophic species, such as *P. guangdongensis*, *G. elata*, and *Gastrodia menghaiensis*, lost some photosynthesis-related genes from their nucleus genomes (Chen et al. 2020b; Jiang et al. 2022). These genes might be under "relaxed selection," where environmental change often eliminates or weakens a selection source that was formerly important for maintaining a particular trait (Lahti et al. 2009). A positive correlation may exist between the degree of heterotrophy in plants and the frequency of nonsynonymous mutations in the genes responsible for the photosynthetic process and plastid and leave functions (Chen et al. 2020b).

How do orchids acquire nutrients from symbionts under the relaxed selection of photosynthetic-related genes? On the genomic side, several studies have shown the expansion of trehalase genes in *Gastrodia* orchids, *Platanthera* orchids, *Dendrobium catenatum*, and *Phalaenopsis aphrodite* (Li et al. 2022; Jiang et al. 2022). The experiments using ^{14}C-labeled glucose by Smith (1967) suggested that orchids synthesize sucrose from fungal-derived trehalose. Ponert et al. (2021) reported that the trehalose analog validamycin A, which has a strong inhibitory effect on trehalases, reduced the growth of symbiotically germinated *Dactylorhiza majalis* (Ponert et al. 2021). Additionally, trehalase activity was increased in symbiotic protocorms (Ponert et al. 2021). They proposed that orchids metabolize and utilize fungal-derived trehalose as a carbon source, corroborating Smith's hypothesis. In transcriptomic studies, high expression of the genes encoding sugar transporters (SWEET) was detected in vitro symbiotic protocorms of *S. vomeracea* inoculated *T. calospora* AL13 (Perotto et al. 2014) and *B. striata* inoculated *Tulasnella* sp. HR1–1 (Miura et al. 2018) and in situ symbiotic roots of *Epipactis helleborine* (Suetsugu et al. 2017) and *Limodorum abortivum* (Valadares et al. 2021). A *Medicago truncatula* SWEET1b transporter contributes to arbuscule maintenance during arbuscular mycorrhizal (AM) symbiosis (An et al. 2019). Additionally, the *SWEET11* gene was highly expressed in *M. truncatula* root nodules (Kryvoruchko et al. 2016). Thus, in addition to the role of nutrient transport

in mycoheterotrophic orchids, SWEET transporters might be involved in maintaining OM symbiotic systems.

In addition to organic carbon, nitrogen is probably a major nutrient transferred to the plant from fungi (Gebauer and Meyer 2003; Hynson et al. 2013; Stöckel et al. 2014; Fochi et al. 2016), but the mechanisms remain largely unknown. According to Li et al. (2022), *Platanthera zijinensis* and *G. elata* lost a nitrate reductase (*NIA*) gene and a nitrite reductase (*NIR*) gene and *P. guangdongensis* lacked the *NIA* gene and exhibited low expression of the *NIR* gene. This suggests that these plants may not directly utilize nitrate from soil. Considering the genome's gene repertoire, nitrate compounds acquired from fungi may be glutamine or ammonium (Li et al. 2022). Gene expression profiles supported the hypothesis that organic nitrogen flows between plants and fungi during symbiosis (Zhao et al. 2014; Valadares et al. 2020, 2021). The transcriptome analysis of *S. vomeraceae* protocorms infected with *T. calospora* by Fochi et al. (2016) revealed that plant and fungal amino acids and peptide transporters were highly expressed during symbiosis establishment. Additionally, the high expression of genes associated with plant and fungal ammonia permeases and the glutamine synthetase-glutamate synthase assimilation pathway were detected in the symbiotic protocorms. The authors suggest that organic nitrogen is mainly transferred to the plant and that ammonium might be taken up by the intracellular fungus from the apoplastic symbiotic interface. Although the reason why fungi infect seeds and protocorms or, in other words, whether there are any merits for colonizing fungi is under debate, Dearnaley and Cameron (2017) proposed a model for bidirectional nutrient transport in OM across intact membranes. The transcriptome analysis of symbiotic protocorms of *G. elata* inoculated with *Mycena dendrobii* revealed significant expression of plant genes involved in clathrin-mediated endocytosis during symbiotic seed germination (Zhang et al. 2017). Future studies should fully elucidate the mechanisms of nutrient transport across interfaces in orchid mycorrhizae.

3.3 Defense System

A delicate balance between plants and fungi creates unstable OM symbiosis. The lady's slipper orchid *Cypripedium macranthos* var. rebunense produces antifungal compounds in seedlings to restrict fungal growth (Shimura et al. 2007). Orchid mycorrhizal fungi act as pathogens to the *B. striata* seeds from which the seed coat had been removed (Miura et al. 2019). These findings have led to the hypothesis that plant defense reactions occur during OM symbiosis and that the fine-tuning of the defense response is essential for maintaining the plant–fungus relationship. In *G. elata*, *Gastrodia* antifungal protein (hereafter GAFP) or also known as gastrodianin genes encoding the monocot mannose-binding lectin antifungal proteins are expanded, and more than 80% of the GAFP genes are highly expressed in protocorms and juvenile tubers harvested from Xiaocaoba in Yunnan Province (Yuan et al. 2018). Additionally, *G. elata* is likely to reduce the number of genes

related to plant pathogen resistance, particularly in salicylic acid (SA) receptor genes, such as *NPR3* and *NPR4*, and SA signaling genes, such as *EDS1*, *PAD4*, *ALD1*, and *FMO1* (Yuan et al. 2018; Xu et al. 2021). Elevated SA-mediated defense responses are generally effective against biotrophic pathogens (Pieterse et al. 2012). Owing to the loss of these genes involved in SA biosynthesis and signaling from the parasitic plant *Cuscuta australis* genome (Xu et al. 2021), a common life strategy may exist for heterotrophic plants.

Moreover, what defense mechanisms are involved in OM symbiosis? Many transcriptome studies of OM symbioses have reported that protocorms and mature roots highly express genes related to reactive oxygen species detoxification during symbiosis (Zhao et al. 2014; Chen et al. 2017; Suetsugu et al. 2017; Gao et al. 2022). These genes play an important role in defense responses against biotic stresses and may be linked to peloton digestion (Blakeman et al. 1976; Suetsugu et al. 2017). The transcriptome analyses further supported the possibility of plant cell–wall remodeling or modification in OM fungal infections, as well as in AM and pathogen colonization (Zhao et al. 2014; Valadares et al. 2021; Balestrini et al. 2022). Orchids, in essence, control these defense responses to the extent that they do not eliminate symbiotic fungi, which Perotto et al. (2014) referred to as "a friendly plant–fungus relationship."

3.4 Phytohormones

Phytohormones play a crucial role in almost every aspect of plant biology, including growth, development, pathogen defense, and microbial symbiosis. For example, exogenous gibberellins (GAs) reduce hyphal colonization and arbuscule formation during AM symbiosis in *Pisum sativum*, rice (*O. sativa*), and *Lotus japonicus* roots, which form typical *Arum*-type arbuscules (El Ghachtouli et al. 1996; Yu et al. 2014; Takeda et al. 2015). However, GA promotes fungal entry and colonization during *Paris*-type AM in *Eustoma grandiflorum* inoculated with *Rhizophagus irregularis* (Tominaga et al. 2020). Interestingly, *Paris*-type colonization is typical of forest floor herbaceous and long-lived, woody, and evergreen plants (Dickson et al. 2007), and some of them are mycoheterotrophic plants (Hynson et al. 2013; Imhof et al. 2013; Giesemann et al. 2020). The symbiotic germination experiment of *Dendrobium officinale* inoculated with *Tulasnella* sp. S6 showed that exogenous GA_3 treatment inhibited fungal colonization in the protocorms and seed germination but did not significantly affect asymbiotic germination in the 4-week-old protocorms (Chen et al. 2020a). Transcriptomic studies have reported high expression of genes related to GA biosynthesis (GA 3-oxidase (ox) and GA20ox) and the GA-GID1-DELLA signaling module in the protocorms of *Cymbidium hybridum* inoculated with *Epulorhiza repens* ML01 and *Anoectochilus roxburghii* inoculated with unknown fungal species, respectively (Zhao et al. 2014; Liu et al. 2015). These findings suggest that GAs play a key role in OM symbiosis.

After recognizing symbiotic factors in each other, the symbiotic process between plants and fungi begins. Strigolactone (SL) is one of the key phytohormones in AM symbiosis initiation. In the rhizosphere, SLs released from plant roots stimulate the hyphal branching of AM fungi, which increases the chances of an encounter with a host plant (Kretzschmar et al. 2012). Yuan et al. (2018) confirmed that SL had similar branch-inducing effects in the OM fungus *Armillaria mellea*. The whole-genome sequences of orchid species have demonstrated the expansion of the genes encoding SL synthesis enzymes and receptors in *G. elata*, *G. menghaiensis*, and *D. officinale* (Wang et al. 2018; Chen et al. 2020b; Jiang et al. 2022). Because the ancestral function of SLs as rhizosphere signaling molecules was already present in the bryophyte *Marchantia paleacea* (Kodama et al. 2022), further studies will determine the role of orchid SLs in OM symbiosis.

Abscisic acid (ABA) is essential for seed dormancy and adaptation to environmental stress (Seki et al. 2007; Miransari and Smith 2014). Herrera-Medina et al. (2007) reported that tomato mutants with reduced ABA concentrations were less susceptible to AM fungus than wild-type plants, suggesting that ABA contributes to the development of the complete arbuscule and its functionality. During the seed germination of *D. officinale*, the ABA concentration was lower in symbiotic protocorms inoculated with *Tulasnella* sp. than in asymbiotic protocorms (Wang et al. 2018), revealing ABA involvement in OM symbiosis. The transcriptome analysis of *Cymbidium hybridum* inoculated with *Epulorhiza repens* ML01 revealed lower expression of 9-cis-epoxycarotenoid dioxygenase (*NCED*) and zeaxanthin epoxidase (*ZEP*) genes, which are related to ABA biosynthesis, in symbiotic roots than in mock-inoculated controls (Zhao et al. 2014). Collaboratively, *ZEP* and *NCED* significantly decreased in the early germination stage of symbiotic *Cremastra appendiculata* inoculated with *Coprinellus disseminatus* compared with those of the *C. appendiculata* seeds at the start of the experiment (Gao et al. 2022). In contrast, Gao et al. (2022) reported that the ABA receptor pyrabactin resistance 1-like genes were upregulated within the same period. Given that three events, germination, symbiotic process, and defense response, could happen simultaneously in symbiotic germination, the network complexity of these events is expected.

3.5 Common Symbiosis Pathway

The first land plants to colonize Earth, possibly cryptophytes, appeared in the Ordovician (approximately 450 million years ago), as confirmed using fossil records (Kenrick and Crane 1997). Fossilized fungal hyphae and spores that resemble modern AM fungi (Glomerales) were found in fossils of the same age (Redecker et al. 2000). Although evidence that these Ordovician fossil fungi were associated with plants is unavailable, the symbiotic association formed with AM-like fungi is thought to support plant terrestrialization (Rensing 2018). Following this founding event, alternative or additional symbioses emerged accompanied by plant diversification (van der Linde et al. 2018; Radhakrishnan et al. 2020). Because AM fungi

were detected in *Borya mirabilis* roots, which belongs to the same order as orchids, Asparagales, (Reiter et al. 2013), and the mycorrhizal fungi of *Apostasia* species, members of the earliest-diverging clade of Orchidaceae, belong to families Botryobasidiaceae and Ceratobasidiaceae (Yukawa et al. 2009), symbiont switching and trophic mode shifts are thought to correlate with the evolutionary success of Orchidaceae (Wang et al. 2021). This section will focus on the common symbiotic pathway (CSP), a putative signal transduction pathway shared by AM and the rhizobium–legume symbiosis, to discuss the mechanisms of OM symbiosis and how the symbiosis has evolved. The transcriptome analysis of symbiotic protocorms of *B. striata* inoculated with *Tulasnella* sp. HR1–1 revealed that the expression patterns of genes related to the signaling pathway of AM symbiosis are partially conserved in *B. striata* (Miura et al. 2018). Additionally, the authors tested whether one of the CSP genes calcium- and calmodulin-dependent protein kinase (*CCaMK*) gene in *B. striata* is functional, by performing a cross-species gene complementation assay using the *Lotus japonicus ccamk-3* mutant (Tirichine et al. 2006). This analysis showed that the *B. striata CCaMK* gene retains the functional characteristics of that in AM-forming plants (Miura et al. 2018). These findings and other studies suggest that orchids possess, at least partly, the molecular mechanisms common to AM-forming plants (Perotto et al. 2014; Suetsugu et al. 2017; Miura et al. 2018). Consistent with this suggestion, the CSP genes, such as symbiosis receptor-like kinase *SymRK*, *CCaMK*, and calcium signal decoding protein *CYCLOPS*, are present in orchid species (Radhakrishnan et al. 2020; Xu et al. 2021). However, the genes encoding the GRAS transcription factor *REQUIRED FOR ARBUSCULE DEVELOPMENT 1* and the half-ATP-binding cassette transporters *STUNTED ARBUSCULE* (*STR*) and *STR2*, which could be involved in the lipid transfer in AM symbiosis, are missing from orchid genomes (Radhakrishnan et al. 2020; Xu et al. 2021). Similarly, the three genes *SymRK*, *CCaMK*, and *CYCLOPS* were found but *RAD1*, *STR*, and *STR2* were not detected in the transcriptome of Ericaceae plants that form ericoid mycorrhiza (Radhakrishnan et al. 2020). Molecular studies of various types of mycorrhizae will help understand mycorrhizal symbiotic evolution.

4 Prospects for Conserving Wild Orchids

Many orchid species are widely known to be endangered. Globally, biodiversity hotspots are facing threats from land conversions, logging, and so on. These changes affect both orchids and other plant species. However, orchids are most likely facing greater threats than other plants if the other organisms they interact with (e.g., pollinators and mycorrhizal fungus) are also affected (Besi et al. 2019; Kolanowska et al. 2021). At a glance, orchid conservation seems to simply preserve the existence of a species, but in fact, orchid conservation requires extensive, complex approaches that should meet their survival requirements, especially during reintroduction into natural habitats. Conservationists and horticulturists worldwide are struggling with this problem, looking for new strategies involving both conventional and modern

biotechnology. Although traditional methods, including symbiotic germination and meristem culture, are commonly preferred for mass seedling production (Knudson 1922; Arditti and Krikorian 1996), reintroduction of seedlings produced from these methods directly into natural habitats could be even more challenging. The difficulty is due to the nature of orchids: Establishing a symbiotic association with appropriate fungi is crucial for orchids, and plant robustness depends on the encounter with the fungal partners.

Consequently, the transplantation of symbiotic seedlings seems to be better in situ growth than asymbiotically grown seedlings. However, only a few orchid species have been successfully cultured in symbiotic environments since Noel Bernard discovered OM symbiosis in 1899. Rapidly developing next- and third-generation sequencing technologies have the potential to make a breakthrough in biodiversity conservation because these sequencers overcome the technological hurdles of analyzing nonmodel plants at the molecular level. In AM symbiosis, the unculturability of AM fungi without plant hosts has been an issue for a long time but is now allowed for their asymbiotic cultures based on past findings and the latest fungal genome information (Kameoka et al. 2019; Sugiura et al. 2020; Tanaka et al. 2022). A former study reported that the cocultivation of the AM fungus *R. irregularis* with bacterial strains of *Paenibacillus validus* induced secondary infective spores without host plants (Hildebrandt et al. 2005). The genomes of AM fungi lack genes encoding type I fatty acid synthases in their genomes but have enzymatic machinery for fatty acid modifications (Tisserant et al. 2013; Tang et al. 2016; Maeda et al. 2018; Kobayashi et al. 2018). Kameoka et al. (2019) corroborated these findings: AM fungi produce spores on palmitoleic acid which is one of the fatty acids containing media. According to Tanaka et al. (2022), the base media containing fatty acids were available for another AM fungus *Rhizophagus clarus*, which lacks type I fatty acid synthase as well as *R. irregularis* (Kobayashi et al. 2018). Tanaka et al. (2022) also suggest that the comparative genome analysis of *Rhizophagus* species can provide essential contributions to establishing custom-made culture methods and identifying key genes involved in fungal diversity (Tanaka et al. 2022). Recent findings in OM symbiosis, such as nitrogen transport, phytohormone signaling, and defense/symbiotic components, will contribute to efficient symbiotic/asymbiotic seed germination and plant growth handling.

Transcriptome and genome analyses provide large datasets and important implications but require additional confirmation. In orchids, obtaining further evidence to support omics data is often difficult owing to the lack of methods for in vitro propagation and gene transfer, a requirement of specific materials and technology to analyze, such as radioisotope and stable-isotope measurements, and some handling problems due to tiny seeds. Advanced technologies and novel ideas from researchers in various fields are required to address these challenges. Orchid species have a huge demand as horticultural and raw materials for Chinese herbal medicines. In addition to the studies of flower formation and asymbiotic mass production of orchids, research on the molecular mechanisms of OM symbiosis is a fascinating subject in that it reveals the symbiotic evolution process and develops a novel in vitro/ex vivo culture system or even in situ transplantation. The application of

information obtained from omics analyses may be unlike untying the Gordian knot: It cannot be directly and completely used to solve challenges in orchid conservation. However, omics information can be used to determine which orchid–fungus pair yields the best outcome for seedling vigor during reintroduction into natural habitats by taking the role of phytohormone/metabolite production. Molecular studies on OM fungi are expected to be implemented in a broader range of orchids, including those of nonmodels.

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Breeding of Orchids Using Conventional and Biotechnological Methods: Advances and Future Prospects

Jean Carlos Cardoso, Joe Abdul Vilcherrez-Atoche, Carla Midori Iiyama, Maria Antonieta Germanà, and Wagner A. Vendrame

1 Introduction to the Family Orchidaceae and Main Commercial Groups Used in the Flower Market

The family Orchidaceae is considered one of the largest groups among angiosperms (along with Asteraceae) in a number of species, with more than 28,000 species distributed in more than 850 genera according to data from World Flora Online and Kew Botanical Garden (WFO 2022; Kew 2022). It is also one of the groups with the widest geographic distribution, with representatives on almost all continents of Planet Earth, including species with epiphytic, terrestrial, and lithophytic growth habits, of which approximately 70% of all epiphytic flora in the world are orchids (Zotz and Winkler 2013).

J. C. Cardoso (✉) · C. M. Iiyama
Department of Biotechnology, Plant and Animal Production, Lab of Plant Physiology and Tissue Culture, Center of Agricultural Sciences, Federal University of Sao Carlos (DBPVA, CCA/UFSCar), Araras, Sao Paulo, Brazil

Graduate Program of Plant Production and Associated Bioprocesses, CCA/UFSCar, Araras, Sao Paulo, Brazil
e-mail: jeancardoso@ufscar.br

J. A. Vilcherrez-Atoche
Graduate Program of Plant Production and Associated Bioprocesses, CCA/UFSCar, Araras, Sao Paulo, Brazil

Graduate Program of Plant Genetics and Breeding, Escola Superior de Agricultura Luiz de Queiroz, University of São Paulo, Piracicaba, Sao Paulo, Brazil

M. A. Germanà
Dipartimento Scienze Agrarie, Alimentari e Forestali (SAAF), Università degli Studi di Palermo, Palermo, Italy

W. A. Vendrame
Environmental Horticulture Department, University of Florida, Gainesville, FL, USA

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In addition to its great ecological importance, diversity, and a high degree of speciation in different regions of the world (Givnish et al. 2015; Pérez-Escobar et al. 2017), this group has been economically exploited worldwide, especially for the purpose of cultivation of ornamental plants. This is mostly due to the high diversity and number of species with inflorescences and flowers with different architectures, colors, and shapes that attract the consumer public in general and move billions of dollars in the world flower market.

A part of this trade can be considered illegal, especially for the exploitation of native species taken from their natural habitat and placed for direct commercialization among collectors of rare species and even commercial cultivation nurseries, reaching high unit economic values for the use of rare species and those at risk of extinction, and which has taken on greater proportions with the online trade, which still has limited capacity to trace the origin and destination of the trade in orchids and other rare species of interest in botanical collecting (Hinsley et al. 2017; Cardoso and Vendrame, 2022).

However, most of the use of orchids as ornamentals has been used legally, using moderate to high technology, and based on the exploitation of native genetic material to obtain hybrid cultivars, the latter including characteristics of horticultural and ornamental interest, in a single plant. In this context of commercial use, it is expected that a cultivar with high potential for use on a large scale will contain as its main characteristics its suitability for large-scale production, which basically requires: (1) rapid flowering that can be controlled under artificial conditions in order to accelerate and facilitate the production that requires flowering plants at different times of the year; (2) uniformity of vegetative and reproductive development, allowing its commercialization to be programmed and delivered in lots (3) compact size plants, a consumer market demand that also allows for an increase in the number of plants per square meter of cultivated area, which is currently expensive to implement and maintain; (4) resilient plants that need less inputs, such as water, fertilizers, and pesticides, especially due to the increase in production costs and the current demands associated with the concept of sustainability; (5) market novelties, which attract the consumer and feed a market marked by innovations and rapid changes in the end consumer's desire. These requirements are of a general order demanded by practically all commercial groups of orchids. However, more specific objectives can be efficient strategies in the development of new cultivars and vary according to the commercial group cultivated.

Since the first artificial orchid hybrid, registered between *Calanthe masuca* × *C. furcata* (De Chandra et al. 2014), there are currently at least a hundred thousand hybrids generated worldwide by collectors and by commercial companies specialized in the development of cultivars and trade of seedlings, also known as "Breeders." Based on this, there are commercial groups of orchids of greater relevance for cultivation as ornamental plants, and from which the genetic improvement for the market of ornamental flowers and plants is quite advanced, being these associated with the genera *Phalaenopsis*, *Dendrobium*, *Cymbidium*, *Oncidium, Vanda*, and *Cattleya* (Fig. 1). The term associated is not by chance, because in the case of *Cattleya* and *Oncidium*, most hybrids used as ornamentals are multigeneric, therefore, originating from crosses containing multiple genera in a single plant. In

Fig. 1 Most important commercial orchids groups used in industrial and trade floriculture

the case of *Cattleya*, crosses compatible with other orchid genera are common, such as *Brassavola*, *Guarianthe*, *Hoffmanseggella, Rycholaelia*, *Sophronitis*, and more recently with the genus *Encyclia*. In *Oncidiinae* subtribe, crosses between *Oncidium* and species of the genera *Brassia, Gomesa, Miltonia*, *Odontoglosssum*, and more recently with the inclusion of other genera, such as *Ionopsis* (Cardoso et al. 2016) and *Rodriguezia* are more conventionally used. These intergeneric Oncidiinae hybrids are commercially called Cambria orchids.

In the other commercial groups, interspecific hybrids prevail, with some commercially relevant intergeneric hybrids, such as *Ascocenda* (*Ascocentrum* × *Vanda*), which allowed the miniaturization of commercial varieties of *Vanda*. Also, using the genus *Rynchonopsis* (*Rhynchostylis* × *Phalaenopsis*) is currently used to achieve the natural blue color in *Phalaenopsis* flowers (Wu et al. 2022).

In contrast, in *Phalaenopsis*, the genus with the greatest commercial importance as an ornamental plant in the world, the greatest consistency is from hybrids obtained within the genus. Due to a large number of *Phalaenopsis* hybrids, the commercial groups or types are divided by the characteristics of their flowers or inflorescences into: standard cultivars, containing inflorescences with a good number of white flowers of medium to large size; multi-flora, characterized by small-sized hybrids or also called mini-phalaenopsis and with multiple, compact and small sized inflorescences; and market novelties, with yellow and red flowers and also the so-called spotted; and the biggest recent novelty called "Harlequins," which present coloration containing the fusion of spots resembling intense and large red spots. In a recent work developed by Lee et al. (2020), it is possible to see different types of cultivars of each of the described *Phalaenopsis* commercial groups. Also, more valued in the market are genotypes capable of synchronously producing two or three inflorescences, which may belong to any commercial group described above.

Also, biotechnological methods have been used more frequently and more effectively in the last decade, contributing to the development of cultivars with specific characteristics, especially using transgenics (Hsieh et al. 2020; Liang et al. 2020). In this context, the advance in knowledge and increase in the efficiency of in vitro regeneration systems, especially through the formation of Protocorm-Like Bodies (PLBs), the increase in the number of sequenced orchid species, and advances in molecular techniques, have resulted in the growing use of these techniques in orchids and other species of ornamental use. Even so, in many countries, the strict regulation related to the release of transgenic cultivars keeps the transgenic cultivars in the field of research by public and private companies and continues to be the main obstacle for these cultivars to reach the final consumer.

2 Basis of Reproductive Biology and Its Application in Conventional Orchid Breeding

Despite the high diversity of species in Orchidaceae, some characteristics are striking and definitive of this group of plants, such as its flowers, which in general consist of three sepals and three petals; one petal is modified and known as lip. In

addition, the reproductive structure is fused into a columnar structure, known as a gynostemium, in which the stigmatic cavity and the pollinia are located, the latter consisting of a mass with millions of pollen grains (Wu et al. 2009).

Most orchid species have hermaphroditic flowers, that is, they contain the female and male reproductive organs in the same flower and fused in the column or gynostemium.

However, there are monoecious species, which therefore produce female flowers separately from male flowers (rarely hermaphroditic), which occur especially in the subtribe Catasetinae (Machnicki-Reis et al. 2015). In this subtribe, there are important genera of orchids used by collectors, such as the genus *Catasetum*, and from which there are important advances in breeding and obtaining hybrid cultivars with exotic colors and hardly found in other orchids subtribes. However, the greatest difficulty in this genus for the expansion of the market aiming at large-scale floriculture has been the long dormancy period of these plants, which lose their leaves in fall-winter, keeping only their pseudobulbs, producing new shoots only in the spring and blooming in spring-summer. In this case, the dormancy of pseudobulbs can be broken by favorable climatic conditions of climatized greenhouses, which would make this group of plants good potential for innovation in the market of flowers and ornamental plants.

However, all the most commercially important groups mentioned have a column containing functional pollinia and stigmatic cavities. Although these structures contribute little to the ornamental aspect, they are essential in conventional breeding, aiming to combine different genomes towards the development of new cultivars of commercial interest.

The process of fertilization in orchids begins with pollination, a process by which pollinia are positioned/inserted in the stigmatic cavity of the flowers. From the pollinia, millions of pollen tubes can emerge containing nuclei that will fertilize the ovules, also in large numbers, and that will give rise to seeds. Embryo development, a process known as embryogenesis, can take from 3 to 18 months depending on the species and type of cross. Even within the same genus, there can be large variations in the period of seed development.

As an example, in *Dendrobium*, one of the genera with the largest number of species, there are two main commercial groups, mostly of hybrid origin, known as Nobile and Den-phal. In the Nobile group, the main species with the greatest genomic contribution to the development of cultivars is *Dendrobium nobile*, and the main characteristic of this group of cultivars is the presence of long pseudobulbs containing short inflorescences with one to four flowers distributed along the pseudobulb (Floricultura 2021). In this group, fruits have a very slow development, and the physiological maturity of seeds, as well as the dehiscence of fruits, occurs from 8 to 14 months after pollination. In the case of the Den-phal group (Fig. 1), *Den. phalaenopsis* and *Den. bigibbum* seem to have the greatest contribution, especially because they have large and round flowers. Despite this, some orchids are classified in the Den-phal group, but in some cases do not have the genome of these two species in their origin. Unlike the previous group, Den-phal orchids are characterized by one or more inflorescences, usually containing numerous flowers,

which arise from the apical region of the pseudobulbs (Cardoso 2012; Fig. 1). In this group of orchids, seed and fruit development is faster, with fruit dehiscence occurring between 4 and 6 months after pollination.

Cattleya and *Vanda* have fruit and seed development time from 8 to 12 months. In the genus *Phalaenopsis*, fruit development, from pollination to natural dehiscence, takes 6–12 months after pollination, like what occurs in *Oncidium*.

Orchid seeds also represent an exclusive characteristic of this family of plants, and the embryos develop in a limited way until the moment of fruit dehiscence and, consequently, their dispersal. Embryos are also devoid of reserves, such as the endosperm and cotyledons, and for effective natural germination, it is necessary the symbiotic association of embryos with mycorrhizal fungi or other microorganisms.

Most likely, partly, or entirely because seeds do not have nutritional reserves for the embryo, this is considered one of the families with the greatest capacity for interspecific hybridization, including multigeneric hybrids, that is, obtained from multiple and successive crosses between different genera and which, in the end, generate fertile hybrids capable of new hybridizations.

An example of this high cross ability is found in the subtribe Laellinae, whose main commercial representative is the genus *Cattleya* and in which, however, most of the cultivars produced and marketed as ornamentals come from interspecific and intergeneric hybrids. In this way, it is possible to cross the genus *Cattleya* with species of the genera *Brassavola* (Ex: *Brassocattleya* Binosa), *Hoffmansegella* (ex *Laelia*) (e.g., *Laeliocattleya* Brazilian Girl, Cardoso 2010; *Laeliocattleya* Nobiles Confetti, Fig. 1), *Sophronitis* (*Sophrocattleya*), *Epidendrum* (e.g., *Epicattleya* "Renne Marques"), *Encyclia* (*Catyclia*), *Broughtonia* (*Cattleytonia*), *Caularthron* (*Caulocattleya*), *Rhincholaelia* (*Rhincholaeliocattleya*), among other multiple combinations of these hybrids.

Thus, if, on the one hand, this high diversity of crosses allows great segregation of traits for breeding, this is a highly complex family in genomic terms. Due to the multiple possible combinations, it can result in a great complexity for molecular and cytogenetic analysis aimed at the identification and origin of chromosomes and genes from these multiple possible combinations, which now difficulty programs to use molecular assisted breeding.

Also, for this reason, and the easy crosses, with good fruit set and seed development, conventional breeding has been used for decades aiming at the improvement of orchids and until today it has been the main method for use in professional programs for breeding and development of new orchid cultivars.

3 Main Methods Used in Conventional Orchid Breeding

Conventional orchid breeding methods are still today, in the era of omics and genetic editing, the main method of orchid breeding aiming at the production of new cultivars.

The prevalence of these methods is currently due to the numerous species diversity and high capacity for interspecific and intergeneric combinations in orchids. Thus, allow the breeder to seek, in a conventional way, genotypes that add different traits to be inserted in commercial cultivars, only using controlled hand pollination to the development of fruit/seeds containing the hybrid progeny.

The other step of this process is the in vitro germination, which has been done through in vitro cultivation techniques, in which seeds, after fruit and seed development, undergo asepsis procedures to eliminate the present microbiota, being placed to germinate in a suitable culture medium containing a carbon source to support the development of embryos into seedlings. After the period of cultivation and in vitro development of the progeny, seedlings are acclimatized in a greenhouse and later selected in a cultivation environment like the one in which they will be grown. Genotypes with desired traits are selected, cloned using micropropagation techniques, and tested on a commercial scale to evaluate clonal stability and cultivar performance under cultivation conditions.

3.1 Creation of Germplasm Banks and Their Relationship with the Objectives of the Breeding Program

Germplasm banks are the main source used to start orchid breeding programs and consist of collections of species, hybrids, or even different genotypes of the same species with characteristics of interest to be inserted and developed in future new cultivars. Most private and public companies with programs for breeding orchids and other ornamental plants have their germplasm bank, and are made up of species from different geographic regions where they naturally occur; the ex situ conservation in protected cultivation is the main method used by breeding companies. That is, species and genotypes of different species of interest are kept outside their natural habitat, in cultivation conditions that simulate this environment and that may involve the use of temperature control technologies (heating and cooling), increase in relative humidity, irrigation, and artificial light. Undoubtedly, the largest orchid breeding programs developed by private companies are in The Netherlands, Taiwan, and Thailand, these are known as "Breeders," and they are responsible for the maintenance of germplasm banks, development, and commercialization of new cultivars, in addition to the production and commercialization of seedlings of these cultivars. Most companies are known as "Breeders" work specifically in the market for cultivars and plantlets production, providing the genetic material for the world flower market. In this way, flower growers who use the technology of these companies, pay as costs the value of the production of plantlets, but also the technology used and associated with the cultivar, also called as royalties. Currently, the plantlets + royalties' has been the most relevant cost among all costs associated with the production of flowers, exceeding in recent years, the cost of labor for cultivation. Thus, to reduce the production costs of these plantlets, large companies have developed production areas and plantlet cultivation systems (owned or

outsourced) within the country where the plantlets are marketed, reducing risks associated with currency fluctuations, high costs and bureaucracy of importing plant material.

In this way, germplasm banks are the main genetic source of traits in these companies, and the collection of species, genotypes, and hybrids is the one that maintains a frequency of production of new cultivars based on diverse and controlled crosses to target-specific traits.

The main characteristics desired and placed as objectives in the current orchid breeding programs can be divided according to the vegetative and reproductive stages of the plants. At the vegetative stage, the main objectives, in general, are compact, rapid, and vigorous vegetative development, good rooting in pot and substrate conditions, and resistance to pests and diseases of the roots and shoots. At the reproductive stage, the most general objectives covering most commercial groups are high adaptability to already established cultivation systems that respond uniformly to the flowering control process; high flowering uniformity and homogeneity of cultivation lots; reduction of the juvenile period and, consequently, faster flowering; natural flowering at different times of the year, therefore, less dependent on specific climatic conditions (Cardoso et al. 2016); the greater number of inflorescences at the same time, which has resulted in higher market value; compact and flexible inflorescence that allows adequate staking; large and round-shaped flowers, and when small, they should be numerous for greater visual filling; novelties about colors and shapes of flowers and inflorescences.

However, specific features must be highlighted, especially for the genus *Phalaenopsis*, which differs in growth habit (monopodial) from most other commercial groups (sympodial). In this case, early flowering is not desired for some reasons: flowering in *Phalaenopsis* occurs after 12–18 months from seedling or plantlet acclimatization in a greenhouse; although it is possible to observe early flowering in some plants, this generally results in reduced inflorescence size and a number of flowers, not being marketed; the early emergence of this inflorescence results in the need for additional management aiming at its elimination, as it delays vegetative development and delays commercial flowering. Regarding the presence of more flexible floral stems to support staking, in *Phalaenopsis* there is also a different demand for inflorescences with high lignification degree and that do not need additional staking, as this would result in reduced plant management. Further, inflorescence lignification is a hereditary trait associated with the type of inflorescence architecture (Pramanik et al. 2022). Also, in *Phalaenopsis*, one of the fungi with the greatest impact on cultivation is the genus *Botrytis*, which causes spots and necrotic spots mainly on flowers. Although this fungus is a problem in all orchids, *Phalaenopsis* seem to have a greater susceptibility and, consequently, the genus in which there is greater damage due to their symptoms reducing the quality and durability of flowers. Thus, the search for more resistant cultivars or sources of resistance should be included in breeding programs, either by conventional crosses, or even biotechnological methods.

In *Dendrobium*, especially in the Nobile group, in addition to innovations in the color of the flowers, above all, plants that flower in almost all nodes of the

pseudobulb are sought, as most cultivars have flowering nodes only in the middle and upper third, and no flowers in the basal third of the elongated pseudobulb. In the Den-phal group, among the objectives are: innovation in relation to colors (Cardoso 2012); increased flowering synchronization, as most cultivars available on the market still have time-dispersed flowering, with less than 60% clonal individuals in a lot with synchronized flowering; production of compact plants with multiple terminal inflorescences or compact plants with 1–5 terminal flowers of large diameter and rounded shape. Due to a large number of species in *Dendrobium*, there is a good potential for the release of new commercial groups, such as those with pendant inflorescences, especially hybrids with *Den. densiflorum*, *Den. thyrsiflorum*, and others from the same group (Teixeira da Silva et al. 2016).

In *Oncidium* and its multigeneric hybrids, the search has been for large plants with multiple inflorescences containing medium- to large-sized flowers and, in the opposite direction, for compact plants with short inflorescences and medium-to-large-sized flowers. Color innovation is one of the central objectives, as most cultivars are between yellow and brown, based on the two groups of great commercial relevance worldwide, which are the groups called "Golden Rains," yellow in color and without fragrance, like *Onc*. Aloha and *Onc*. Sweet Sugar, and the *Onc*. "Sharry Baby," with brown tone flowers with intense fragrance, which resembles the smell of chocolate (Cardoso et al. 2016). Another group that has gained commercial importance is commonly called Cambrias and is grouped by different intergeneric hybrids, such as *Colmanara*, a multigeneric hybrid (*Oncidium* × *Odontoglossum* × *Miltonia*), *Vuylstekeara* (*Cochlioda* × *Miltonia* × *Odontoglossum*), *Beallara* (*Cochlioda* × *Miltonia* × *Oncidium* × *Odontoglossum*) and which results in multiple inflorescences with a good number, size, and color of the flowers. One of the successful examples of this hybrid and cultivated worldwide is *Colmanara* "Wild Cat," with yellow flowers and brown spots, and *Beallara* "Tahoma Glacier," with compact inflorescence, large and star-shape white flowers with red spots (Fig. 1).

In *Oncidium* and *Dendrobium*, as well as their hybrids, high-impact rust has emerged more recently, causing spots on pseudobulbs and leaves, these spots are also called shotgun blasts, as they are characterized by several necrotic spots and which together have a more or less circular shape. The causal agent of the disease is not yet fully elucidated, but it is probably due to phytopathogenic fungi of the *Cercospora* and *Alternaria* genera. However, for all genera, the main pathogenic fungus actually is from the genus *Botrytis*, which causes numerous brown color spots in the petals and sepals, which reduces the quality and impedes their commercialization.

In the genus *Cattleya* and its hybrids, the main problem associated with cultivation are the time from cultivation to the first flowering, which often exceeds 3–5 years, putting these plants at a disadvantage in relation to the other genera mentioned above, which normally flower at 18–24 months of cultivation; the low shelf life of its flowers, which rarely exceeds 20 days in the best hybrids, and; the high sensitivity of flower buds to stresses caused by handling, transport, and change of environment. These characteristics put this plant at a disadvantage in relation to other genera used as ornamentals, such as *Phalaenopsis*, *Dendrobium*, and

Oncidium, since in these plants, the time from cultivation to commercial flowering is 18–24 months, with a shelf life of around 30 days or more, with *Phalaenopsis* hybrids that can last longer than 60 days of shelflife, with good resistance to handling and transport.

3.2 Crosses by Controlled and Directed Pollination

After the creation of the germplasm bank based on the objectives of the breeding program, the process of directed crosses begins, in which pollinia of one genotype are taken to the stigmatic cavity of the other. In this process, in addition to the choice of parents for the purpose of breeding, there is also a strong influence on orchids in the choice of the plant to be used as a parent. In crosses carried out with different genotypes by our research group and breeding program with *Cattleya*, we have observed a vegetative development (e.g., type and intensity of rooting, type of leaves and pseudobulbs of the progenies) with a greater genetic inheritance of traits from the mother parent.

Preferably, pollinia taken from the paternal parent should be removed and immediately brought to the stigma for pollination. Nevertheless, due to the difficulties of synchrony in flowering or even obtaining plants with different flowering times from the parents, it is possible to store pollinia. Pollinia lose their viability very quickly after they are removed from the flowers at room temperature, but they can be stored for a few weeks or even a few months at low temperatures, ranging from − 20 °C to 8 °C (Yuan et al. 2018).

After pollination, the germination of pollen grains in the stigma and the long way to the inferior ovary and eggs for fertilization begins, which usually takes a few days to occur. After fertilization, the process of zygote development begins and culminates in embryogenesis, which, as previously mentioned, can take from 90 to more than 360 days depending on the genus and species of orchids used in the crosses.

Problems related to incorrect pollination and/or non-occurrence of fertilization can result in flower abortion, early fruit abortion after a period of development, or even in the formation of seeds without embryos. These anomalies related to reproduction may be associated with the non-viability of pollinia caused by different factors, the incompatibility in the crossing, and as observed in our studies, the genetic factors contained in different genotypes, which result in different degrees of fruit and seed production in orchids.

Interestingly in the case of orchids, most commercial hybrids, even after successive generations of interspecific hybridization, maintain different levels of fertility. Crosses, therefore, can be species × species, hybrid × hybrid, or hybrid × species, mostly with success in obtaining progenies.

After 24–48 h of pollination, senescence of flowers due to pollination can be observed, with important changes such as wilting and forward bending of petals, sepals, and lip (Fig. 2a, b), as if they were protecting the reproductive organ, until the moment they dry completely and are detached from the gynostemium/ovary. At the

Fig. 2 (**a**) Intergeneric cultivar from *Cattleya* hybrid group (*Potinara* Free Spirit) within one pollinated flower (p-f) showing the closing of petals and sepals and the green and swelling of the inferior ovary. (**b**) Pollinated flowers of Den-phal hybrid showing the swelling of its ovaries. (**c**) Green fruit (g-f) and fruit starting dehiscence (fsd) and yellowish color, with 8 months after pollination. (**d**) Fruits and seeds of *Oncidium* orchid 8 months after pollination. (**e**) In vitro culture of F1 hybrid progeny of *Cattleya* orchids. (**f**) Greenhouse cultivation of different F1 progenies seedlings of *Cattleya* intergeneric orchid

same time, there is a clear increase in the green color and swelling of the ovary (Fig. 2a, b). This process of fruit swelling continues throughout fruit development, until the moment of dehiscence or natural opening of the fruit (Fig. 2c), at which time the three infertile valves detach from the fertile valves of the capsule (Dirks-Mulder et al. 2019) with seed dispersal by wind.

Fruit from the directed crossing, also called capsules, can be harvested shortly before (unripe fruits) or at the beginning of the dehiscence, also called ripe fruits (Fig. 2c, d). Harvesting unripe fruits require care, especially regarding knowledge about the seed maturation time, which is very variable in orchids and is subject to the risk of an early harvest, which results in abortion and non-germination of most seeds obtained from the cross. When ripe, part of the seeds is loose inside the fruits and this coincides with the maturation and dehiscence of the capsule.

After being removed from the capsule, seeds are ready to be placed for germination. As a standard procedure performed at the Laboratory of Plant Physiology and Tissue Culture of the Federal University of São Carlos, fruit from directed crosses are harvested at the beginning of the dehiscence, when capsules change from green to yellowish-green, or even when it is noticed the beginning of the dehiscence, which starts in the distal region of the fruit, close to the column (Fig. 2c). After harvesting, fruits are opened, and seeds are exposed and kept to dry for at least 24 h (Fig. 2d), followed by removing all the seeds with the aid of a brush. Seeds are then stored in plastic tubes under a low temperature (8 °C). In this way, it is possible to store the seeds, with good viability for at least 6 months. This is extremely valid when working with many crosses and there is a need for reseeding due to non-germination or other problems that arise from the first seeding attempt.

3.3 Asymbiotic Cultivation as the Main Means for Obtaining Progenies

Germination of orchid seeds under natural conditions, due to the limited or abscence of nutrient reserves associated with seeds, is dependent on relationships with microorganisms that make a symbiotic association with orchids, especially mycorrhizal fungi and rhizobacteria (Tsavkelova et al. 2016; Chen and Nargar 2020). Although it is possible to isolate, cultivate and, later, subculture these microorganisms together with orchid seeds to promote germination, a technique known as "seed baiting," this is a tiring technique, of more difficult implementation, which requires care with the microorganism, with the seed and with the interaction of the two organisms. These characteristics hardly meet the objectives of a breeding program, in which the main objective is to germinate many progenies to select new cultivars with superior characteristics. Symbiotic cultivation has shown good applicability in projects to understand the interaction microbiota and orchid species, in orchid species in which symbiotic germination does not seem to result in success as with terrestrial species, and in conservation and restoration projects with orchid species (Yang et al. 2020).

Thus, the studies that began with Knudson (1922) greatly helped the breeding programs, by developing a technique for cultivating and germinating orchid seeds in an asymbiotic way, that is, without the need for microorganisms. This technique uses a culture medium containing a nutrient solution, a source of sugar and agar. Culture media such as those of Knudson (1922) or Murashige and Skoog (1962) containing half the concentration of macronutrients (MS1/2) have been the most used culture media for germination of seeds of different orchid genera and meet the need to obtain high germination rates (Teixeira da Silva et al. 2015; Chen and Nargar 2020). A critical point for culture media to promote high germination rates is that the pH of the media should be adjusted to values between 5.5 and 6.0.

Asymbiotic germination under in vitro conditions can allow germination above 90% and allows the germination of seedlings that would hardly germinate or survive the stresses associated with the natural environment, in which less than 1% of the seeds germinate. At the same time, in a breeding program looking for high-performance plants, the objective is to obtain a large number of plants for post-germination selection of plants with interesting horticultural and ornamental characteristics, under conditions very different from those in nature, using technologies such as environmental thermal control, availability of water and fertilizers based on balanced irrigation and nutrition programs available throughout the life of the plant. That is, the cultivation conditions of a new cultivar, including the plant selection process, take place in a very different environment from the natural one, in which plants are evaluated and selected according to their performance under artificial cultivation, similar to the conditions in the which large-scale flowering plants are produced.

After in vitro germination, not infrequently, several hundred seedlings are germinated in a single cultivation flask, which requires a process of subcultures and plant selection from the beginning. In hybrids of the genus *Cattleya*, Cardoso et al. (2016) developed a methodology for the systematic selection of seedlings, which starts from in vitro cultivation and ends at the time of the second flowering. This process consists of, at each subculture and from the first in vitro subculture, initiating the selection of seedlings with better vegetative performance. Soon after the germination of orchid seeds, the so-called protocorms are formed, globular structures from the multiple cell divisions of the embryo placed for in vitro germination. It is notable for most asymbiotically germinated progenies, the production of two types of structures, one of which remains as protocorms and another group of progenies directly originates or evolves to the formation of seedlings, containing leaves, roots and sometimes, pseudobulbs. In this first selection, which occurs after 90–120 days of cultivation, only seedlings are selected. These seedlings are then transferred to subculture 1, from which after 90–120 days of cultivation, there is a second selection based on plants with a good shoot and root development. It is possible that some genotypes still need a third subculture before reaching 3–8 cm in length (Fig. 2e), when the seedlings selected in vitro are taken to the acclimatization process, with the removal of the plants from the in vitro conditions with cultivation in culture medium to ex vitro environment with cultivation in a substrate.

Acclimatization can last for 90 to 180 days and is carried out in trays with the adequate substrate under greenhouse conditions. As the most common substrates,

sphagnum, peat moss, and coconut fiber are used, but different mixtures prepared by specialized companies can be found. Here, fertigation programs also begin, in which fertilization or plant nutrition is offered together with irrigation. After this period of cultivation, a new round of selection of superior plants is carried out, with a selection of plants with vigorous vegetative development and absence of symptoms of pests and diseases, excluding those with inferior development. These selected plants are transplanted into an intermediate pot size (*Cattleya* and *Oncidium*), with 6–9 cm in diameter (Fig. 2f), or even into the definitive and large pot size (*Oncidium*, *Phalaenopsis*, *Dendrobium*, *Vanda*), with 9, 12, or 15 cm in diameter, depending on plant size and cultivation objective, and in which plants are finally selected for faster flowering characteristics and ornamental attributes (Cardoso et al. 2016). This methodology has been used effectively in different commercial genera of orchids and new cultivars have already been obtained in the commercial groups of *Cattleya* (Cardoso 2009; Cardoso et al. 2016), Denphal, a *Dendrobium*-type orchid (Cardoso 2012) and *Oncidium* (Cardoso 2017).

Importantly, in this case, the selection of plants occurs more for characteristics of horticultural interest, to the detriment of the ornamental aspect, aiming at the selection of plants with rapid development and early flowering. Ornamental traits are selected from only those plants that flower the fastest. Although this seems to be a limitation of the technique, the choice of parents is an important step towards the selection of plants that include rapid development and abundant flowering and with a shape/color of interest to the market.

4 Biotechnological Approaches Used for Breeding Orchids

Although conventional breeding prevails over other methods in the development of new orchid cultivars, in the last decade there has been an important growth in the contribution of biotechnological techniques that resulted in the development of new groups of orchid cultivars. The advancement of research in the areas of sequencing, omics, and genetic engineering has currently allowed advances in the application of biotechnological tools for breeding ornamental plants, including orchids. The most relevant cases with the greatest commercial impact have occurred in genera used as ornamental plants and of greater commercial importance, such as *Cymbidium* and *Phalaenopsis* (Balilashaki et al. 2022; Cai et al. 2015 Yang et al. 2021a, b), and have made possible the identification of genes correlated to pathways of great relevance for breeding orchids, such as the floral scent in *Cymbidium goeringii* (Ramya et al. 2019) and the color of the flowers in *Phal. equestris* and *Phal. aphrodite* (Hsu et al. 2022).

The most used techniques for this purpose include the induction and selection of somaclonal variants from in vitro culture, in vitro polyploidization using mutagens, transgenics, and the isolation and fusion of protoplasts. The main biotechnological tools used for orchid breeding, as well as the target traits achieved for each technique, are resumed in Fig. 3.

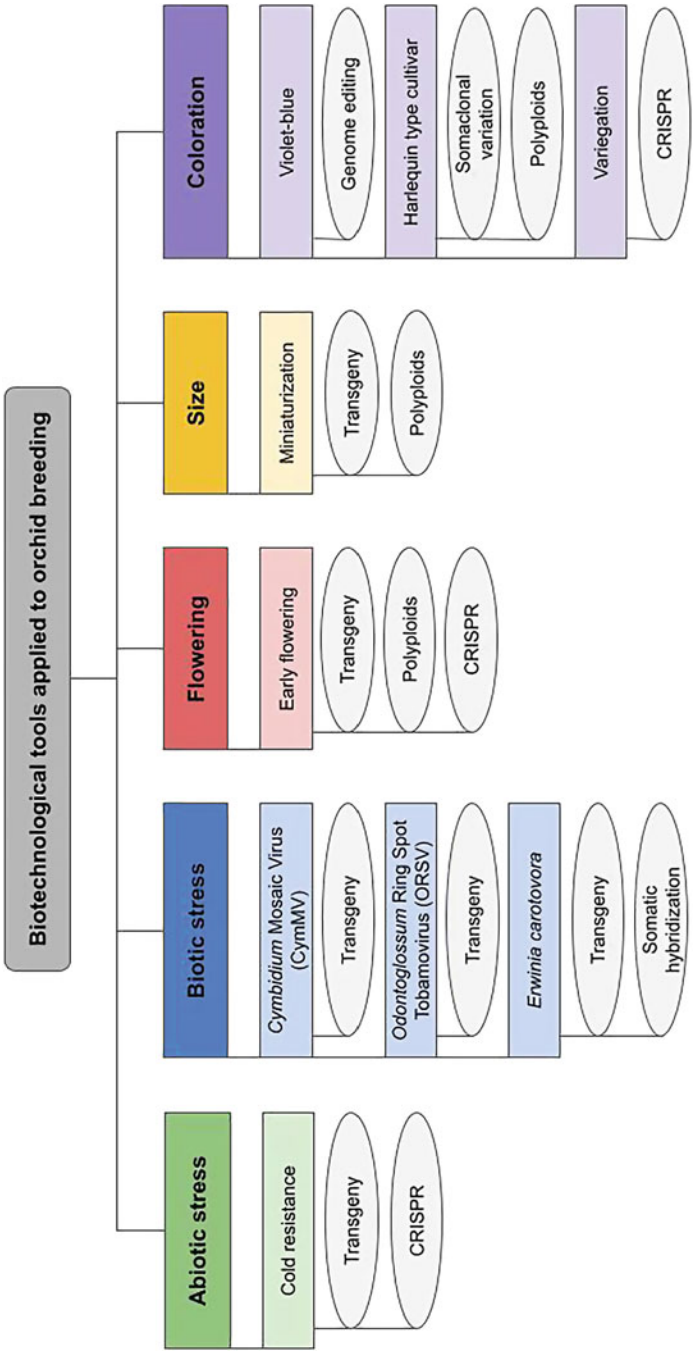

Fig. 3 Target traits achieved using biotechnological tools. Rectangle forms represent the target traits achieved and elliptical forms showed the techniques used to induce the traits achieved

4.1 Induction of In Vitro Somaclonal Variations

In vitro somaclonal variation consists of genotypic and phenotypic variation that occurs in in vitro tissues. Somaclonal variations are more frequently observed in Protocorm-Like Bodies (PLBs), which are globular structures similar to protocorms but of somatic-origin in orchids. The PLBs are used for large-scale clonal propagation of different orchids genera. However, this technique of propagation, also called Induction, Proliferation, and Regeneration of Protocorm-Like Bodies (IPR-PLBs) is a source of in vitro somaclonal variation in orchids, such as in *Phalaenopsis*, *Dendrobium,* and *Oncidium* (Cardoso et al. 2020).

Otherwise, somaclonal variation can be a genetic variation source of new traits of interest such as biotic and abiotic stress resistance, and morphological and physiological variations in flowers (Wang et al. 2019). The occurrence of punctual genetic mutation derived from PLBs is interesting in ornamental breeding because the cultivar maintains the main characteristics of originals with changes in one or a few traits. Therefore, somaclonal variation can be strategically applied to meet the demand for novelties in the orchid flower market, not requiring a long period of hybridization and progeny selection.

As an example, one of the most actual, interesting, and commercial novelty in orchids are the Harlequin-type cultivars of *Phalaenopsis*, attractive for the color of their flowers with red or magenta-black fused spots, initially obtained and derived from an in vitro SV from the clonal system for propagation of *Phalaenopsis* Golden Peoker "Brother" (Hsu et al. 2019; Lee et al. 2020). Currently, the Harlequin-type cultivars represent an innovative and attractive cultivar group used as a new source of genes for the color of flowers, due to the heredity of this characteristic in crossings with other *Phalaenopsis* groups (Lee and Chung 2021).

However, somaclonal variation is random and spontaneous and can result in morphological abnormalities of plants, for example, the occurrence of creased leaves (Tokuhara and Mii 2001) or the occurrence of deformity in flower structures, such as the absence of the labellum (Cardoso et al. 2020), which are not desired for clonal mass propagation or breeding purposes.

Some of the factors that can affect the frequency of somaclonal variation (SV) in orchids are the species or genotype used, the type and concentration of phytorregulators in the culture medium, the origin of explant and the system used for regeneration, the age of the in vitro culture, and also the number and environmental conditions of subcultures (Chin et al. 2019; Cardoso et al. 2020).

Long-term cultures are one of the main factors leading to somaclonal variation in orchids. In vitro somaclonal variation was observed in *Doritis pulcherrima* derived from PLBs after 2 years of in vitro culture. The main changes observed between the original and SV-derived were the color of leaves, purple in the somaclones, and green in the original-type plantlets, in addition to differences observed in the size and content of chlorophylls, which were higher in the original-type ones (Thipwong et al. 2022). Long-term subculture also resulted in the presence of somaclonal variation in

Oncidium "Milliongolds," which was detected by SLAF-seq in PLBs-derived clones after 10 years of in vitro culture (Wang et al. 2019).

Among phytoregulators, most reports point to cytokinins as the main cause of SVs in orchids. Somaclonal variation was reported in PLBs of *Dendrobium* "Sabin Blue" cultured for 2 years in a medium containing kinetin as phytoregulator, and detected by ISSR and DAMD molecular markers (Chin et al. 2019) and in PLBs of *Dendrobium nobile* cultured in medium containing thidiazuron (Bhattacharyya et al. 2016), a cytokinin-like component.

Besides PLBs, the use of indirect organogenesis by callus proliferation resulted in increased somaclonal variation frequency in *Vanilla planifolia* Jacks, producing chlorophyll-variegated plantlets regenerated from callus (Ramírez-Mosqueda and Iglesias-Andreu 2015).

The presence of SVs in orchids can be detected by molecular markers, such as ISSR (Inter Simple Sequence Repeats), RAPD (Random Amplified Polymorphic DNA), SCoT (Start Codon Targeted), DAMD (Direct Amplification of Minisatellite DNA region), SLAF-seq (Specific-Locus Amplified Fragment Sequencing) (Cardoso et al. 2020; Li et al. 2021a), or using morphological of adult plants until their flowering (Zanello and Cardoso 2019).

4.2 Transgeny

Transgenic technology is an efficient breeding technique that allows the transference of foreign new genes into a plant genome (Belarmino and Mii 2000). For orchids, two methods have been employed: the particle bombardment or biolistic consists of a physical and direct approach to transfer exogenous genes into plant tissues delivered by microparticles of gold or tungsten, which penetrate plant cell wall; the *Agrobacterium tumefaciens*-mediated transformation, which is a biological method based on the infection of plant tissue with specific strains of *Agrobacterium tumefaciens*, a soil bacterium, that has the capacity of transferring genes into the host plant (Mii and Chin 2018).

Although *Agrobacterium*-mediated transformation was considered a method only for dicotyledonous plants, since monocots are not a natural host of *Agrobacterium*, monocots as orchids have been successfully transformed (Mirzaee et al. 2022). The particle bombardment has as its advantage the independence of hostage limitation. However, *Agrobacterium*-mediated system is preferred for the ease and high-repeatability of the technique.

In the family Orchidaceae, genetic transformation has been established in all main commercial orchid genera (Li et al. 2021a; Zhang et al. 2022), and some factors are important to achieve a successful genetic transformation system. *Agrobacterium*-mediated transformation requires bacteria strains with an efficient infection of orchid cells, followed by the later regeneration of cells and tissues under in vitro culture. Thus, the use of super virulent strains, such as EHA101 and EHA105 (Subramaniam and Rathinam 2010; Mirzaee et al. 2022) has contributed to improving the

transformation efficiency. Strain EHA101 is the most used in *Phalaenopsis*, *Cattleya*, *Cymbidium*, *Dendrobium,* and *Vanda* orchids. Strain EHA101 is more frequently reported in *Phalaenopsis*, but recent studies involving genetic transformation in this genus only used strain EHA105. In addition, EHA105 was also the most used strain in *Oncidium* and *Erycina* (Mii and Chin 2018). The target explant used for *Agrobacterium* transformation is also high important, and most studies focused on PLBs or protocorms.

After the infection of plants using *Agrobacterium*, it is important to eliminate bacteria from plant cells and tissues by using antibiotics. The main antibiotics for this purpose are hygromycin, kanamycin, cefotaxime, and meropenem. Some antibiotics are also helpful to select the transformants, when a marker gene was used for transformation. Usually, antibiotic-resistance genes are used to certify the occurrence of transgeny and to select transformants from the non-transformed tissues and individuals. Thus, transformants containing the antibiotic-resistance gene will survive when exposed to antibiotics while the non-transformants will be eliminated (Mii and Chin 2018; Ozyigit and Yucebilgili Kurtoglu 2020).

In the family Orchidaceae, the first reports of genetic transformation focused on testing and improving the efficiency of the method, using only marker or reporter genes to demonstrate that explants were successfully transformed. However, genetic transformation has enabled the change in flower color, the induction of early flowering, the resistance to pathogens, such as *Cymbidium* Mosaic Virus (CymMV) and *Odontoglossum* Ring Spot Tobamovirus (ORSV), and more recently, the resistance to *Erwinia carotovora* (Li et al. 2021a), the production of miniaturized *Phalaenopsis* by overexpression of the OsGA2ox6 gene (Hsieh et al. 2020), and the modification of the color of flowers, such as violet-blue in the white-flower *Phalaenopsis* cultivar (Liang et al. 2020).

The main limitations of genetic transformation in orchids are the low efficiency of transgeny; the limited results until now, especially with changes in the color of flowers of transgenic plants, and the difficulties with the release of transgenic cultivars in the flower market.

4.3 In Vitro Polyploidization and Self-Duplication of Genomic DNA

Polyploidy is a biological event in which eukaryotic organisms have more than two complete sets of chromosomes, thus generating changes from the genetic level to their relationship and adaptation with the environment (Fox et al. 2020; Soltis et al. 2009) and which are observed from humid tropical forests, desert regions, and extremely cold environments. This characteristic of increased vigor in polyploid organisms is due to genetic redundancy, which also serves as a defense mechanism against the negative effects of mutations and heterosis (Comai 2005). Genetic studies have analyzed genome duplication events in angiosperms, revealing that they all

have a paleopolyploid ancestor, as their genome has undergone at least one duplication event during evolution (Jiao et al. 2011; Renny-Byfield and Wendel 2014).

There are two mechanisms for natural polyploid formation in plants: the production of unreduced gametes (2*n*) and somatic duplication (Sattler et al. 2016). Somatic duplication in plants is called endoreduplication, which is modulated by hormonal, environmental, and nutritional factors. Endoreduplication is caused by errors during the endocycle of mitosis, where cells replicate their genome but do not undergo cytokinesis, generating different levels of ploidy within them (Maluszynska et al. 2013). Diploid organisms can also produce unreduced gametes due to errors during the first or second division of the restitution phase of meiosis (Sattler et al. 2016).

From the establishment of in vitro plant cultivation by Haberlandt (1902) and with the first report of polyploidization using this cultivation system (Murashige and Nakano 1966), it was possible to determine that plant tissue culture could be used not only for mass propagation of plants but also as a new and efficient tool for artificially obtaining polyploid plants (Dhooghe et al. 2011). Currently, the use of in vitro cultivation system using colchicine as an antimitotic agent has become the most common and popular strategy for plant polyploidization (Eng and Ho 2019).

In orchids, the chromosome number of more than 90% of species is the result of at least one polyploidy event (Mondin and Neto 2006). Thus, natural polyploidy events, such as endopolyploidy and the formation of unreduced gametes have been reported in many orchid genera, especially those used as ornamentals (Vilcherrez-Atoche et al. 2022). Endopolyploid tissues have been reported in almost all commercial orchid genera, with DNA content ranging from 2C to 16C in *Cymbidium*, 2C to 32C in *Dendrobium*, and 2C to 64C in hybrids of *Phalaenopsis* (e.g., *Doritaenopsis*) and *Vanda* (Vilcherrez-Atoche et al. 2022). Regarding the frequency of occurrence of unreduced gametes in orchids, this is not naturally high, being observed in some commercial cultivars of *Cymbidium*, between 0.15–4.03% (Zeng et al. 2020).

Although the natural production of polyploid plants from non-reduced gametes or tissues with high rates of endopolyploidy is possible, polyploidization with the use of antimitotics is the most used technique for the artificial induction and increase of the frequency of polyploids for orchid breeding (Vilcherrez-Atoche et al. 2022).

The artificial induction of polyploidy in orchids has already been reported, at least once, in the main genera used as ornamental plants, and in more than 80% of polyploidization studies, the mutagen used was colchicine (Vilcherrez-Atoche et al. 2022). *Dendrobium*, *Cymbidium,* and *Phalaenopsis* were the first genera used for artificial chromosome duplication in Orchidaceae. Menninger (1963) first performed the induction of a tetraploid of *Cymbidium* using colchicine, followed by Griesbach (1981) who exposed protocorms of *Phal. equestris*, *Phal. fasciata, Phal.* "Betty Hauaserman" to colchicine, resulting in the generation of polyploid plants with an average frequency of 46%. Chaicharoen and Saejew 1981 also successfully performed artificial autopolyploidy of *Dendrobium phalaenopsis* using colchicine.

The procedure for autopolyploidization in orchids using colchicine basically consists of choosing the tissues to be treated; in orchids protocorms, and PLBs are the most used, with the concentration (50–500 mg L^{-1} colchicine) and explant

exposure time (1–7 days) to the antimitotic agent (Vilcherrez-Atoche et al. 2022). After this process, tissues are immersed or exposed in a culture medium containing the antimitotic agent, followed by washing the tissues with deionized water and transferring the treated explants in a culture medium without the antimitotic agent, in order to reduce phytotoxic effects and regenerate polyploidized individuals. Subsequently, there is a need to select polyploidized individuals, which has been performed more frequently using flow cytometry, which is a more practical method that allows the analysis of a large number of plants in a short time, being more effective than chromosome counting using microscopy (Vilcherrez-Atoche et al. 2022).

Plant polyploidization results in a change in the architecture of polyploidized plants, including stem size and diameter, as well as leaf dimensions, shape, and color (Eng et al. 2021). Polyploid plants of *Phal. amabilis* var. *grandiflora* showed a reduction in plant size and an increase in the number of leaves (Mohammadi et al. 2021). Likewise, polyploid plants of *Den. nobile* showed a decrease in size, pseudobulb diameter and leaf width/length (Vichiato et al. 2007) in relation to diploids. In *Cym. lowianum*, artificial chromosome duplication generated plants with slow growth, short stems, and darker and thicker leaves (Xuejiao et al. 2010). In the reproductive part, such as leaves and inflorescences, changes such as increased size, color intensity, aroma, and durability of flowers are also observed (Sattler et al. 2016; Vichiato et al. 2007). Changes like those described have already been observed in polyploid plants of *Phal*. Golden Sands "Canary" showed an increase in the size of the flowers, in addition to a darker and more intense color (Griesbach 1985). Likewise, polyploid *Den*. *officinale* plants generated flowers with increased lip length and gynostemium width (Zhang and Gao 2020).

Most commercial cultivars of *Dendrobium*, *Cymbidium,* and *Phalaenopsis* originate from interspecific crosses, in which it has been observed that many of these hybrids have different levels of infertility due to irregularities during meiosis (Bolaños-Villegas and Chen 2007; Sattler et al. 2016). Triploid hybrids are those with the greatest infertility problems (De et al. 2014a, b), limiting their use in orchid breeding programs. Artificial polyploidization of these triploid genomes may result in the restoration of fertility in these hybrids (Sattler et al. 2016). An example of fertility restoration in orchids was observed in the triploid hybrid *Phal*. Golden Sands "Canary", in which colchicine-treated protocorms generated fertile hexaploid plants that were later used as progenitors for the development of new cultivars, such as *Phal*. Meadowlark (Griesbach 1985). On the other hand, the development of triploid plants (3×), from the crossing of polyploidized plants (4×) with diploid plants (2×), could result in hybrids of interest to companies focused on breeding, since the infertility of these hybrids could limit its use by other competing companies' genetic improvement programs.

4.4 Isolation, Culture, Regeneration, and Fusion of Protoplasts in Orchids

Protoplasts are plant cells free of the cell wall, which can be obtained from different plant tissues and organs, and which have the biological mechanisms necessary for the reconstruction of a new cell wall aiming at the regeneration of a complete plant (Naing et al. 2021).

The first stage in the protoplast culture system is the isolation of cells from the tissue or organ of the donor plant. There are two methods for removing the cell wall from plant cells, either by mechanical procedures used to obtain small amounts of protoplasts from larger cells (Davey et al. 2005) and by enzymatic digestion treatments (Davey et al. 2003). Currently, treatments using enzymes are the most used, in which intrinsic factors specific to the explant and extrinsic factors are considered important during the release and acquisition of protoplasts (Giles 2013; Sinha et al. 2003).

There are some efficient protocols for the isolation of protoplasts in different orchid genera, as observed by Teo and Neumann (1978), in which they used enzymatic treatment with cellulase (2%), macerosyme (1%), pectinase (0.5%), and 0.7 M sorbitol for the isolation of protoplasts from protocorms, leaves, plantlets, and shoots of *Renantanda* "Rosalind Cheok," *Phalaenopsis, Cattleya, Dendrobium,* and *Paphiopedilum*, respectively. Price and Earle (1984) also used isolated enzymatic treatments with 2% cellulase or in combination with driselase (0.5%) and macerosyme (1%) and 0.2 M and 0.5 M sorbitol for the isolation of protoplasts in *Angraecum, Brassia, Cattleya, Dendrobium, Odontonia, Paphiopedilum,* and *Vanilla*.

After the isolation of protoplasts, it is necessary to determine some important parameters such as density, viability, and yield of the isolated material to increase the chances of establishing the culture and achieving a high efficiency of fusion and regeneration (Naing et al. 2021). In orchids, protoplast/cell viability and isolated protoplast density were determined by the fluorescein diacetate and hemocytometer method, respectively (Yasugi et al. 1986; Shrestha et al. 2007; Ren et al. 2020a, b). These two parameters analyzed during the isolation of protoplasts and their possible regeneration can be influenced by the type and endogenous characteristics of the explant (Naing et al. 2021). Ren et al. 2020a, b observed in *Cymbidium* that the highest yield (~2.50 × 107/g FW), viability (~92.09%) and durability (>70% intact protoplasts for up to 3 days) of protoplasts were obtained with the use of leaf base tissues, compared to flower pedicels and young root tips. Explant age can also influence protoplast yield (Khentry et al. 2006), in which two-month-old *Dendrobium* Sonia "Boom 17" leaves generated a greater number of protoplasts per fresh weight (g) compared to 1-month-old leaves. In *Dendrobium* Pompadour, it was determined that the protoplast size and yield of leaves from plantlets >2.5 cm in length (31.12×10^5/g FW) were higher compared to leaves smaller than 2.5 cm in length (28.33×10^5/g FW) (Kanchanapoom et al. 2001). In a *Cymbidium* hybrid, a difference in protoplast isolation efficiency was found using in vitro (5.2×10^4/

g FW) and ex vitro (4.4×10^4/g FW) leaves (Pindel 2007). Similar results were reported by Kang et al. (2020).

In orchids, extrinsic factors such as low temperature (5 °C) caused a decrease in the percentage of isolation of protoplasts/cells after enzymatic treatment of leaves and petals of *Dendrobium* Yukidahura "Rainha" (Yasugi 1986).

Once the protoplasts are obtained, they are suspended to obtain an optimal density that allows theirs in vitro cultivation. In orchids, protoplast density is an important factor during its cultivation, as observed in protoplasts of *Dendrobium* Sonia "Bom 17," in which a density of 2×10^5 protoplasts/mL showed a higher division rate (20.18%) for the highest density, 5×10^5 protoplasts/mL (6.45%) (Khentry et al. 2006). The authors attributed this lower division rate to excess protoplasts, which would cause a rapid decrease in nutrients, interfering with cell wall regeneration and normal protoplast division.

In orchids, there are studies on the cultivation of protoplasts in some genera, such as *Aranda* (Kanchanapoom and Tongseedam 1994), *Cymbidium* (Pindel 2007), *Dendrobium* (Khentry et al. 2006; Yasugi 1986; Kunasakdakul and Smitamana 2003), *Phalaenopsis* (Shrestha et al. 2007; Kobayashi et al. 1993; Ichihashi and Shigemura 2002), *Rhyncholaelia* (Mota-Narvaez et al. 2018), and *Vanilla* (Montero-Carmona and Jiménez 2015).

Among the factors with the greatest effect on the cultivation and regeneration of *Phalaenopsis* and *Dendrobium* protoplasts are the culture medium, phytoregulators and the gelling agent. Shrestha et al. (2007) reported that the use of sodium alginate beads allowed a greater production of compact colonies of cells and that they presented a high capacity of callus induction and plant regeneration in *Phalaenopsis* when compared to the method with gellan gum or standard with agar. Plant regeneration from protoplast culture can follow the organogenic or embryogenic pathway. About 70% of ornamental species follow the organogenic regeneration pathway (Tomiczak 2020), but in orchids, regeneration via embryogenesis and the subsequent formation of PLBs has been more frequently observed (Shrestha et al. 2007; Kobayashi et al. 1993; Mota-Narvaez et al. 2018; Kunasakdakul and Smitamana 2003).

Isolation and cultivation of protoplasts have different purposes and applications in modern agriculture. Among these applications, the fusion of protoplasts from different species, also called somatic hybridization, is a biotechnological tool that allows the formation of different types of somatic hybrids depending on the degree of fusion between two protoplasts of different origins (Grosser et al. 2010). This technique has been used to hybridize species that cannot transmit their genetic characteristics through conventional breeding techniques via sexual hybridization (Grosser et al. 2010). The formation of somatic hybrids by protoplast fusion has been applied in ornamental plants (Naing et al. 2021) and in some orchid genera, such as *Dendrobium* (Kanchanapoom et al. 2001; Thomas et al. 2017; Yasugi 1989), *Phalaenopsis* (Sumardi and Indrianto 1991), and *Vanilla* (Montero-Carmona and Jiménez 2015; Divakaran et al. 2008; Macareno et al. 2016).

There are two mechanisms to carry out the fusion of protoplasts in plants by physical means—via electrofusion, and by chemical means—via polyethylene

glycol (PEG). Electrofusion allows the fusion of organelles and maintains the viability and integrity of the protoplast (Davey et al. 2005). Polyethylene glycol is a high molecular weight reagent that dehydrates and alters cell membranes, increasing their fluidity and affinity between membranes (Begna 2020) and has been the most used compound for the fusion of orchid protoplasts (Divakaran et al. 2008; Sumardi and Indrianto 1991; Yasugi 1989). Yasugi (1989) managed to form somatic hybrids of *Dendrobium* with *Epidendrum*, *Cattleya,* and *Paphiopedilum,* and Sumardi and Indrianto (1991) performed the fusion of *Dendrobium* and *Phalaenopsis* protoplasts using PEG. In the *Vanilla* genus of orchids, electrofusion was also used for somatic hybridization, which generated a high number of fusion events (8.9%) (Montero-Carmona and Jiménez 2015).

4.5 Production of Haploid and Double-Haploid Plants

The production of haploid and double-haploid plants is one of the most promising techniques in breeding programs for allogamous plants, with a high heterozygosity rate. Obtaining intervarietal hybrids from homozygous lines has supported world agriculture since the end of the last century, serving as the basis for high yields of crops such as corn, and more currently in the cultivation of vegetables, from the cultivation of hybrids originating from the F1 generation of crosses between homozygous lines.

Conventionally, obtaining homozygous lines from heterozygous plants can take between 7–9 generations of self-fertilization, making the process slow and tedious. On the other hand, the techniques of in vitro culture of gamete tissues, such as the culture of anthers or isolated microspores or the culture of eggs or ovaries in vitro are strategies of great value in different crops and currently support the production of homozygous strains in different species of agronomic and horticultural interest (Chaikam et al. 2019; Germanà 2011).

Basically, in a process of obtaining haploid and double-haploid plantlets, gamete cells from microspores or eggs are induced to enter consecutive cell divisions, without the need for fertilization. Thus, through the embryogenic pathway, there would be the formation of embryos and haploid plantlets, from a change from the gametophytic to the sporophytic pathway. In this regeneration process, there may be maintenance (haploid) or natural duplication of the haploid genome (double-haploid), resulting in completely homozygous plants, which can be used to obtain intervarietal hybrids.

Despite the wide applicability, there are few reports of obtaining haploid and double-haploid plants in orchids. Kato and Ichihashi (2018) observed the formation of haploid and double-haploid plantlets in orchids of the genus *Bletilla* via parthenogenesis, that is, by the use and regeneration of plantlets from unfertilized eggs. Sporophytic cell division has also been reported, due to the occurrence of symmetrical divisions and multicellular structures from microspores of *Dendrobium* hybrid and *Spathoglottis* orchids (Indrianto et al. 2015).

Despite the few studies in this area, the development of breeding programs based on obtaining haploid and double-haploid plants, this technology can be of great value both to expand the diversity of genotypes available for breeding programs and to produce hybrid seeds from homozygous lines, which could change, simplify, and cheapen the entire propagation system for ornamental orchids. This is because using this technology, seeds of the F1 generation of homozygous lines are used as the main source of propagation material, instead of complex systems conventionally used on a large scale and which involve the regeneration and proliferation of somatic tissues in vitro for cloning orchids (Cardoso et al. 2020).

If, on the one hand, each orchid fruit generates hundreds of thousands or even a million seeds, and directed pollination is a simple method to be used in orchids, on the other hand, some difficulties can limit the use of this technique, such as high polyploidy rate of current commercial cultivars, which would result in the regeneration of dihaploid ($n = 2x$, from $2n = 4x$) and non-haploid ($n = x$, from $2n = 2x$) tissues. Thus, for this technology to be properly employed, it requires the need to identify cultivars with commercial potential that have not yet undergone polyploidization, resulting in haploid and double-haploid plants that can be evaluated as homozygous lines in crosses aiming at obtaining elite hybrids.

5 Orchid Breeding in the Generation of Genome Sequencing and Editing

5.1 The Transient Gene Expression System in Orchids

Advances in genetic sequencing in some orchid genera (Chao et al. 2017; Hsiao et al. 2021) have allowed for the beginning of work on the identification and functional characterization of genes; this information is essential to understand the complex mechanisms of development, flowering, adaptation, nutrition, and reproduction in this plant family (Hsieh et al. 2013, 2020; Su and Hsu 2003; Tan et al. 2005).

The transient gene expression system using protoplasts is a technique that combines plant tissue culture through the isolation of protoplasts with genetic transformation mediated by the incorporation of genetic material through a vector using polyethylene glycol (PEG) or electroporation for the study of biological activity of different genes or proteins in plant cells (Ren et al. 2021; Davey et al. 2005).

It is a tool that allows for characterizing and studying the behavior, regulation, interaction, and expression of genes of interest within the plant transcriptome (Lin et al. 2018). There are studies in the literature on some orchid genera, such as *Cymbidium* (Ren, Gao, et al. 2020; Ren et al. 2021; Yang et al. 2021a, b; Ren et al. 2020a, b), *Dendrobium* (Li et al. 2021a, b, c) and *Phalaenopsis* (Li et al. 2018; Lin et al. 2018), which use the transient gene expression system using protoplasts.

In *Cymbidium*, the polyethylene glycol (PEG)-mediated transient gene expression system using leaf-based protoplasts was used to analyze the CsDELLA gene responsible for gibberellin (GA) regulation for flowering-related genes. Among the results of this work is the high efficiency of protoplast transfection (80%), which allowed the subcellular localization of the CsDELLA-GFP protein. The analyses showed that the overexpression of the CsDELLA gene caused a decrease in the expression of some genes related to flowering and the CsSOC1 and CsFT genes, while its silencing generated an upregulated expression of these aforementioned genes. The expression of genes related to flowering promoted by gibberellic acid (GA) also caused the suppression of the CsDELLA gene (Ren et al. 2021).

Yang et al. (2021a) used protoplasts from young leaves and petals of *Cym. ensifolium* to generate overexpression of the Ce-miR396 gene through the transient gene expression system using polyethylene glycol (PEG). The results showed that the overexpression of the Ce-miR396 gene generated a decrease in the transcription of the CeGRF gene in both types of protoplasts, indicating that the Ce-miR396 gene plays a key role in the development of plant organs and that regulatory differences in each CeGRF gene are due to different tissue-specific expression patterns.

In *Cymbidium*, the transient gene expression system by protoplasts allowed not only studies related to plant development but also virus–plant interaction studies, as observed in Ren et al. (2020b), where it was possible to observe in protoplasts that *Cymbidium* mosaic virus (CymMV) infection increased the expression of three proteins (CsNPR1–2, CsPR1–1, and CsPR1–2) and when salicylic acid (SA) was present, it increased the expression of CsNPR1–2. This SA-dependent protein CsNPR1–2 response is a defense mechanism that *Cymbidium* has against CymMV infection.

The PEG-mediated transient gene expression system also allowed the subcellular localization (cytoplasm and nucleus) of the DOTFL1 protein in protoplasts of the leaf mesophyll of *Dendrobium* "Chao Praya Smile." The DOTFL1 protein is an ortholog of TFL1 in *Arabidopsis thaliana* and is involved in vegetative growth as well as floral transition events necessary for reproductive success in *Dendrobium* (Li et al. 2021a, b, c).

In *Phalaenopsis* "Ruili Beauty" and *Phalaenopsis aphrotide* subsp. *formosa*, a transient gene expression system mediated by PEG was developed using protoplasts from young leaves and petals, respectively; in which this genetic tool allowed the subcellular localization of fluorescence-labeled proteins in the cell nucleus and membrane. Furthermore, the modified protoplasts served to provide specific information, such as protein–protein interactions, transcription factor activity, and response mechanisms to plant growth regulators (Li et al. 2018; Lin et al. 2018).

5.2 CRISPR Gene Editing

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/Cas9) is a gene editing technique that has been efficiently used in plant genetics (Semiarti

et al. 2020). One of its advantages in relation to genetic transformation is the fact that it allows the production of non-transgenic plants, which facilitates their regulation regarding genetic modification. CRISPR can perform gene editing without transferring exogenous DNA, but silencing endogenous genes to achieve a trait of interest (Corte et al. 2019).

Advances in genome sequencing in the family Orchidaceae have an impact on the development of this technique. Although some species of the genera *Phalaenopsis*, *Dendrobium*, *Cymbidium*, *Gastrodia, Bletilla, Platanthera, Vanilla,* and *Apostasia* have their genome sequenced (Zhang et al. 2022), the CRISPR/Cas 9 technique was reported only in *Phalaenopsis* (Nopitasari et al. 2020; Tong et al. 2020) and *Dendrobium* (Kui et al. 2017).

In *Phalaenopsis amabilis*, a successful CRISPR/Cas9 KO system was developed using protocorms and PDS3 as target genes, in which transformant plants showed albino phenotype in leaf tissues (Semiarti et al. 2020). A gene editing system was developed using *Agrobacterium*-delivered CRISPR/Cas9 carrying the VAR2 gene into *Phal. amabilis* protocorms, resulting in variegate patterns in the leaves of transformant plants (Nopitasari et al. 2020). In *Phal. equestris,* CRISPR was used to produce mutants, combined with *Agrobacterium*-mediated transformation with MADS genes, which are important for flower development (Tong et al. 2020). In *Dendrobium officinale*, CRISPR/Cas9 was applied for editing endogenous genes associated with the lignocellulose biosynthesis pathway (Kui et al. 2017). In *Dendrobium* Chao Praya Smile, the DOTFL1 was knockout aiming at rapid flowering and bulb formation, studying the role of DOTFL1 in plant development (Li et al. 2021a, b, c).

These results demonstrate that CRISPR gene editing systems are promising for the molecular breeding of orchids, enabling the transference of exogenous genes and the deletion of endogenous genes, as well as the development of new cultivars with characteristics of interest in the family Orchidaceae, regardless of the disadvantages of conventional breeding techniques.

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Biotechnological Interventions and Societal Impacts of Some Medicinal Orchids

Kalpataru Dutta Mudoi, Papori Borah, Dipti Gorh, Tanmita Gupta, Prasanna Sarmah, Suparna Bhattacharjee, Priyanka Roy, and Siddhartha Proteem Saikia

1 Introduction

Orchids are extremely fascinating plants that surpass all the plant groups in the "Plant kingdom." It belongs to the Orchidaceae family, which is the second largest as well as the highly advanced family among flowering plants. It encompasses approximately 850 genera and 35 thousand species (Stewart and Griffith 1995; Gutierrez 2010). Orchids are better known for their alluring, enchanting attractive floweret, which are extremely precious globally in floricultural trades. Orchids became the second most top-selling cut flowers as well as potted floricultural products due to their increasing demand in the globe for trading. Their aristocratic, adorable, and wonderful colors, sometimes-intricate forms, have enchanted men and women through the ages. Orchids lend a charming beauty with their extraordinary flower heterogeneity, in terms of size, shape, structure, number, density, color, and fragrance. Besides their adorning values, the orchids are also mentioned specially for their therapeutic medicinal properties as well as economic importance especially in the traditional pharmacopeias extensively since time immemorial (Withner 1959; Kaushik 1983). Earlier in China and Japan orchids were used as herbal medicine for different illnesses nearly 3000–4000 years ago, respectively (Reinikka 1995; Bulpitt 2005; Jalal et al. 2008).

Many species of *Vanda, Dendrobium, Habenaria, Malaxis, Cymbidium, Coelogyne, Cypripedium, Anoctochilus*, *Bletilla, Calanthe,* and *Cymbidium,* etc. are significantly important for having medicinal importance. Medicinal orchid

K. D. Mudoi (✉) · P. Borah · D. Gorh · T. Gupta · P. Sarmah · S. Bhattacharjee · S. P. Saikia
Agrotechnology and Rural Development Division, CSIR-North East Institute of Science and Technology, Jorhat, Assam, India

P. Roy
Centre for Infectious Disease, CSIR-North East Institute of Science and Technology, Jorhat, Assam, India

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plays an outstanding part in therapeutics with the presence of important phytochemicals such as alkaloids, flavonoids, carotenoids, sterols, saponins, anthocyanins, and polyphenols either in their pseudo bulb, tubers, leaves, stems, flowers, roots, or in the complete plant (Okamoto et al. 1966; Williams 1979; Majumder and Sen 1991; Majumder et al. 1996; Zhao et al. 2003; Yang et al. 2006; Singh and Duggal 2009). Several ailments like arthritis, tumors, fever, malaria, snakebite, scorpion bite, depression, tuberculosis, cervical carcinoma, diabetes, and biliousness, etc. are cured by medicinal orchids (Szlachetko 2001). These orchids were also employed as food and fodder, and local medicine by rural communities for their livelihoods and revenue generation. Moreover, uprooting the whole plant from its habitat for sale to the traders as well as over-exploitation by rural communities causes the extinction of many important orchid species (Kala 2004). Other than that native environment of many orchids is rapidly declining due to hefty desertification, habitat loss, urban sprawl, and usage of land for farming and cultivation. Therefore in medicinal orchids, it leads to a wide gap between booms and busts.

In recent years, in Western countries, the growing use of herbal medicine and its demand is increasing. Ultimately, this type of over-exploitation requisites an intense protection measure. But in situ or ex situ of medicinal orchids conservation in their natural habitat is not sufficient for propagation as their rate is low. Orchid seeds are small, have no endosperm, and require fungal pathogens to germinate; therefore, germination rates in nature are very low (Arditti 1992). It takes a long time to obtain the desired number of orchids through asexual reproduction by rhizomes, bulbs, or rooting branches. Hence, it needs proactive mass distribution and re-establishing them in nature. To meet their growing pressure and to reduce collection pressure on wild species, biotechnological approaches such as the plant tissue culture technique has contributed immensely to plantlets production on large scale and developed different protocols for rapid cloning of desired genotypes using various types of explants. This technique has come up as a key drive in the production of planting quality material for commercially and medicinally important orchids to fulfill the increasing demand and to reduce the collection pressure on wild orchids.

Under the above circumstances, biotechnological approaches enhance the in vitro propagation as well as conservation and mass multiplication of important medicinal orchids has raised high hopes by adopting asymbiotic seed germination, vegetative explants materials, artificial seed technology and secondary metabolites production, in vitro acclimatization of raised plantlets and their establishment in nature, etc. This chapter briefly endows the state-of-the-art information mediated on tissue culture with biotechnological interventions in some medicinal orchids through micropropagation, along with its societal impacts such as ethnomedicinal properties, phytochemistry, biological activities, and economics that being the need of the hour.

2 In Vitro Propagation

To establish a successful propagation of orchids explants type selection is the most crucial factor. Among the various vegetative explants materials, the leaf has been utilized as a potent and potential source of explants for the mass multiplication of orchids. Leaf has the viability for producing a large number of uniform plantlets from a single leaf or leaf segment through direct embryogenesis or organogenesis. Knudson (1922) explored the asymbiotic seed germination in orchids under the aseptic condition, which was the first feasible technique of in vitro propagation that formed the base of modern biotechnology (Knudson 1922). Later on, Rotor (1949) developed a method to culture *Phalaenopsis* using uni-nodal flower stalk cuttings but all credit goes to George Morel for developing a micropropagation technique for orchids at a large scale (Rotor 1949). Virus-free *Cymbidium* clones were obtained from in vitro shoot meristem culture (Morel 1960). Later on, Morel (1964) reported that it was possible to produce million of plantlets within a year using a single bud by frequent sub-culturing of protocorm-like bodies (PLBs) that motivated the orchid growers (Morel 1964). The present-day micropropagation in both basic and practical aspects is much more organized than it was in the beginning. Though shoot-tips have remained the most commonly used explants for propagating orchids, the regeneration potential of other explants like axillary buds, stem discs, inflorescence segments, floral stalks, leaves, leaf peels, perennating organs (pseudobulbs, rhizomes, tubers), and roots has also been utilized successfully (Vij et al. 2004; Arditti 2008).

2.1 Seed Germination

To produce firm seeds and flowers, it takes 5–10 years for an orchid plant. Orchid seeds are one of the most distinctive features of the Orchidaceae family. They are tiny, very small, and powdered, and are produced in large quantities, with 1300–4000,000 seeds per capsule (Harley 1951; Arditti 1961). Very fragile, relatively undifferentiated, and without endosperms or cotyledons, seeds are produced from the majority of orchid species (Mitra 1971).

Due to a lack of metabolic machinery and functional endosperm, the natural germination rate of orchid seeds is very poor. Only 0.2–0.3% germinates in natural conditions (Prasad and Mitra 1975). It is well known that the seeds of almost all orchids are entrusted to mycorrhizal fungi for germination in natural conditions. Symbiotic fungi have been extensively exhibited to induce seed germination in both terrestrial and epiphytic orchids for seedling development. But, asymbiotic seed germination has imparted a systematic way for the mass multiplication of orchids (Chen et al. 2022).

2.1.1 Asymbiotic Seed Germination

The ability of orchid seeds to germinate asymbiotically by in vitro means was demonstrated for the first time by Knudson in *Cattleya* species (Knudson 1922). Asymbiotic in vitro seed germination of orchids occurred by culturing immature ovules often known as either embryo, fruit, or pod (Fig. 1a–d). The germination potential of immature embryos was much better than that of mature ones and varied with their developmental stages. Due to pH, dormancy, and other metabolic factors, very young orchid oocytes cannot germinate and thus cannot form suitable explants (Withner 1953). During in vitro seed germination of orchids, the intermediate protocorm stage is followed by subsequent seedling development (Fig. 1e–f). A protocorm is a chlorophyll-like, hairy, and pear-like bulbous or oblong structure that originates from the apical or lateral suture of the seed coat and provides nutrients like cotyledons during embryonic development and subsequent seedling growth (Lee 1987). Protocorms have been inconsistently assessed as uniform callus structures or distinct shoots (Kanase et al. 1993). The protocorm-like body specified the orchids for the regeneration of multiple plantlets which is a blessing to the world floricultural market (Fig. 1g–j).

Asymbiotic seed germination of orchids was exploited for in vitro mass production of orchids with commercial and medicinal importance for conservation and ecorestoration. It was reported by several investigators from time to time.

Half strength of Murashige and Skoog (MS) medium (Murashige and Skoog 1962) were used for seed germination of *Bletia purpurea* (Dutra et al. 2008), *Coelogyne stricta* (Parmar and Pant 2016), *Cymbidium giganteum* (Hossain et al. 2010), *Cymbidium goeringii* (Gong et al. 2018), and *Spathoglottis plicata* (Aswathi et al. 2017; Hossain and Dey 2013). Accordingly, *Cymbidium aloifolium* was germinated in 1.0 mg/L 6-benzylaminopurine (BAP) and 0.5 mg/L α-naphthaleneacetic acid (NAA) supplemented (Paul et al. 2019). However, a modified half-strength MS medium was tested for in vitro germination of *Dendrobium ovatum* (Shetty et al. 2015).

Six different media compositions for testing were examined for their effectiveness towards the growth of *Dactylorhiza hatagirea* (Warghat et al. 2014) and *Bletia purpurea* seeds in BM-1 (Van Waes and Debergh 1986); 1/2 MS, Vacin and Went modified (VW) medium (Vacin and Went 1949); Malmgren modified terrestrial orchid medium (MM) (Malmgren 1996) and Knudson C (KC) medium (Knudson 1946). *Dendrobium macrostachyum* seeds were accomplished on MS, VW, and KC medium having different accumulation, amalgamation of growth hormones, and other additives. Among them, VW basal medium tested with 0.5 mg/L BAP and 5 mg/L NAA was more acceptable for plantlet formation (Li et al. 2018). *Dactylorhiza hatagirea* was cultured on Heller and Lindemann (LD) medium (Warghat et al. 2014), MM, VW, MS, and KC media. Both MS and KC medium were examined for asymbiotic seed germination of *Eria bambusifolia* (Basker and Bai 2010). MS, KC, and KC-modified Morel medium were used for *Satyrium nepalense* (Mahendran and Bai 2009) seed germination. Seeds from mature capsules

Fig. 1 In vitro micropropagation of *Cymbidium aloifolium*, (**a**) Mother plant, (**b**) Seed capsule, (**c**) In vitro seed germination, (**d**) Swelling of seeds, (**e**) PLBs formation, (**f**) Enlargement of PLBs, (**g**) Shoot formation, (**h**) Formation of shoot and root, (**i**) Shoot elongation, (**j**) Shoot multiplication, (**k**) Hardening and acclimatization, (**l**, **m**) Acclimatized plantlets ready for ecorestoration

of *Dendrobium trigonopus* were augmented in B_5, MS, and 1/2 MS with NAA, BAP, and bark powder for in vitro germination (Pan and Ao 2014). MS + 1.0 mg/L BAP + Phytamax™ were provided for seed germination of *Dendrobium aphyllum* (Hossain et al. 2013).

Vacin and Went (1949) medium was alone tested for seed germination of *Dendrobium parishii* (Vacin and Went 1949; Kaewduangta and Reamkatog 2011). Likely, on VW medium mature seeds of *Dendrobium lasianthera* were enhanced with the incorporation of different concentrations of peptone of 1, 2, and 3 gm/L (Utami et al. 2017). Mature seeds of *Cypripedium macranthos* were sown on hyponex-peptone (HP) medium that contained 1 μM NAA and BAP after sterilization (Shimura and Koda 2004). Mature capsules of *Ansellia africana* were tested on Vasudevan and Van Staden (2010) medium for seed germination in vitro (Vasudevan and Van Staden 2010; Bhattacharyya et al. 2017a). However, in vitro germination of *Dendrobium nobile* Lindl. (Bhattacharyya et al. 2014), *D. thyrsiflorum* (Bhattacharyya et al. 2015), *D. heterocarpum* (Longchar and Deb 2022), *Cymbidium iridioides* (Pant and Swar 2011), *C. kanran* (Shimasaki and Uemoto 1990), *Cypripedium debile* (Hsu and Lee 2021), and *C. macranthos* (Shimura and Koda 2004) was reported in MS medium of full strength. *Cymbidium iridioides* young pods were cultured on MS medium containing 1 mg/L of NAA and BAP (Longchar and Deb 2022). Immature seeds of *Cymbidium kanran* were inoculated on MS medium for shoot multiplication (Shimasaki and Uemoto 1990). Young pods of *Cymbidium iridioides* were cultured on MS medium having NAA (1 mg/L) + BAP (1 mg/L) for micropropagation (Pant and Swar 2011).

2.2 *Micropropagation of Orchids Via Vegetative Explants Materials*

In orchids, as a result of out crossing, heterozygous offspring were produced from seeds. Therefore, it is necessary to augment various vegetative parts of mature plants to validate micropropagation protocols in orchids. Georges Morel was the pioneer for culturing *Cymbidium* shoot tips and attained protocorm-like bodies (PLBs) from contaminated plants to regenerate mosaic virus-free plants (Morel 1960). He introduced the term "protobulb (PLB)" in his work published in the Bulletin of the American Orchid Society (Arditti 2010). At the same time, a number of orchid species have yielded fruitful results, including *Lycaste, Cattleya, Ondontoglossum, Dendrobium, Phaius, Miltonia*, and *Vanda* (Arditti and Ernst 1993).

Large-scale propagation of medicinal orchids through in vitro method, different vegetative explants sources such as shoot tip, axillary bud, leaves, nodal segments, and inflorescence were augmented through callus formation or PLB mediation or direct shoot bud formation as described below:

2.2.1 Shoot Tip Culture

To induce efficient clonal propagation of medicinal orchids, shoot tips have been efficiently cultured. It was first implemented in *Cymbidium* by Morel (Morel 1960). This technique enables the rapid propagation of *Vanda coerulea (*Seeni and Latha 2000*)*. Response of bud formation is obtained from the shoot tips in vitro and mature plants in a medium having 8.8 μM BAP and 4.1 μM NAA. For forming multiple shoots in *Vanda tessellate* BAP and NAA combination was found to be more effectual as compared to indole-3-acetic acid (IAA), NAA, and kinetin at single action (Rahman et al. 2009). Shoot primordium of *Doritis pulcherrima* was cultured for rapid propagation and regeneration of plantlets (Mondal et al. 2013). In VW medium, *Dendrobium* shoot tip was cultured containing 15% coconut water plus 10 ppm NAA for a rapid proliferation of PLB and plantlet formation as well as the growth of seedlings (Soediono 1983). Sixty days old *Dendrobium chrysotoxum* shoot tips was inoculated on MS + 0.1 mg/L NAA + 3% sucrose + 0.5 mg/L BAP for proliferation, shoot induction (Gantait et al. 2009).

2.2.2 Nodal/Internodal Culture

Dendrobium fimbriatum segments were conferred for shoot induction, and proliferation in MS + 0.2–0.5 mg/L NAA + 1.0–4.0 mg/L BAP (Huang et al. 2008). But MS medium with NAA and BAP at 17.76 μM recorded maximal regeneration (14.0 ± 0.47) of shoots (Paul et al. 2017). Stem nodes of *Dendrobium devonianum* cultured at MS + 0.01–0.5 mg/L NAA + 1.0–4.0 mg/L BAP for PLB and shoot induction and proliferation in vitro (Li et al. 2011, 2013a). 0.5–1.0 cm nodal segments excised with axillary buds from 4–5-month-old *Dendrobium chrysanthum* seedlings grown in vitro, half strength MS + 0.1 mg/L NAA + 6 mg/L BAP + 3% sucrose + 0.65% agar (Mohanty et al. 2013a).

Nodal explants of *Malaxis acuminata* were cultured on MS + sucrose (3% w/v) + 3 μM NAA + 3 μM BAP and resulted in well-developed plantlets with shoots and root growth (Arenmongla and Deb 2012). Young healthy nodal shoot segments from the newly grown branches of wild *Bulbophyllum odoratissimum* were taken and cultured on BAP (4.0 mg/L) and IBA (0.5 mg/L) fortified MS medium for producing maximum shoot proliferation (Prasad et al. 2021). Nodal cultures of *Ansellia africana* were tested in an MS medium supplemented with 5 μM NAA and 10 μM of meta-topolin (mT) for multiple shoot induction (Bhattacharyya et al. 2017a). Pseudo-stem segments of *Dendrobium nobile* with nodes (0.5–1 cm) was used as explants for induction of PLBs with varied concentration of thidiazuron (TDZ) for culture (Bhattacharyya et al. 2014). *Malaxis acuminata* internode cultures responded to MS + 0.5 mg/L NAA + 3 mg/L TDZ; MS + 0.5 mg/L NAA + 3 mg/L TDZ + 0.4 mM spermidine (spd); MS + 1.5 mg/L activated carbon (AC) + 4 mg/L IBA was used for shoot induction (Cheruvathur et al. 2010).

2.2.3 Leaf Culture

Leaves and leaf tips of young orchids were cultured in vitro for PLB initiation and shoot proliferation. Wimber (1965) showed the potential of *Cymbidium* leaves (Wimber 1965). Growth stimulation in the nutrient pool, donor axis location, and physiological age of the mother plant strongly determine the regeneration potential (Trunjaruen and Taratima 2018). Therefore, factors like growth hormones, medium nutrients composition, leaf part, leaf source (in vivo/in vitro), explants preparation, leaf maturity, etc. determine the efficiency of a leaf explants micropropagation protocol (Chugh et al. 2009).

The leaf base of Vandaceous orchids evinced greater proliferative potential than leaf tips (Na and Knodo 1995; Jena et al. 2013; Seeni and Latha 1992; Nayak et al. 1997). Younger leaves perform better than older leaves. Leaves of mature *Vanda coerulea* did not respond to bud formation or PLB in vitro (Seeni and Latha 2000). Whereas, mature plants of *V. spathulata* (L.) Spreng the regeneration potential of leaf explants was noticed with 28.5 μM IAA + 66.6 μM BAP medium (Mitra et al. 1976).

2.2.4 Axillary Bud Culture

Axillary bud culture also played a very important role in medicinal orchid micropropagation. *Cymbidium elegans*'s axillary buds were responsive to PLBs formation (Pant and Pradhan 2010). Axillary bud culture of *Dendrobium longicornu* was tested in MS medium with 0.8% agar + 3% sucrose + 5 μM NAA and 15 μM BAP (Dohling et al. 2012). In *Cypripedium formosanum* a quarter concentration of MS medium containing 22.2 or 44.4 mM BAP was sufficient to propagate 6.3 and 7.1 shoots per explant with an average length of 10.6–11.7 mm to produce cultures after 90 days (Lee 2010). Five species of *Dendrobium* (*D. crumenatum*, *D. fimbriatum, D. moschatum, D. nobile,* and *D. parishii*) induced multiple shoots when axillary buds were cultured in vitro (Sobhana and Rajeevan 1993). Field-grown axillary buds of *Lycaste* hybrids were grown in half-strength MS basal medium supplemented with 0.5 mg/L BAP and 1.0 mg/L TDZ and 2% (w/v) sucrose (Huang and Chung 2011). Six to seven millimeter long shoot tips of *Aranda* Deborah hybrids grown in VW medium supplemented with coconut water (20% v/v) produced an average of 2.7 PLB after 45 days (Lakshmanan et al. 1995).

2.2.5 Pseudobulb Culture

The pseudobulb of *Coelogyne cristata* was cultured with basal medium + BAP (1–10 mg/L) + kinetin (1–10 mg/L) alone and in combination with NAA (1–10 mg/L). In parallel sets of experiments, 0.2% AC was used in the medium for shoot multiplication (Sharma 2021); 6-BAP (2.0 mg/L) + NAA (0.5 mg/L) induced shoot

proliferation in *C. flaccid* (Parmar and Pant 2016). The pseudobulb of *Malaxis acuminata* was cultured on MS + 1.0 mg/L BAP + 1.0 mg/L NAA + 2.0 g/L AC for PLB formation (Suyal et al. 2020).

2.2.6 Flower Bud Culture

Ascofinetia, Neostylis, and Vascostylis were the first species to culture the young flower buds or inflorescence for medicinal orchid micropropagation (Intuwong and Sagawa 1973). Similarly, *Phalaenopsis*, *Phragmipedium,* and *Cymbidium* were also cultured equivalently (Kim and Kako 1984). The floral buds were exposed to either higher auxin levels or higher cytokinin levels and anti-auxin levels (Zimmer and Pieper 1977; Tanaka and Sakanishi 1978; Reisinger et al. 1976). Younger floral buds or inflorescence were more responsible than the matured ones in terms of shoot or PLB proliferation in *Oncidium Gower Ramsey*, *Phalaenopsis capitola*, *Dendrobium Miss Hawaii*, *Ascofinetia (*Intuwong and Sagawa 1973*;* Mitsukuri et al. 2009*;* Nuraini and Shaib 1992*)*.

2.2.7 Root and Rhizome Segment Culture

The in vitro root culture was so far attempted with success in a few species of medicinal orchids. The capacity of orchid root to induce shoot regeneration was very low as reported earlier (Kerbauy 1984). Thereafter roots of *Catasetum*, *Cyrtopodium*, and *Rhyncostylis* were utilized to regenerate plantlets a very high proliferation rates (Kerbauy 1984; Sanchez 1988; Sood and Vij 1986). Root tips excised from *Vanda* hybrids and *Rhyncostylis* were cultured in 1.0 mg/L IAA, 1.0 mg/L BAP and 200 mg/L of casein hydrolysate for a speedy shoot proliferation rate (Chaturvedi and Sharma 1986). Rhizome of *Cymbidium goeringii* responded to MS + 0.2% (w/v) AC, 3% (w/v) sucrose, 0.2% (v/v) coconut water, and 0.8% (w/v) agar powder (Park et al. 2018). Moreover, auxin, particularly NAA was responsible for stimulating rhizome formation of some medicinal orchids and ultimately new shoots were developed from a rhizome in a cytokinin-enriched medium of *C. kanran* Makino (Shimasaki and Uemoto 1990), *C. forrestii* (Paek and Yeung 1991), and *Geodorum densiflorum* (Roy and Banerjee 2002).

Rhizome tips were also tested for PLB formation and shoot development (Udea and Torikata 1972). In a few cases, cytokinins were inductive for stimulation of shoots from rhizome segments of medicinal orchids such as *Cymbidium forrestii (*Paek and Yeung 1991*)* and *Geodorum densiflorum* (Lam.) Schltr. (Roy and Banerjee 2002; Sheelavantmath et al. 2000). Sometimes BAP was responsible for the reduction of rhizome growth and branching but induced certain rhizome tips gradually into shoots (Paek and Yeung 1991).

2.2.8 Thin Cell Layer Culture

Longitudinal or transverse sections of the thin cell layers are isolated from different plant parts such as leaves, floral primordia, stems, or PLBs. The efficiency of normal plant tissue culture and thin cell layer culture techniques is compared very methodically (Rout et al. 2006). In vitro raised seedlings of *Dendrobium chrysotoxum,* cross-section (2 mm thickness) of stem-nodes is grown in MS medium (semi-solid and liquid) supplemented with BAP 4.44 μM and Kinetin 4.65 μM induced shoot buds (Kaur 2017).

2.2.9 Protoplasts Culture

Different explants of orchids like stem, root, leaf disc, petal, and protocorm were used for the isolation of protoplasts. Chris K. H. Teo (Malaysian scientist) and K. Neumann (German botanist) first introduced the induction and synthesis of orchid protoplasts (Teo and Neumann 1978a, b). Since then studies were carried out in this field for the isolation of orchid protoplasts. However, during the screening of more than 24 orchid species, from bases of juvenile leaves of medicinal orchid *Cymbidium aloifolium* protoplast culture was achieved (Seeni and Abraham 1986).

2.3 Root Induction

Concentrations of different auxins were incorporated into basal media either singularly or in combination for testing their root-promoting efficiency in medicinal orchids. For root induction of *Dendrobium fimbriatum* with 100% rooting frequency, MS + 0.5 mg/L NAA or 0.3–1.0 mg/L IBA and a combination of 0.5 mg/L IBA and NAA were used (Huang et al. 2008). IBA, IAA, and phenolic elicitor PG containing MS medium were responsible for root induction of *Ansellia africana* within 6 weeks interval (Bhattacharyya et al. 2017a). IBA was responsible for root promotion of medicinal orchids viz., 1.0 mg L/1 IBA in *Acampe praemorsa* (Nayak et al. 1997) and *Cymbidium iridioides* (Pant and Swar 2011), and 1.5 mg L/1 IBA in *Dendrobium densiflorum* (Pradhan et al. 2013).

A decline in root number and length was reported with increased concentration of IBA. In *Dendrobium nobile*, IBA was better than NAA in maximizing root numbers (Asghar et al. 2011). MS + 3% sucrose + 2 g/L AC + 0.2 mg/L IBA was used in *Dendrobium chrysotoxum* (Gantait et al. 2009). Whereas, in the root formation of *Vanilla planifolia* and *Geodorum densiflorum*, NAA exhibited a conducive effect (Sheelavantmath et al. 2000; Tan et al. 2011).

In *Dendrobium transparens* (Sunitibala and Kishor 2009) and *Dendrobium primulinum* (Pant and Thapa 2012) supplementation of IAA increased the rate of root proliferation whereas its affectivity was poor during root formation. However,

rooting of *Vanda spathulata* shoots was observed within 3–9 weeks in a medium containing 75 g/L banana pulp and 5.7 μm IAA. In vitro shoots of 2–5 cm in length developed two to five roots easily in pots at 80–90% survival rates instead of hardening (Decruse et al. 2003).

2.4 Photoperiodic Condition

In vitro seed culture and micropropagation of medicinal orchids were influenced by ambiance conditions, like photoperiod (PP) for efficient early culture development.

Cool white light, 16/8-h PP, 1000 lux light intensity, 25 ± 2 ° C, and pH 5.2 have been reported for *Dendrobium moschatum* (Kanjilal et al. 1999). Fluorescent light, 12/12-h PP, 60 μL mol m^{-2} s^{-1}, 25 ± 2 °C was provided in *D. parishii (*Kaewduangta and Reamkatog 2011*). D. trigonopus* was probably supplemented with 14/12-h PP, 25 ± 2 °C, 50 μL mol m^{-2} s^{-1} (Pan and Ao 2014). In *D. aphyllum* provide 14/12-h PP, 60 μL mol m^{-2} s^{-1}, cool white fluorescent, 25 ± 2 °C (Hossain et al. 2013). 1000–1500 lux, 12/12-h PP, white fluorescent tube, 25 ± 1 °C extended to *D. candidum* (Zhao et al. 2008). 50 μL mol m^{-2} s^{-1}, 12/12-h PP, 25 ± 2 °C was furnished in *D. chrysanthum* (Mohanty et al. 2013a). In *D. chrysotoxum* 16/8-h PP, 30 μL mol $m^{-2}s^{-1}$, white fluorescent tube, 60% RH, 25 ± 2 °C was supplied (Gantait et al. 2009). Originally, 25 ± 2 °C in the dark for 2 weeks, 23 μL mol $m^{-2}s^{-1}$ 25 ± 2 ° C, 16/8-h PP, (callus + PLB) was described in *D. crumenatum* (Kaewubon et al. 2015). 350–500 lux 16/8-h PP, 25 ± 2 °C was supplied in *D. densiflorum* (Pradhan et al. 2013). 1500–2000 lux, 12/12-h PP, 25 ± 2 °C and pH 6.0 was suitable for *D. devonianum* (Li et al. 2011, 2013a). Cool white fluorescent tubes, 12/12-h PP, 40 μL mol m^{-2} s^{-1}, 25 ± 2 °C were used in *D. draconis* (Rangsayatorn 2009). 2000 lux, 12/12-h PP, 25 °C and pH 5.4–5.6 was reported in *D. fimbriatum* (Huang et al. 2008). Cultures of *Ansellia africana* were maintained in cool white fluorescent tubes in a culture room with a light intensity of 40 μ mol m^{-2} s^{-1} at 25 ± 2 °C under a dark and light cycle of 12 h (Bhattacharyya et al. 2017a). *D. fimbriatum* was cultured under a photoperiod of 14 h with a light intensity of 50 μ mol m^{-2} s^{-1} using cool-fluorescent tube lights, at 25 ± 2 °C (Paul et al. 2017).

2.5 Hardening and Acclimatization

Hardening and acclimation of in vitro cultured plantlets are important steps of micropropagation for better survival and successful plant establishment under ex vitro conditions. The percentage of plant loss or damage is higher during the transfer of in vitro growing plants to ex vitro conditions. Regenerates have to adapt to many abnormal conditions such as high irradiance, low humidity, and water hydraulic conductivity of the root and root-stem connections in an ex vitro environment (Fila

et al. 1998). Acclimatization of regenerates with gradually reducing humidity will overcome this threat (Bolar et al. 1998).

Well-rooted micropropagated orchid plantlets were ready for acclimatization after attaining sufficient growth in terms of root or shoot length. After removal from flasks, the well-rooted plants were cleaned thoroughly to remove the remnant of artificial media such as sucrose and nutrient agar. Thereafter, clean plantlets were soaked in an effective fungicide solution before shifting them into pots or poly sleeves having a potting mixture. The blending of various potting mixtures plays an important part in the survivability of orchid plantlets raised in vitro. A combination of the potting mixture was pounded of dried coconut husk or coco peat, tiny pieces of tree cortex, peat moss or sphagnum moss, and pieces of broken bricks or charcoals in various ratios. The ideal potting mixture should have water retaining capacity along with draining out of extra water and aeration for proper hardening and acclimatization of plants (Diaz et al. 2010; Kang et al. 2020) (Fig. 1k–m).

Brick pieces and charcoal chunks (1:1) mixture were fruitful for acclimatization of *Dendrobium chrysanthum* with a topmost cover of moss (Mohanty et al. 2013a). Plantlets of *Dendrobium moschatum* were shifted for hardening to a blending of charcoal, brick, coal, sand, and soil (1:1:1:1:1) with 48% survivability (Kanjilal et al. 1999). Rooted shoots of *Dendrobium macrostachyum* were provided with a perlite and peat moss mixture and kept in the green house for acclimatization (Li et al. 2018). In the mixture of coco peat, litter, and clay in the ratio of 2:1:1 with a covering of sphagnum moss *Cymbidium aloifolium* plantlets were acclimatized with an 85% survival rate (Pradhan et al. 2013). Acclimatization was carried out for hardening plantlets of *Dendrobium draconis* and shifted to cocopeat and perlite (1:1) composition with 92% achievement (Rangsayatorn 2009). In *Coelogyne cristata, the* composition of pine bark, brick, moss, and charcoal pieces (1:1:1:1) was used for transplanting (Sharma 2021). In *Coelogyne finlaysonianum*, brick, charcoal, coco peat and litter (1:1:1:1); brick, charcoal, litter, and saw dust (1:1:1:1); brick, charcoal, and litter (1:1:1); and brick and charcoal (1:1) were utilized for survival (Islam et al. 2015). A mixture of humus and sand (1:1) was tested in *Changnienia amoena* (Jiang et al. 2011). A composition of brick, charcoal, coconut husk, and sand (1:1:1:1) was provided for acclimatization of *Spathoglottis plicata (*Grell et al. 1988*)*. In *Cymbidium iridioides*, plantlets were acclimatized by using cocopeat, peat moss, and brick (Pant and Swar 2011). In the ratio of 1:1:1 substrate of brick, charcoal, shredded bark, and a moss cover were imparted for the survivability of *Dendrobium longicornu* in a greenhouse (Jaime et al. 2015). *Eria bambusifolia* was tested on coconut husk, charcoal, brick pieces, broken tiles, and perlite (Basker and Bai 2010). Hardening plantlets of *Satyrium nepalense* were transferred to a 1:1:1 ratio of a mixture containing vermicompost, sand, and coconut husk in plastic pots (Mahendran and Bai 2009). *Rhynchostylis retusa* was adapted in small plastic pots containing (2:1) moss and bark (Naing et al. 2010). *Cypripedium macranthos* was hardened in a plastic bag that contain wet vermiculite and acclimatized in a soil mixture of coarse volcano ash and clay granules (Shimura and Koda 2004). *Dactylorhiza hatagirea* was survived in a potting mixture consisting of (1:1:1) cocopeat, vermiculite, and perlite (Warghat et al. 2014). Rooted plantlets of

Dendrobium lasianthera were planted in a composition of coconut husk and sphagnum moss (3:1) and achieved a 90% survivability rate (Utami et al. 2017). In vitro rooted *Ansellia africana* plantlets were tested with a mixture of vermiculite, sand, and decaying litter (1:1:1) and found 87% survivability after 60 days (Bhattacharyya et al. 2017a). *Dendrobium nobile* plantlets were acclimatized with various compositions of mixture viz., (1) charcoal and bricks in the ratio 1:1; (2) in the ratio 1:1 of decaying litter and brick; (3) in the ratio 1:1:1 of brick chips, leaf litter, and charcoal; and (4) brick chips, leaf litter, and charcoal in the ratio 1:1:1 in addition to the topmost coating of moss. Among various compositions brick, charcoal, and decaying litter treatment as well as moss covering received the highest 84.3% survivability (Bhattacharyya et al. 2014). Composition of (a) brick and charcoal (1:1) (b) brick and coco peat in the ratio 1:1 (c) coco peat, brick, charcoal pieces in the ratio 1:1:1; and (d) leaf mold, brick chips, and cocopeat in the ratio 1:1:1 were supplied for transplantation of *Bulbophyllum odoratissimum* in Green house condition with 90% relative humidity (RH) and 91.66% survival rate. Among the different treatments, brick chips, charcoal, and coco peat (1:1:1) containing the mixture was best for high water retention as well as good aeration capacity (Prasad et al. 2021).

3 Ecorestoration

Ecosphere restoration is the "task reconstructing of an ecosystem that has been damaged due to manmade catastrophe" (Libini et al. 2008). The main objective of restoration is to re-establish the environmental system that is disturbed by various factors with respect to its structure and functional properties.

After successful acclimatization, in vitro-raised *Vanda coerulea* plantlets were transferred to tree trunks of forest segments, for successful ex situ harbor by using the binding medium like moss and coconut husk with 70–80% survivability rate for ecorestoration. Such a study commencing in India for restoring the natural habitat is of great interest from a horticultural and conservation point of view (Seeni and Latha 2000). Similarly, *Epidendrum ilense* and *Bletia urbana* were also shifted to the forest ecosystem or typical natural habitat for ecorestoration (Christenson 1989; Rublo et al. 1989). During the lab to land transfer strategy, it was observed that host trees with rough bark were selected and the in vitro-raised orchids were fixed either to the tree trunks with the roots or tree bark for ecorestoration efforts (Decruse et al. 2003; Aggarwal and Zettler 2010; Aggarwal et al. 2012; Gangaprasad et al. 1999; Grell et al. 1988; Kaur et al. 2017). Micropropagated plantlets of *Smithsonia maculate* showed 48% survival after one year reinforced at Karamana river of Peppara Wildlife Sanctuary, Kerala, India. The pilot trial on restoration through micropropagation was useful for further reintroduction and population enhancement for the practical conservation of this orchid (Decruse and Gangaprasad 2018). In vitro rooted plantlets of *Vanda spathulata* were observed with a 50–70% survival rate, which were introduced into forest segments at Ponmudi and Palo de in the Southern Western Ghats of India (Decruse et al. 2003).

The reintroduction trials of orchid plantlets should be conducted with well-established in vitro-rooted plantlets during the monsoon period to corroborate the maximum survival rate of the plantlets for ecorestoration or eco-rehabilitation study.

4 Artificial Seed Technology

The concept of artificial or synthetic seed was first coined by Murashige and at present it is well known by some different names such as manufactured seed, synthetic seed, or synseed (Murashige 1977). Artificial seeds were originally defined as "encapsulated single somatic embryos" by Murashige (1978), i.e., a clonal product that can grow into plantlets at in vitro or ex vivo conditions if used as real seeds for sowing, storage, and transport (Murashige 1978). Gray and Purohit (1991) also define somatic embryos with practical usage in commercial plant production (Gray et al. 1991). Therefore, the production of synthetic seeds has previously been restricted to those plants where somatic embryogenesis has been reported. Although somatic embryogenesis is restricted to selective plant species, to overcome this limitation, exploration of a suitable alternative to somatic embryos, i.e., non-embryogenic vegetative propagules like shoot tips, segmental/axillary buds, protocorm-like bodies (PLBs), organs or embryogenic callus is practiced (Ahmad and Anis 2010; Ara et al. 2000; Danso and Ford-Llyod 2003).

However, artificial/synthetic seeds or beads production was reported first time by Kitto and Janick (Kitto and Janick 1985). Since then, several flowering plant species have extensively utilized this technique including orchids. Production of synthetic seeds opens a new vista in plant tissue culture technology by adding many fruitful improvements on a commercial scale. Artificial seeds were utilized for transformation into plantlets under in vitro and in vivo circumstances. It was applied for the multiplication of rare, threatened, and endangered plant species which are hard to propagate by normal propagation process and by natural seeds.

Synthetic seed production in orchids is especially important as they produce minute non-endosperm seeds. Corrie and Tandon (1993) have used protocorms to produce synthetic seeds of *Cymbidium giganteum* which are transferred to a nutrient medium or sterile sand and soil medium developed healthy seedlings (Corrie and Tandon 1993). Comparable conversion frequencies of 100%, 88%, and 64% were obtained on in vitro, sand, and sand-soil mixture condition, respectively. These observations enable the direct transplantation of aseptically grown protocorms into the soil as well as reduce the cost of growing plantlets in vitro and subsequent acclimatization. As orchids produce tiny and non-endospermic seeds, the production of artificial seeds was beneficial.

Several reports on encapsulation using somatic embryos have been carried out (Ara et al. 2000; Danso and Ford-Llyod 2003; Castillo et al. 1998; Ganapati et al. 1992). For synthetic seed production, meristematic shoot tips or axillary buds were also utilized in orchids along with somatic embryos or PLBs (Ganapati et al. 1992; Bapat et al. 1987; Piccioni and Standardi 1995). Encapsulation of PLBs is well

reported in many orchids such as *Cymbidium giganteum*, *Dendrobium wardianum*, *Dendrobium densiflorum*, *Phaius tonkervillae*, and *Spathoglottis plicata* (Danso and Ford-Llyod 2003; Saiprasad and Polisetty 2003; Vij et al. 2001).

In *Dendrobium* orchid, Saiprasad and Polisetty found that fractionated PLB was best suited for encapsulation at leaf primordia stage 13–15 days after culture (Saiprasad and Polisetty 2003). Encapsulation matrices prepared with MS medium (3/4 strength) + 0.44 μMB BAP + 0.54 μM NAA result in 100% conversion of encapsulated PLBs when cultured on MS medium + 0.44 μMB BAP + 0.54 μM NAA (*Dendrobium*). Sarmah et al. (Sarmah et al. 2010) production of synthetic seeds in an endangered monopod orchid, i.e., *Vanda coerulea* by leaf-based encapsulating PLBs with 94.9% conversion frequency on immediate inoculation in Ichihashi and Yamashita (IY) medium (Ichihashi and Yamashita 1977). 95% conversion was achieved on encapsulating PLB of *Flickingeria nodosa* in Burgeff medium (Withner 1955) + 2% sucrose + 2 mg/L Adenine sulfate + 1 mg/L IAA at 4 °C for 3 months (Nagananda et al. 2011). Alginate encapsulation of *Aranda* × *Vanda* PLB was also reported (Gantait et al. 2012). Three percent sodium alginate and 75 mM calcium chloride support better encapsulation of individual PLBs (4 mm long). Plant growth regulator (PGR)-free MS medium (1/2 strength) reported 96.4% of conversion. Likely, short-term storage of PLBs of *Dendrobium shavin* (Bustam et al. 2012); 60-day-old PLBs in *Dendrobium nobile* (Mohanty et al. 2013b) and *Coelogyne breviscapa* (Mohanraj et al. 2009); 30-day-old PLBs in *Geodorum densiflorum* (Datta et al. 1999); PLB of *Spathoglottis plicata* Blume (Haque and Ghosh 2017); somatic embryos in *Dendrobium candidum* (Guo et al. 1994) were used for encapsulation with varied binding solution, polymerization time, and conversion percentage. During the sowing of artificial seeds contamination is one of the main barriers to the commercialization of encapsulation technology. However, Chitosan was used as a fungal growth retardant.

5 Genetic Stability

The somaclonal variations are a phenomenon of plant tissue culture that is dependent on medium composition, multiplication, explants type, adventitious shoots formation, culture period, and plant genotype (Côte et al. 2001). Despite several experiences of in vitro regeneration, either genetic uniformity or variability was observed in micropropagated plantlets (Larkin and Scowcroft 1981). Micropropagation provides a feasible substitute to seed propagation as it entitles rapid propagation of elite stock cultivars in a fairly short duration of time. For the raising of quality plant material, the genetic consistency of micropropagated plants is a prerequisite factor. In contrast, genetic instability occurs in the in vitro-regenerated plants (somaclonal variation) due to the use of hyper-optimum potency of growth regulators and continuous sub-culturing. Orchid micropropagation was interrupted with an intervening callus phase, which interfered with the integrity of the regenerated clonal

plantlets (Nookaraju and Agrawal 2012); on the other hand, micropropagation via meristem culture was considered as uniform culture (Rani and Raina 2000).

To examine the in vitro protocols, whether propagation was either true-to-type or not clonal fidelity was tested with various Single Primer Amplification Reaction (SPAR)-based methods such as Inter Simple Sequence Repeats (ISSR), Random Amplified Polymorphic DNA (RAPD), and Direct Amplification of Minisatellite DNA (DAMD) markers (Zietkiewicz et al. 1994; Williams et al. 1990; Heath et al. 1993). In addition, a recently invented molecular marker, the Start Codon-Targeted (SCoT) polymorphism (Collard and Mackill 2009) has gained popularity as a powerful tool for the evaluation of clonal fidelity or genetic diversity in regenerated orchid plants (Bhattacharya et al. 2005; Ranade et al. 2009) (Table 1).

Very few studies were endured for testing of clonal fidelity of micropropagated orchids. Among them, the genetic stability of micropropagated *Dendrobium* plantlets was screened by Random Amplified Polymorphic DNA (RAPD) marker (Ferreira et al. 2006). Likely, in *Habenaria edgeworthii* (Giri et al. 2012a); *Aerides crispa* (Srivastava et al. 2018); *Anoectochilus elatus* (Sherif et al. 2017); *Changnienia amoena* (Li and Ge 2006); *Cymbedium finlaysonianum* (Worrachottiyanon and Bunnag 2018); *Cymbidium giganteum* (Roy 2012); *Cymbidium aloifolium* (Sharma et al. 2011; Choi et al. 2006); *Dendrobium densiflorum* (Mohanty and Das 2013); *Dendrobium chrysotoxum* (Tikendra et al. 2019a); *Dendrobium fimbriatum* (Tikendra et al. 2021); *Dendrobium heterocarpum* (Longchar and Deb 2022); *Dendrobium moschatum* (Tikendra et al. 2019b); *Dendrobium nobile* (Bhattacharyya et al. 2014); *Eulophia dabia* (Panwar et al. 2022); *Rhynchostylis retusa* (Oliya et al. 2021); *Spathoglottis plicata* (Auvira et al. 2021); *Vanda coerulea* (Manners et al. 2013) and in *Vanilla planifolia* (Sreedhar et al. 2007) genetic uniformity was tested by RAPD marker.

Moreover, Inter Simple Sequence Repeats (ISSR) marker was tested in *Anoectochilus elatus* (Sherif et al. 2017, 2018); *Anoectochilus formosanus* (Lin et al. 2007; Zhang et al. 2010); *Bletilla striata* (Wang and Tian 2014); *Bulbophyllum odoratissimum* (Prasad et al. 2021); *Cymbidium aloifolium* (Sharma et al. 2011, 2013; Choi et al. 2006); *Dendrobium aphyllum* (Bhattacharyya et al. 2018); *Dendrobium chrysotoxum* (Tikendra et al. 2019a); *Dendrobium crepidatum* (Bhattacharyya et al. 2016a); *Dendrobium fimbriatum* (Tikendra et al. 2021); and in *Dendrobium nobile* (Bhattacharyya et al. 2014); *Dendrobium thyrsiflorum* (Bhattacharyya et al. 2015); *Habenaria edgeworthii* (Giri et al. 2012a); *Platanus acerifolia* (Huang et al. 2009); *Vanda coerulea* (Manners et al. 2013; Gantait and Sinniah 2013); and *Vanilla planifolia* (Gantait et al. 2009; Sreedhar et al. 2007; Bautista-Aguilar et al. 2021) for studying the effectiveness of in vitro protocol. Simple Sequence Repeats (SSR) marker was tested in *Vanilla planifolia* (Bautista-Aguilar et al. 2021). Amplified Fragment Length Polymorphism (AFLP) marker was tested in *Anoectochilus formosanus* (Zhang et al. 2010) and *Dendrobium thyrsiflorum* (Bhattacharyya et al. 2017b). Inter-Retrotransposon Amplified Polymorphism (IRAP) marker was tested in *Bletilla striata* (Guo et al. 2018) and *Dendrobium aphyllum* (Huang et al. 2009). Directed Amplification of Minisatellite-region DNA (DAMD) marker was tested on *Cymbidium aloifolium*

Table 1 Genetic stability analysis of some medicinal orchids with various markers

Sl no	Plant species	Markers	Findings	References
1	*Aerides crispa*	RAPD	RAPD was used to confirm the genetic variations among 52 in vitro morphological variants. Among these, only 15 mutant lines were established based on genetic diversity	Srivastava et al. (2018)
2	*Anoectochilus elatus*	ISSR	2.38% polymorphism and 97.61% monomorphism with genomic uniformity that of the mother plant was revealed with band patterns using ISSR	Sherif et al. (2017)
		ISSR	Using ISSR, homogeneity in direct somatic embryo regenerated plants was found to be 94.22% whereas 93.05% from plants elevated from an indirect somatic embryo	Sherif et al. (2018)
3	*Anoectochilus formosanus*	ISSR and AFLP	Among the regenerated shoots, the range of genetic variation was from 0.00% to 5.43%	Lin et al. (2007)
		ISSR	Among the total 1810 scorable bands, 94% were genetically similar whereas only 2.76% polymorphism was observed	Zhang et al. (2010)
4	*Ansellia africana*	SCoT	Using SCoT in micropropagated plants, an increment in clonal variability with a higher gene flow value (Nm = 1.596) was recorded	Bhattacharyya et al. (2017a)
5	*Bletilla striata*	SCoT and IRAP	96.17% polymorphic bands were recorded using the SCoT marker and 94% polymorphic bands were recorded using the IRAP marker	Guo et al. (2018)
		ISSR	Clonal fidelity assessment by ISSR markers revealed 99.8–100.0 % similarity between the regenerants and their mother plants and 99.5–100.0 % similarity among the regenerants	Wang and Tian (2014)
6	*Bulbophyllum odoratissimum*	ISSR	The genetic homogeneity degree using ISSR markers was high among the clones	Prasad et al. (2021)
7	*Changnienia amoena*	RAPD	Percentage of polymorphic bands at the species level was 76.5% and at the population level it was 37.2%	Li and Ge (2006)
8	*Cymbidium aloifolium*	ISSR	At the inter-specific level, 90% of polymorphism was observed. Among the species, the average cumulative genetic similarity was 66%. The	Sharma et al. (2013)

(continued)

Table 1 (continued)

Sl no	Plant species	Markers	Findings	References
			range of average polymorphism at the intra-specific level was 29.8–69.9 % within five Cymbidium species	
		RAPD, ISSR, and DAMD	Polymorphism in five species of *Cymbidium* viz., *C. aloifolium*, *C. mastersii, C. elegans*, *C. eburneum,* and *C. tigrinum* was found to be 96.6% at an inter-specific level and 51.2–77.1% at an intra-specific level	Sharma et al. (2011)
		RAPD	Similarity values for total bands score analysis ranged from 0.501 for *Cymbidium aloifoilu*m and *C. kanran* to 0.935 for *Cymbidium ensifolium* and *Cymbidium marginatum*	Choi et al. (2006)
9	*Cymbedium finlaysonianum*	RAPD	The genetic stability of the cryopreserved synthetic seeds was confirmed with a similar index value of 0.998	Worrachottiyanon and Bunnag (2018)
10	*Cymbidium giganteum*	RAPD	5.81% molecular variation was detected in the regenerants	Roy (2012)
11	*Dendrobium aphyllum*	IRAP and ISSR	Among the regenerants, the pooled data revealed 5.26% clonal variability whereas individually 7.69% (IRAP) and 4% (ISSR) variability was detected	Bhattacharyya et al. (2018)
12	*Dendrobium chrysotoxum*	RAPD and ISSR	Among the *in vitro* clones and mother plants, 96.30% of monomorphism, and 3.6% of polymorphism was detected	Tikendra et al. (2019a)
13	*Dendrobium crepidatum*	SCoT and ISSR	Cumulative ISSR and SCoT data revealed high genetic fidelity among the regenerates with 6.25% clonal variability. Whereas within the micropropagated plants SCoT data revealed a 10% total variability	Bhattacharyya et al. (2016a)
14	*Dendrobium densiflorum*	RAPD	No genetic variation was observed	Mohanty and Das (2013)
15	*Dendrobium fimbriatum*	RAPD, ISSR & SCoT	Among the regenerants, 100% monomorphism was observed, while low genetic polymorphism of 1.52%, 1.19%, and 3.97% with RAPD, ISSR, and SCoT markers, respectively, was exhibited	Tikendra et al. (2021)
16	*Dendrobium heterocarpum*	RAPD, DAMD, and SCoT	Genetic homogeneity of the regenerates was confirmed with 96.89% monomorphism and 3.11% polymorphism	Longchar and Deb (2022)

17	*Dendrobium nobile*	RAPD and SCoT	94.04% monomorphism and 5.95% polymorphism confirmed the high degree of genetic stability within the *in vitro* propagated plants	Bhattacharyya et al. (2014)
		SCoT	The very high degree of clonal fidelity within the propagated plantlets was confirmed	Bhattacharyya et al. (2016b)
18	*Dendrobium thyrsiflorum*	ISSR and SCoT	In detecting clonal variability, SCoT is more efficient compared to ISSR	Bhattacharyya et al. (2015)
		AFLP	High genetic diversity with 98.50% polymorphism was observed	Bhattacharyya et al. (2017b)
19	*Eulophia dabia*	RAPD	Genetic stability was evaluated which proved true to typesets of the in vitro-raised plants	Panwar et al. (2022)
20	*Habenaria edgeworthii*	RAPD	Genetic stability was confirmed among regenerates	Giri et al. (2012a)
21	*Platanus acerifolia*	ISSR	A genetically stable micropropagated line of *P. acerifolia* was confirmed with 2.88% polymorphism	Huang et al. (2009)
22	*Rhynchostylis retusa*	RAPD	Genetic uniformity among all the analyzed in vitro samples and with the mother plant was confirmed	Oliya et al. (2021)
23	*Spathoglottis plicata*	RAPD	53.28% polymorphism was reported in the orchid variants	Auvira et al. (2021)
		SCoT	Genetic uniformity of the regenerates with the mother plant was confirmed	Manokari et al. (2022)
24	*Vanda coerulea*	ISSR	Genetic stability was confirmed in plantlets from converted capsules stored in 4 and 25 °C	Gantait and Sinniah (2013)
		RAPD and ISSR	Natural genetic diversity with 58.88% polymorphism was shown at the intra-specific level	Manners et al. (2013)
25	*Vanilla plantifolia*	RAPD & ISSR	No genetic diversity was recorded among the micropropagated plants	Sreedhar et al. (2007)
		SSR & ISSR	High genetic stability with low polymorphism percentages was detected	Bautista-Aguilar et al. (2021)

(Sharma et al. 2011) and *Dendrobium heterocarpum* (Longchar and Deb 2022). Start Codon-Targeted Polymorphism (SCoT) was performed in micropropagated plantlets of *Anseilla africana* (Vasudevan and Van Staden 2010); *Bletilla striata* (Guo et al. 2018); *Dendrobium crepidatum* (Bhattacharyya et al. 2016a); *Dendrobium fimbriatum* (Tikendra et al. 2021); *Dendrobium heterocarpum* (Longchar and Deb 2022); *Dendrobium nobile* (Bhattacharyya et al. 2014, 2016b); *Dendrobium thyrsiflorum* (Bhattacharyya et al. 2015), and *Spathoglottis plicata* (Manokari et al. 2022) for homogeneity demonstration.

Genetic variation or polymorphism was analyzed in *Bulbophyllum odoratissimum* as 3.94% (Prasad et al. 2021); 2.76% in *Anoectochilus formosanus* (Zhang et al. 2010); 2.53% in *Dendrobium chrysotoxum*; 2% in *Dendrobium moschatum* (Tikendra et al. 2019a, b); 2.38% in *Anoectochilus elatus* (Sherif et al. 2018); and 2.88% in *Platanus acerifolia* (Huang et al. 2009). The results of the ISSR analysis confirmed the feasibility of the micropropagation protocol of orchids although tiny dissimilarity in genomic constituents was noticed. Such negligible variation may be due to the maintenance of in vitro culture for a longer duration, concentration of growth regulators, and in vitro stress conditions that lead to clonal variations (Tikendra et al. 2019a; Razaq et al. 2013; Devarumath et al. 2002).

6 Ethno-Medicinal Properties

Orchids are the backbone of traditional herbal medicines and have been extensively studied because of their pharmacological importance. From ancient times orchids are being used in traditional systems of medicine like Ayurveda, Siddha, Yunani, Homeopathy, Traditional Chinese Medicine (TCM), etc. Chinese described a *Dendrobium* species and *Bletilla striata* in *Materia Medica* of Shen-Nung (twenty-eighth century B.C.) and in many other Chinese writings orchids symbolize friendship, perfection, numerous progeny, noble, and elegant (Reinikka 1995). In India, there are nearly 1600 species that constitute about 9% of the total flora (Medhi and Chakrabarti 2009). The therapeutic importance of Indian orchids in treating ailments is well documented in the literature (Lawler 1984; Handa 1986) (Table 2).

Several orchid species have important ingredients in various traditional medicinal formulations. Whole plants or their parts are used as a paste or in boiled form, single or mixed with other food stuffs as therapeutics in several ailments (Pant 2013; Gopalakrishnan and Seeni 1987).

The roots of *Acampe papillosa* are used in rheumatism, burning, boils, expectorant, biliousness, asthma, bronchitis, eyes, and blood, and help in curing infections, curing secondary syphilis, uterine diseases, tuberculosis, fever, and throat troubles (Hossain 2009; Zhan et al. 2016; Chopra et al. 1969). The root of *Acampe praemorsa* is used as a tonic for rheumatism and treats neuralgia, sciatica, syphilis, and uterine disorders. Various parts of this orchid are used for the treatment of cough, stomach-ache, ear-ache, and eyes diseases, reduce body temperature, antibiotic for wounds, traumatic pain, backache, menstruation pain, burning sensation, asthma, bronchitis,

Table 2 Distribution and therapeutic importance of some medicinal orchids

Sl. No.	Species	Common Name and Local Name	Habitat and Distribution	Part Used	Therapeutic Importance	References
1	*Acampe papillosa*	Small Warty Acampe	Epiphytic Bangladesh, Bhutan, India (North West Himalaya, Sikkim, West Bengal); Laos, Myanmar, Nepal, Thailand, and Vietnam	Root	Asthma, bronchitis, eyes, and blood Helps to cure syphilis and uterine diseases, tuberculosis, poisonous infections, throat troubles, and fever. Also used as a cooling agent, astringent, and expectorant Crusted roots are used as a tonic; pasted roots are used for rheumatic pains, sciatica, and neuralgia	Piri et al. (2013), Hossain (2009), Chopra et al. (1969)
2	*Acampe praemorsa*	Wight's Acampe, Brittle Orchid Kannada: Seete hoo, Seete dande; Konkani: Kanphoden	Epiphytic Tropical Africa, India, eastwards to China and southwards to Malaya, Indonesia, The Philippines, and New Guinea	Root	Used as a tonic for arthritis, rheumatism, sciatica, neuralgia, syphilis, and uterine disorders. Pulverized plant mixed with egg white and calcium heal fractured limbs. Freshly prepared paste of its roots along with *Asparagus recemosus* root paste cures arthritis	Suja and Williams (2016), Perfume workshop (n.d.-a), Hossain (2009), Leander and Lüning (1967), Shanavaskhan et al. (2012), Devi et al. (2015), Panda and Mandal (2013), Nongdam (2014), Mishra et al. (2008)
3	*Aerides crispa*	Curled aerides Marathi: Pan Shing	*Epiphytic* Karnataka: Districts of Hassan, Mysuru, Ballari, Chikkamagaluru, Chitradurga, Kodagu (Coorg), Shivamogga,	-	2–3 drops of boiled pulverized plant with neem is used to treat earache	Jayashankar and Darsha (2021), Perfume workshop (n.d.-a)

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Table 2 (continued)

Sl. No.	Species	Common Name and Local Name	Habitat and Distribution	Part Used	Therapeutic Importance	References
			Uttara Kannada, Dakshina Kannada			
4	*Aerides multiflorum*	The Multi-Flowered Aerides—In Thailand—Aiyaret—Phuang Malai, Fox brush orchid, Maana	Terrestrial, epiphytic, saprophytic Found in Bangladesh, eastern Himalayas, India, Nepal, western Himalayas, Andaman Islands, Myanmar, Thailand, Laos, Cambodia, and Vietnam Elevation: 1100 m	Whole plant	Leaf paste is applied on wounds and earaches. The powdered leaf is used as a tonic. In vitro tubers and leaves have an antibacterial effect and antimicrobial effects, respectively	Lal et al. (2020), Perfume workshop (n.d.-a), Baral and Kurmi (2006), Basu et al. (1971), Behera et al. (2013), Bhattacharjee (1998)
5	*Aerides odorata*	Fragrant Fox Brush Orchid, Fragrant Aerides, Fragrant Cat's-tail Orchid Mizo: Nau-ban	Epiphyte Native to South-Central and South-East China, Bangladesh, East Himalaya, West Himalaya, Nepal, India, Cambodia, Laos, Myanmar, Thailand, Vietnam, Borneo, Jawa, Lesser Sunda Islands, Malaya, Philippines, Sulawesi, and Sumatera	Roots, leaves, fruits	Leaf paste and Fruits are used to heal wounds and cure tuberculosis. Leave juice and seeds are used for treating boils in the ear, nose and other skin disorders. Combination of the fresh root of *A. odorata*, root powder from *Saraca asoca*, bark from *Azadirachta indica* and common salt used as an oral medicine for painful swollen joints	Hongthongkham and Bunnag (2014), Devi et al. (2013), Perfume workshop (n.d.-a), Leander and Lüning (1967), Hossain (2009), Baral and Kurmi (2006), Behera et al. (2013)
6	*Anacamptis pyramidalis*	Pyramidal Orchid	Terrestrial Throughout the UK, many European countries	-	For skin whitening; exhibits antioxidant and scavenging capacities	Parker (2016), Perfume workshop (n.d.-a)

			including Slovenia, in North Africa and the Near East Elevation: 0–1600 m			
7	*Anocetochilus elatus*	South Indian Jewel Orchid Malayalam: Nagathali Assamese: Boga-kopou-phul	Terrestrial Distributed along Southern Western Ghats of India	Whole Plant	Used in the chest and abdominal pain and to treat snake bites	Sherif et al. (2012, 2018)
8	*Anocetochilus formosanus*	Jewel orchid	Terrestrial Widely distributed in Taiwan and Fujian Province of China, and Japan	Whole plant	The whole plant is used as a cooling agent, an antipyretic, for relieving pain in the waist and knee, and for treating tuberculosis, diabetes, bronchitis, renal infections, snake bites, and stomach aches. The plant also possesses anti-cancerous properties	Jiang et al. (2015), Perfume workshop (n.d.-a), Aswandi and Kholibrina (2021), Nandkarni (1976)
9	*Ansellia africana*	Leopard orchid	Perennial, and epiphyte, or sometimes terrestrial Tropical and subtropical areas of southern Africa	Whole plant	Stem infusion is used as an antidote to bad dreams. Leaves and stems are used for treating madness	Bhattacharyya and Staden (2016), Saleh-E-In et al. (2021)
10	*Arundina graminifolia*	Bamboo orchid, Bird Orchid, Kinta Weed Manipuri: Kongyamba lei; Mizo: Le-len	Terrestrial Myanmar, India, Sri Lanka, Nepal, Thailand, Vietnam, the Ryukyu Islands, Malaysia, Singapore, China to Indonesia, the Philippines and New Guinea	Whole plant	It possesses anti-bacterial activity. The root is used as a pain reliever. The scrapped bulbous stem is applied on the foot heels to treat the cracks	Hu et al. (2013), Aswandi and Kholibrina (2021), Hossain (2009), Kumar (2002), Dakpa (2007)

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Table 2 (continued)

Sl. No.	Species	Common Name and Local Name	Habitat and Distribution	Part Used	Therapeutic Importance	References
11	*Bletilla striata*	*Hyacinth orchid or Chinese ground orchid*	Terrestrial Japan, Korea, Myanmar (Burma), and China (Anhui, Fujian, Gansu, Guangdong, Guangxi, Gui zhou, Hubei, Hunan, Jiangsu, Jiangxi, Shaanxi, Sichuan, Zhejiang)	Tuber, Root	Tubers are used in treating hemorrhage, tuberculosis, and bleeding. It promotes the regeneration of muscle and other tissues. They are used to treat sores, chapped skin, dysentery, fever, malignant ulcers, gastrointestinal disorders, hemorrhoids, anthrax, malaria, eye diseases, etc. The powdered roots mixed with oil are applied to burns and skin diseases. Effective against leucorrhea. Leaves are used to cure lung disease	He et al. (2017), Perfume workshop (n.d.-a), Kong et al. (2003), Bulpitt et al. (2007)
12	*Bulbophyllum odoratissimum*		Lithophytic *China, India* *Native to:* Andaman Is., Assam, Cambodia, China South-Central, China Southeast, East Himalaya, India, Laos, Myanmar, Nepal, Thailand, Tibet, Vietnam	Whole plant	Fractures, pulmonary tuberculosis, hernia pain	Perfume workshop (n.d.-a), Bhattacharjee (1998)
13	*Calanthe discolor*	*Japanese Hardy Orchid*	Terrestrial Korea, Japan, and China	Whole plant	The entire plant is used to improve blood circulation, heal abscesses,	Suetsugu and Fukushima (2014), Perfume

					rheumatism, bone pain, and traumatic injuries as well as treat skin ulcers and hemorrhoids	workshop (n.d.-a), Yoshikawa et al. (1998)
14	*Changnienia amoena*			Whole plant, roots		Teoh (2019)
15	*Coelogyne cristata*	*Swarna Jibanti;* Jibanti India: *Hadjojen* (bone joiner) Nepal: *ban maiser, jhyanpate*	Found in moss forests associated with tree bark and rocks, often exposed to sun India, Bhutan, Nepal, Tibet and mountainous regions of Northern Thailand Elevation: 1500–2600 m	Pseudobulbs	Pseudo bulbs are used for constipation and aphrodisiac. The juice is used for healing wounds, boils, and sores	Sharmaa et al. (2014), Mitra et al. (2018), Perfume workshop (n.d.-a), Pant and Raskoti (2013), Subedi et al. (2011), Pamarthi et al. (2019)
16	*Coelogyne flaccida*	Bearded Coelogyne, loose Coelogyne China: *Lilinbeimu Lan*, *Guishangye*	Epiphyte or lithophyte Himalayas, Nepal, North India, Bhutan, China, and Myanmar Elevations: 900–1400 m	Pseudo bulb	Used to treat headache, fever, and indigestion	Kaur and Bhutani (2013), Pant and Raskoti (2013), Teoh (2016), Pamarthi et al. (2019), Perfume workshop (n.d.-a)
17	*Coelogyne nervosa*	Veined coelogyne	Epiphytic Southern Western Ghats of Kerala and Tamil Nadu	Whole plant	Has potential antimicrobial, antioxidant, and anticancer properties	Sathiyadash et al. (2014), Ranjitha et al. (2016)
18	*Coelogyne stricta*	The Rigid Coelogyne Pseudobulb India: Harjojan	Found on tree trunks or lithophytes on mossy rocks Elevations: 1400–2000 m North-East India, Sikkim, Bhutan, Myanmar, and Nepal	Pseudobulbs	The paste is used to cure headaches and fever	Perfume workshop (n.d.-a), Basker and Bai (2006), Yonzone et al. (2012), Pamarthi et al. (2019)
19	*Cymbidium aloifolium*	Malanga, aloe-leafed cymbidium Boat Orchid	Epiphytic herb Global Distribution India, Sri Lanka, Thailand,	Rhizome, root, pseudo bulbs	The paste is used to treat fractured and dislocated bones	Behera et al. (2013), Perfume workshop (n.d.-a), Pamarthi et al. (2019)

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Table 2 (continued)

Sl. No.	Species	Common Name and Local Name	Habitat and Distribution	Part Used	Therapeutic Importance	References
		Tamil Nadu*:* *panaipulluruvi* Assam: Kopou-Phul	Indonesia, Java, Indo-Malaysia			
20	*Cymbidium ensifolium*	Golden-thread orchid, burned-apex orchid, spring orchid, and rock orchid	Epiphytic *Global Distribution: India and Sri Lanka* *Native to:* Assam, Cambodia, China South-Central, China Southeast, Hainan, Japan, Korea, Laos, Myanmar, Philippines, Taiwan, Thailand, Tibet, Vietnam	Root, flower	Root decoction is used to treat gonorrhea. Flower decoction used in eye sore disorders	Chang and Chang (1998), Tsering et al. (2017)
21	*Cymbidium finlaysonianum*	Finlayson's Cymbidium Malay: *Sepuleh* Thai: *Ka Re ka Ron Pak Pet*	Terrestrial (Primary Rainforest, Secondary Rainforest, Coastal Forest) Thailand, Vietnam, Cambodia, Peninsular Malaysia, Java, Borneo and the Philippines Elevation: 0–1200 m	-	Restore health	Islam et al. (2015), Perfume workshop (n.d.-a)
22	*Cymbidium giganteum*	Iris-like Cymbidium	Epiphytic Chinese Himalayas, India, eastern Himalayas, Nepal, western Himalayas, Myanmar, and Vietnam Elevation: 0–1200 m	Leaves	Wounds	Hossain et al. (2010), Bulpitt (2005), Fonge et al. (2019), Linthoingambi et al. (2013)

23	*Cymbidium goeringii*	Noble orchid *Japan: Chun Lan (spring orchid)*	Terrestrial East Asia including Japan, China, Taiwan, and South Korea Elevation: 300–3000 m	Seed, whole plant	Seeds are used to cure wounds and injuries and also in curing fractures, and traumatic soft tissue injuries	Perfume workshop (n.d.-a), Teoh (2016)
24	*Cymbidium iridioides*	Iris Cymbidium Chinese: *Huang chan Lan*	Epiphytic China, India, Bhutan, Nepal, Myanmar; and Vietnam Elevation: 900–2,800 m	Leaves, pseudo bulbs, roots	Fresh juice of this plant is used to stop bleeding. The powder is used as a tonic. During diarrhea, pseudo bulbs and roots are consumed	Perfume workshop (n.d.-a), Aggarwal and Zettler (2010), Arditti et al. (1982), Arditti and Ernst (1984), Medhi and Chakrabarti (2009)
25	*Cymbidium kanran*	The Cold Growing Cymbidium	Terrestrial Exclusively distributed in Northeast Asia including China, Japan, and Korea	Whole plant	Cures coughs and asthma. Roots are used to cure ascariasis and gastroenteritis	Perfume workshop (n.d.-a), Jeong et al. (2017)
26	*Cymbidium lancifolium*	Lance leafed Cymbidium	Grows in broad-leaved forests where the soil is rich in humus and also plenty of leaf litter In the Himalayas, India, Nepal, Bhutan, China, Taiwan, Japan Elevation: 300–2300 m	Whole plant	Used to cure rheumatism, improve blood circulation and treat traumatic injuries	Perfume workshop (n.d.-a)
27	*Cymbidium longifolium*	Red-Spotted Lip Cymbidium; In China Chang Ye Lan	Epiphytic, lithophytic, or terrestrial Found in China, Eastern Himalayas, Nepal, Bhutan, Burma, and India Elevation: 1000–2500 m	Pseudo bulb	The fresh shoot is used for nervous disorders, madness, epilepsy, hysteria, rheumatism, and spasms. Salep used as demulcent. An aqueous solution of powdered pseudo bulbs is taken orally on an empty stomach	Nongdam (2014), Sood et al. (2006), Yonzone et al. (2013), Zhan et al. (2016)

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Table 2 (continued)

Sl. No.	Species	Common Name and Local Name	Habitat and Distribution	Part Used	Therapeutic Importance	References
28	*Cymbidium sinense*	Japan—Hosai-Ran—Taiwan-Ran—In China Mo Lan	Terrestrial Found in India, Myanmar, northern Thailand, Vietnam and east China, Japan	Whole plant	Used in purifying heart, lungs, treating cough and asthma	Perfume workshop (n.d.-a)
29	*Cypripedium calceolus*	Lady's-slipper orchid Japanese: Ko-atsumori-sou	Shady, deciduous and mixed woodland, predominantly on calcareous soils Spain, Europe, China, Siberia, Sakhalin Island, and Japan Elevation: 2000 m	Root, rhizome	It acts as a sedative, promotes sleep, and reduces pain when powdered roots are mixed with sugar water. A tea prepared from roots is used to treat jangling nerves and headaches	Kull (1999), Kolanowska and Busse (2020), Singh and Dey (2005)
30	*Cypripedium debile*	Frail lady's slipper ***Lan*** (two leaf spoon orchid)	Japan, Korea, Taiwan, and China	Whole plant	Used to improve blood circulation, reduce swelling, relieves pain, and act as a diuretic	Perfume workshop (n.d.-a)
31	*Cypripedium formosanum*	Formosa lady's slipper	Terrestrial Found on sandy floor of the forest and in open areas in Taiwan Elevation: 2000–3000 m	Whole plant	Improves blood circulation, regulates menses, and relieves pain and itching. Roots and stems are used to treat malaria, snake bites, traumatic injury, and rheumatism	Perfume workshop (n.d.-a)
32	*Cypripedium guttatum*	Spotted lady's slipper	Hardy terrestrial European Russia to Korea, Alaska to Yukon Elevation: 1000–4100 m	Roots and leaves	Used to treat epilepsy	Zhang et al. (2007), Perfume workshop (n.d.-a)
33	*Cypripedium macranthos*	Large flowered lady's slipper		Rhizome, flower,	Used to treat skin disease, roots and stem promote	Shimura and Koda (2004), Shimura et al. (2007),

			Terrestrial East Belarus to temperate East Asia	stem, and root	dieresis, reduce swelling, expel gas, relieve pain and improve blood flow. Dried flowers are used to stop in wound bleeding	Perfume workshop (n.d.-a)
34	*Cypripedium parviflora*	Yellow lady's slipper or moccasin flower	Terrestrial *Native to:* Delaware, Nebraska, North Dakota, Québec, Rhode I., Elevation: 1400 m	Rhizome	Cures insomnia, anxiety, headache, emotional tension, fever, palpitations, tumors, irritable bowel syndrome, neuralgia, and reduces menstrual and labor pain	Meier et al. (2018), Moerman (1986), Grieve (1998), Kumar et al. (2005)
35	*Cypripedium pubescens*	Yellow lady's slipper	Deciduous and coniferous forest, meadows, fens Newfoundland to British-Columbia, south to Georgia, Arizona, Washington, and Europe Elevation: 5750–11,000 ft.	Root	The plant is diaphoretic, hypnotic, nervine, antispasmodic, sedative, and tonic. Used in diabetes, diarrhea, dysentery, paralysis, joint pain, convalescence, impotence, and malnutrition	Pant and Rinchen (2012), Wani et al. (2020), Shrestha et al. (2021), Perfume workshop (n.d.-a), Singh and Duggal (2009), Khory (1982)
36	*Dactylorhiza hatagirea*	Himalayan Marsh Orchid India: Munjataka in Ayurveda	Terrestrial India, Pakistan, Afghanistan, Nepal, Tibet, and Bhutan. Elevation: 2500–5000 ft.	Tubers	Used as a tonic, heals wound, fever, and control burns and bleeding. Also used as food due to the presence of starch	Pant and Rinchen (2012), Wani et al. (2020), Shrestha et al. (2021), Perfume workshop (n.d.-a), Aggarwal and Zettler (2010), Arditti (1967, 1968, 1992), Arditti et al. (1982), Arditti and Ernst (1984)
37	*Dendrobium amoeneum*	The Lovely Dendrobium	Epiphytic Western Himalayas, India,			Venkateswarlu et al. (2002)

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Table 2 (continued)

Sl. No.	Species	Common Name and Local Name	Habitat and Distribution	Part Used	Therapeutic Importance	References
			eastern Himalayas, Nepal, Bhutan, Sikkim, Bangladesh, Myanmar Elevation: 600–2000 m	Pseudo bulbs, leaves	The freshly prepared paste is used to cure skin diseases and dislocated bones	
38	*Dendrobium aphyllum*	Thai names: Uean sai, Ueang sai long laeng, etc. Assamese: Haliki-thutia-phul	Epiphytic Continental Southeast Asia, Southwest China, Sikkim, and Nepal	Pseudo bulbs	Leaf paste is applied on the abnormal and deformed head parts of the newly born baby to get a normal shape	Liu et al. (2018), Perfume workshop (n.d.-b), Pant (2013)
39	*Dendrobium candidum*	Shihu in Chinese and Sekkoku in Japanese	Epiphytic Southern China, Taiwan, Nepal, Thailand, Vietnam, India, Myanmar Elevation: 2000–3000 m	Leaves	Used to treat diabetes	Nongdam (2014), Wu et al. (2004)
40	*Dendrobium chrysanthum*	Golden yellow-flowered dendrobium	Epiphytic and Lithophytic India, Nepal, Bhutan, Burma, China, Thailand, Laos, and Vietnam Elevation of 450–2000 m	Stem, leaf	The stem is used as a tonic to enhance the immune system, promote body fluid production, and reduce fever. The leaf is used as an antipyretic and mild skin disease as well as benefits the eyes	Nongdam (2014), Bulpitt (2005), Jalal et al. (2008, 2010), Li et al. (2016)
41	*Dendrobium chrysotoxum*	Golden Orchid Thai: Uang Khan Vietnam: Kim diep	Epiphytic North-East India, Nepal, Bhutan, Burma, China, Thailand, Laos, and Vietnam	Whole plant	The whole plant possesses antitumoral and anticancerous properties. Stem and flower extract is used as tonic and leaf extract as antipyretic	Nongdam (2014), Perfume workshop (n.d.-b), Sood et al. (2006), Bulpitt et al. (2007), Joshi et al. (2009)

42	*Dendrobium crepidatum*	Shoe-Lip Dendrobium China: Meigui Shihu (rose Dendrobium)	Epiphytic	Pseudo bulbs, stem	Pseudo bulb paste is used to treat the fracture and dislocated bones. Stems are used as a tonic for treating arthritis and rheumatism	Perfume workshop (n.d.-b), Joshi et al. (2009), Joshi and Joshi (2001), Hu et al. (2016)
43	*Dendrobium crumenatum*	Pigeon orchid, Dove orchid India: Jivanti Malay: bunga angin (wind orchid)	Malaysia, Singapore	Leaf	Leaves are used to treat boils and pimples	Perfume workshop (n.d.-b), Joshi and Joshi (2001), Topriyani (2013)
44	*Dendrobium densiflorum*	Pineapple Orchid Thai: Ueang Mon Kai Liam Vietnam: Thy-tien	Epiphytic China, Bhutan, NE India, Myanmar, Nepal, Thailand Elevation: 400–1000 m	Pseudo bulbs, leaf	Pulps of the pseudo bulbs are used to treat boils, pimples, and other skin eruptions. Leaf paste is used on fractured bones, to relieve sprains and inflammations	Perfume workshop (n.d.-b), Arditti (1992), Arditti et al. (1982), Arditti and Ernst (1984), Keerthiga and Anand (2014), Pant et al. (2022)
45	*Dendrobium devonianum*	Devon's Dendrobium China: Chiban Shihu (teeth pedal Dendrobium)	Epiphytic Native to south China, the eastern Himalayas (Bhutan, Assam), Myanmar, Thailand, Laos, Vietnam	Stem	Dried stems are used as an immune system enhancer	Li et al. (2011, 2013a), Perfume workshop (n.d.-b), Cakova et al. (2017)
46	*Dendrobium draconis*	Thai names: Ueang ngoen, ueang ngum Myanmar Name: Kein na ri	Terrestrial India, Cambodia, Laos, Myanmar, Thailand, and Vietnam	Stem	Used in antipyretic and hematinic	Rangsayatorn (2009), Perfume workshop (n.d.-b)
47	*Dendrobium fimbriatum*	Fringe Lipped Dendrobium China: Liusushihu (tasseled stone orchid)	Epiphytic, lithophytic and terrestrial China, Western Himalayas, Bangladesh, Eastern Himalayas, India, Nepal,	Whole plant	Used in upset of liver and severe anxiety. Leaves are used for treating fractured bone, the pseudo bulbs are used in fever	Huang et al. (2008), Nongdam (2014), Perfume workshop (n.d.-b), Arditti et al. (1982)

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Table 2 (continued)

Sl. No.	Species	Common Name and Local Name	Habitat and Distribution	Part Used	Therapeutic Importance	References
		India: Fringed lip Dendrobium	Bhutan, Laos, and Vietnam Elevation: 800–2400 m			
48	*Dendrobium heterocarpum*	*Golden-Lip Dendrobium* Thailand: Ueang Si Tan in	Epiphyte Native to: China, Nepal, Bhutan, the Indian subcontinent and Southeast Asia	Pseudo bulb	The paste is used to treat fractured and dislocated bones	Arditti and Ernst (1984), Warinhomhoun et al. (2022)
49	*Dendrobium lasianthera*	Sepik Blue Orchid	Epiphyte New Guinea, Papuasia, Asia Tropical	Roots, stem, leaves	Anticancer	Utami et al. (2017)
50	*Dendrobium longicornu*	Long-horned dendrobium	Epiphyte or terrestro-litho-phyte Native to southern China, the Himalayas (Nepal, northeastern India, Bhutan, Bangladesh) and northern Indo-China region Elevation : 1200–3000 m	Whole plant	The plant juice mixed with lukewarm water is used for treating children with fever. The boiled root is used to feed the livestock to remove cough	Dohling et al. (2012), Perfume workshop (n.d.-b)
51	*Dendrobium macrostachyum*	Fringed Tree Dendrobium	Epiphytic India, Myanmar, Sri Lanka and on the Cape York Peninsula Native to Australia, tropical Asia, and eastern Malaysia	Tender shoot tip	Tender shoot tip juice is used for earaches	Pyati et al. (2002), Perfume workshop (n.d.-b), Reddy et al. (2001)
52	*Dendrobium moschatum*	Musk Dendrobium Thai: Ueang Champa	Epiphytic Northeast India, Bhutan and Nepal across Myanmar	Pseudo bulb	Pseudo bulb paste is used to treat dislocated and fractured bones	Kanjilal et al. (1999), Perfume workshop (n.d.-b)

			and Thailand to Laos, Vietnam, and China			
53	*Dendrobium nobile*	Noble Dendrobium China: Jinchashihu (gold hairpin Dendrobium) Japanese name: Koki	Epiphytes or lithophytes Himalayas and China	Pseudo bulb, seed, Stem	The pseudo bulb extracts cure eye infections and burns; the plant is used to treat pulmonary tuberculosis, flatulence, and dyspepsia, and reduce salivation, night sweats, fever, and anorexia. Also used as an antiphlogistic, tonic. Seeds are used to heal wounds; stems to cure fever and tongue dryness; stems are used for longevity	Bhattacharyya et al. (2014), Asghar et al. (2011), Luo et al. (2010), Singh and Duggal (2009), Perfume workshop (n.d.-b), Arditti (1967), Arditti et al. (1982)
54	*Dendrobium ovatum*	Green Lipped Dendrobium India: Anantali Maravara	Epiphytic Global Distribution: Western Ghats of India	Whole plant	Fresh plant juice cures stomach ache, excites bile, also acts as a laxative to the intestines, and cures constipation	Pujari et al. (2021), Shetty et al. (2015), Perfume workshop (n.d.-b), Kirtikar and Basu (1981), Caius (1986)
55	*Dendrobium parishii*	Parish's Dendrobium Thai: Ueang Khrang Sai San	Epiphyte. Native to the Eastern Himalayas, China, Thailand, Myanmar, Laos, Cambodia, and Vietnam	Pseudo bulbs	Antipyretic encourages the secretion of body fluids	Kongkatitham et al. (2018), Perfume workshop (n.d.-b)
56	*Dendrobium primulinum*	Primrose Yellow Dendrobium	Epiphyte Assam, Himalayas, Nepal, Andaman Islands, Myanmar, Thailand, China, and Vietnam	Dried stems	Immune system enhancer	Pant and Thapa (2012)

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Table 2 (continued)

Sl. No.	Species	Common Name and Local Name	Habitat and Distribution	Part Used	Therapeutic Importance	References
57	*Dendrobium thyrsiflorum*	Pinecone-like raceme dendrobium	Epiphytic, lithophytic, or terrestrial Native to the Eastern Himalayas, China, Thailand, Myanmar, Laos, Cambodia, and Vietnam Elevation: 1200–2000 m	Stem	Used to resist heat, benefits the stomach, and promotes the production of body fluid	Wrigley (1960), Ruixuan et al. (2015), Perfume workshop (n.d.-b)
58	*Dendrobium transparens*	Translucent Dendrobium	Epiphytic Western Himalayas, Bangladesh, eastern Himalayas, India, Nepal, Bhutan, Sikkim, Myanmar, China, and Vietnam Elevation: 500–2100 m	Pseudo bulb	The paste is used to treat fractures and dislocated bones	Sunitibala and Kishor (2009), Arditti and Ernst (1984)
59	*Dendrobium trigonopus*	Thailand: Triangular Column Foot Dendrobium	Epiphyte The plant grows in the forest of Burma, Thailand, SW China, Laos and Vietnam Elevations: 300–1500 m	Stem	Used to cure fever and anemia	Hu et al. (2008a), Perfume workshop (n.d.-b)
60	*Doritis pulcherrima*	Beautiful Moth Orchid	Terrestrial, epiphytic Myanmar, Thailand, China, Laos, and Vietnam Elevation: 1000–4900 ft.	Leaves	Used to treat ear infections	Perfume workshop (n.d.-c)
61	*Eria bambusifolia*	Bamboo-Leaf Eria	Epiphytic World distribution: India, Thailand Elevation: 1000–1300 m	Whole plant parts	Treating hyperacidity and stomach disorders	Basker and Bai (2010), Zhan et al. (2016)

62	*Eulophia dabia*	Dubious Eulophia Salibmisri, Sung Misrie	Terrestrial Afghanistan, Baluchistan, Uzbekistan, Southern Himalayas, South China	Tubers	Stimulate appetite, cures stomach ache, and stimulates blood flow	Pant (2013), Perfume workshop (n.d.-b), Panwar et al. (2022)
63	*Eulophia epidendraea*	Epidendrum Eulophia Katou kaida maravara	Terrestrial South India, Sri Lanka, Bangladesh	Tubers	Cure tumor, and diarrhea; acts as an appetizer, anthelmintic, aphrodisiac, stomachic, and worm infestation, stimulate appetite, and purifies blood during heart troubles	Perfume workshop (n.d.-d), Narkhede et al. (2016)
64	*Eulophia graminea*	Grass Eulophia Kattuvegaya	Terrestrial India, Sri Lanka, Southeast Asia, China, and Japan	Whole plant	Juice to treat earache	Perfume workshop (n.d.-d)
65	*Eulophia nuda*		Terrestrial Found in the Western Ghats of India, tropical Himalayas, Myanmar and South China, Indochina, Malaysia, Indonesia, Philippines and the Pacific Islands	Whole plant	A thick paste of tubers is applied on the stomach to kill intestinal worms, cure rheumatoid arthritis, bronchitis, scrofulous glands, and tumors, purify the blood, and used as a tonic, acts as an anti-aphrodisiac, demulcent and anthelmintic. The leaf is used as a vermifuge, the whole plant is used in stomachache and snake bites, and the stem is used to stop bleeding and pain from trauma	Hada et al. (2020)
66	*Gastrodia elata*	Tianma China: Ming Tianma, Japan: Tenma, Korean name: Cheon ma	Saprophytic Nepal, Bhutan, India, Japan, North Korea,	Tuber	Used in stroke, tetanus, migraine, malaise, generalized dermatitis dizziness,	Perfume workshop (n.d.-d), Chen et al. (2014)

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Table 2 (continued)

Sl. No.	Species	Common Name and Local Name	Habitat and Distribution	Part Used	Therapeutic Importance	References
			Siberia, Taiwan, and China Elevations: 400–3200 m		sleepiness, insomnia, high blood pressure, blood circulation, rheumatism, numbness, paralysis, backache, skin boils, ulcers, and body pain	
67	*Geodorum densiflorum*	Nodding Swamp Orchid Bangladesh: Kukurmuria China: Dibao Lan India: Kukurmuria	Terrestrial Japan, China, Taiwan, Sri Lanka, Myanmar, Philippines, Indochina, Thailand, Malaysia, Ryukyu Islands, Indonesia, Nepal, India	Pseudo bulbs, roots	Used as a disinfectant. Root paste mix with ghee and honey in menstrual disorders and root paste is applied on insect bites and wounds	Nongdam (2014), Perfume workshop (n.d.-d), Sheelavantmath et al. (2000)
68	*Gymnadenia conopsea*	China: shou shen, Shouzhangshen Japan: Tegata-chidori	Lithophytes Russia, Europe, Japan, Korea	Stem	Treat kidney disorders, cough, dysfunction, discharge, traumatic injuries, thrombosis, chronic hepatitis, lactation failure stops bleeding, and fever	Perfume workshop (n.d.-d), Gustafsson (2000)
69	*Habenaria edgeworthii*		Terrestrial	Leaves and roots	Cooling and spermophytic	Singh and Duggal (2009)
70	*Habenaria pectinata*	Comb Habenaria	Terrestrial Assam, China South Central, East Himalaya, Myanmar, Nepal, Pakistan, West Himalaya	Bulb	Bleeding diathesis, burning sensation, fever, and phthisis	Singh and Duggal (2009)
71	*Herminium lanceum*	Lanceleaf Herminum China: Shuangchunjiaopan Lan	Terrestrial Shandong, Tibet, Dongbei,	Roots	The root is beneficial for the lungs and kidney, strengthen muscles and	Perfume workshop (n.d.-d)

			Guangxi, Taiwan Elevation: 1100–3500 m		bones, stops bleeding, and treats tuberculosis	
72	*Liparis odorata*	Fragrant Liparis	Terrestrial *Global distribution: Widespread* *Native to: Japan, Bangladesh, Cambodia, China South-Central, China Southeast, East Himalaya, India, Laos, Myanmar, Nansei-shoto, Nepal, Sri Lanka, Thailand, Tibet, West Himalaya*	Whole part	The whole plant is used for external use, tubers are used to treat stomach disorders and its paste is for chronic ulcers	Perfume workshop (n.d.-e)
73	*Malaxis acuminata*	Jeevak	Terrestrial Bangladesh, India, Nepal, Myanmar, Thailand, Laos, Cambodia, Vietnam, Malaysia, and Philippines Elevation: 1500–2100 m	Pseudo bulb	Used as tonic, Aphrodisiac, styptic, antidysentery and febrifuge. The paste is applied on insect bites, and treats rheumatism, bleeding, burning sensation, and lungs disease	Pushpa et al. (2011)
74	*Oberonia ensiformis*	Word-Leaf Oberonia China: Jian Ye Yuan Wei Lan	Lithophytic, epiphytic Nepal, India, China, Myanmar, Thailand, Laos, and Vietnam Elevation: 600–1000 m		Used to encourage diuresis, treat cystitis, urethritis, injuries, and fractures and improve blood circulation	Perfume workshop (n.d.-c)
75	*Papilionanthe teres*	Cylindrical Vanda, Parrot Flower China: Banghua Lan, India: Chaitek Lei in	Epiphytic India, Andaman Island., Bangladesh, China South-Central, East Himalaya, India, Laos, Myanmar,	Stem and leaves	Stem and leaves are used to improve blood flow and reduce swelling. The paste is used to treat dislocated bone. Leaf paste is applied	Perfume workshop (n.d.-c)

(continued)

Table 2 (continued)

Sl. No.	Species	Common Name and Local Name	Habitat and Distribution	Part Used	Therapeutic Importance	References
		Manipuri, cylindrical Vanda	Nepal, Thailand, Vietnam Elevation: 600 m		to reduce fever. Stem juice protects from coughs and colds	
76	*Pholidota articulata*	Rattlesnake orchids India: Harjojan; Jivanti Myanmar: Kwyet mee pan myo kywe Nepal: Thurjo, Pathakera	Epiphytic Montane to submontane zones, Uttarakhand Himalayas, Arunachal Pradesh, and Indo-China to Malaysia India, Nepal, Bhutan Myanmar, Thailand, Cambodia, Vietnam, Malaysia, and Indonesia	Whole plant	Enriched in remove gas and reduce swelling, treat coughs, headache, dizziness, traumatic injuries, sores and ulcers, irregular menses and uterine problems, and fractures, used as a stimulant, demulcent, and tonic. Pseudo bulbs paste is applied on dislocated bones. Powdered root treat cancer and capsule juice are used to treat skin eruptions and ulcers	Perfume workshop (n.d.-c)
77	*Pholidota pallida*	China: Eumaishixiantao	Epiphytic Bhutan, Central Nepal, Northeast India	Root and pseudo bulb	Its powder induces sleep and juice to remove abdominal pain. Root and pseudo bulb paste is used to cure fever	Perfume workshop (n.d.-c)
78	*Platanthera chlorantha*	Greater butterfly-orchid	France, Germany, Great Britain, Albania, Austria, Baltic States, Belarus, Belgium, Bulgaria, Denmark, Finland, Greece, Hungary, Iran, Iraq, Ireland, Italy,	Whole Plant	The whole plant is used in strengthening the kidneys and lungs, and cures sexual dysfunction, hernia, and enuresis affecting children	Perfume workshop (n.d.-c)

			Krym, Netherlands, North Caucasus, Norway, Poland, Romania, Sicilia, Spain, Sweden, Switzerland, Turkey, Ukraine, Yugoslavia			
79	*Rhynchostylis retusa*	Foxtail orchid Blunt Rhynchostylis India: Kopou phool, draupadi mala, panas keli Nepal: ghoge gava	Epiphytic Global Distribution: Indo-Malaysia, India	Leaf, root, flower	Leaves and roots paste are used in rheumatism. Leaf juice is used in constipation, gastritis, acidity, and as an emollient. Root juice is used to heal cuts and wounds, and root is used in menstrual pain and arthritis. Dry flowers are used as an emetic	Basu et al. (1971), Bhattacharjee (1998), Bulpitt et al. (2007), Dakpa (2007) Dash et al. (2008)
80	*Satyrium nepalense*	Nepal Satyrium	Terrestrial Sri Lanka, India, Bhutan, and Myanmar Elevation: 2400–5000 m	Tubers	Treats diarrhea, dysentery, and malaria. Tubers are consumed as an aphrodisiac and used as children's growth supplements. Juice is used in cuts and wounds. The powder is used as a tonic and to treat colds, coughs, and fever	Baral and Kurmi (2006); Behera et al. (2013), Bulpitt et al. (2007), Gutierrez (2010)
81	*Spathoglottis plicata*	Philippine ground orchid, Large purple orchid	Terrestrial Taiwan, Southern India, Indonesia, Japan, Malaysia, New Guinea, Philippines, Sri Lanka, Thailand, Vietnam, Australia, Tonga and Samoa	Pseudo bulb	Treat rheumatic swelling, relieve pain, and uplift blood circulation	Teng et al. (1997), Friesen and Friesen (2012)

(continued)

Table 2 (continued)

Sl. No.	Species	Common Name and Local Name	Habitat and Distribution	Part Used	Therapeutic Importance	References
82	*Thunia alba*	White Thunia	Epiphytic India, China, and Southeast Asia Elevation: 2000 m	Whole plant	Cough pneumonia, bronchitis, bone break treatment, and injury	Xu et al. (2019a)
83	*Vanda coerulea*	Blue Orchid, blue vanda, autumn lady's tresses India: Kwaklei Lawhlei Vandara	Epiphytic Native to: North East India Elevation: 2500–4000 ft.	Flower	Flower juice is used in treating glaucoma, cataract, and blindness	Roy et al. (2011)
84	*Vanda roxburghii*	Rasna	Epiphytic Widely distributed throughout Bangladesh	Root	Treat fever, nervous system disease dyspepsia, snake bites bronchitis, hiccough, piles, rheumatism, allied disorders	Uddin et al. (2015), Uprety et al. (2010)
85	*Vanda spathulata*	Spoon-Leaf Vanda India: Ponnampumaravara	Terrestrial South India and Sri Lanka India: Karnataka, Kerala, and Tamil Nadu	Dried flowers	Dried flower powdered juice is used to treat asthma, and depression, enhance memory, and antioxidant activity, and alleviate chronic disease, and degenerative ailments such as cancer, autoimmune disorders, hypertension, delay the aging process, and atherosclerosis	Decruse et al. (2003), Jeline et al. (2021), Gupta and Katewa (2012)
86	*Vanda tessellata*	Grey orchid or Checkered Vanda		Leaves		Chowdhury et al. (2014)

			Epiphytic India, Myanmar, China, and Sri Lanka		Inflammations, rheumatism, dysentery, bronchitis, dyspepsia, and fever	
87	*Vanda testacea*	Small flowered Vanda	Epiphytic India, Myanmar, and Sri Lanka	Roots, leaves, and flowers	The powdered extract is used in nervous disorders, piles, inflammations, rheumatism, bronchitis, and anticancerous drugs	Kaur and Bhutani (2009)
88	*Vanilla planifolia*	Flat-leaved vanilla	Terrestrial or epiphytic South America Native to: Mexico and Central America	Fruits	Treats intestinal gas and fever, increases sexual desire, used as flavoring syrup and perfume fragrance	Rxlist (n.d.)

and mild uterine diseases (Pant 2013; Perfume workshop n.d.-a; Leander and Lüning 1967; Shanavaskhan et al. 2012; Devi et al. 2015; Panda and Mandal 2013; Nongdam 2014; Mishra et al. 2008). The paste of leaves of *Aerides multiflorum* is used for wounds, cuts, earaches, and consumed as a tonic (Perfume workshop n.d.-a; Baral and Kurmi 2006; Basu et al. 1971; Behera et al. 2013; Raja 2017). The leaf of *Aerides odorata* is applied in cuts, wounds, and tuberculosis, the fruit is used to heal the wound. Leave juice and seeds are used in treating boils in ear, nose, and skin disorders (Pant 2013; Perfume workshop n.d.-a; Leander and Lüning 1967; Baral and Kurmi 2006; Basu et al. 1971; Behera et al. 2013). The whole plant of *Anocetochilus elatus* is used to relief chest and abdominal pain and treats snake bites (Raja 2017; Sherif et al. 2012; Jiang et al. 2015).

The whole plant of *Anocetochilus formosanus* is used as an antipyretic, in detoxification, and treats tuberculosis, diabetes, bronchitis, infections in the kidney, bladder, cramps, snake bites, stomach ache, inflammation, hematemesis, nocturnal emission, nephritis, vaginal discharge, hepatitis, hypertension, and convulsions The plant possesses antioxidant, anti-hyperglycemic, hepatoprotective, anticancerous properties, and pharmacological effects, such as antiosteoporosis, antihyperliposis, and antifatigue (Perfume workshop n.d.-a; Aswandi and Kholibrina 2021; Nandkarni 1976). The leaf and stem of *Ansellia africana* are used for treating madness. Besides it also possesses anti-acetylcholinesterase activity in treating Alzheimer's disease (Saleh-E-In et al. 2021; Bhattacharyya and Staden 2016). The whole plant of *Arundina graminifolia* is used for curing rheumatic, trauma, bleeding, and snake bites. To relieve body aches root is used. In cracks scrapped bulbous stem is applied on the foot-heels (Pant 2013; Aswandi and Kholibrina 2021; Kumar 2002; Dakpa 2007).

Bletilla striata is used for tonic, against leucorrhea; leaves are used in treating lung disease; tubers are used for regeneration of muscle and other tissues, in hemorrhage dyspepsia, dysentery, fever, malignant ulcers, gastrointestinal disorders, anthrax, malaria, eye diseases, ringworm, tumors, necrosis, silicosis, traumatic injuries, coughs, chest pain, cures tuberculosis, sores, scaling, chapped skin, blood purification, strengthening, and lungs consolidation, malignant swellings, breast cancer, pustules ulcers, demulcent, and expectorant (Perfume workshop n.d.-a; Kong et al. 2003; Bulpitt et al. 2007). The *Bulbophyllum odoratissimum* plant is used to cure fractures, pulmonary tuberculosis, hernia pain, infusion, or decoction is used to treat tuberculosis and chronic inflammation (Perfume workshop n.d.-a; Chen et al. 2008; Bhattacharjee 1998). The entire plant of *Calanthe discolor* is used for improving blood flow, circulation, abscesses, scrofula, rheumatism, bone pain, and traumatic injuries, treating skin ulcers and hemorrhoids (Perfume workshop n.d.-a; Yoshikawa et al. 1998). *Changnienia amoena* plant cools the blood, acts as anti-heat and antitoxic, cures coughs, blood-streaked sputum, sores, and furuncles (Teoh 2016). The pseudo bulbs of *Coelogyne cristata* are used in constipation and aphrodisiac (Pant and Raskoti 2013; Subedi et al. 2011; Pamarthi et al. 2019). *Coelogyne stricta* pseudo bulb paste cures headaches and fever (Pamarthi et al. 2019; Yonzone et al. 2012). *Coelogyne flaccida* pseudo bulb paste cures headache and fever, juice helps in indigestion (Teoh 2016; Pant and Raskoti 2013; Pamarthi et al. 2019).

The rhizome paste of *Cymbidium aloifolium* is applied on fractured and dislocated bones. Bulbs are used as demulcent agents (Pamarthi et al. 2019). The root of *Cymbidium ensifolium* decoction used to treat gonorrhea and flower decoction used in eye sore disorders (Tsering et al. 2017). The leaves of *Cymbidium giganteum* are applied over wounds (Bulpitt 2005; Fonge et al. 2019; Linthoingambi et al. 2013). The seed of *Cymbidium goeringii* is used to treat cuts and injuries; entire plant parts are used in curing fractures (Teoh 2016). The leaf juice of *Cymbidium iridioides* is used to cease blood; its powder as a tonic; pseudo bulbs and roots are consumed in diarrhea (Aggarwal and Zettler 2010; Medhi and Chakrabarti 2009; Arditti et al. 1982; Arditti and Ernst 1984). The whole plant of *Cymbidium kanran* is used in heart purification, cures cough and asthmatic problems, and its roots are used to cure ascariasis and gastroenteritis. The whole plant of *Cymbidium lancifolium* is used in the treatment of rheumatism, improves blood flow, and traumatic injuries. The whole plant of *Cymbidium sinense* is used in purifying the heart, lungs; treat cough and asthma (Perfume workshop n.d.-a). The dried powdered pseudo bulb of *Cymbidium longifolium* is consumed on an empty stomach and fresh shoot is used for nervous disorders, madness, epilepsy, hysteria, rheumatism, and spasms. Salep used as demulcent (Zhan et al. 2016; Teoh 2016; Yonzone et al. 2013).

The powdered roots of *Cypripedium calceolus* promote sleep and reduce pain and tea prepared by the roots cures nerves and headaches (Singh and Dey 2005). The whole plant of *Cypripedium debile* is used for improving blood flow, swellings, pain, and diuretic. Likely, *Cypripedium formosanum* is used to improve blood flow, menses, expels gas, pain and itching whereas roots along with stems are used in treating malaria, snake bites, traumatic injury, and rheumatism. The roots and leaves of *Cypripedium guttatum* are used in treating epilepsy (Perfume workshop n.d.-a). The rhizomes, roots, and stems of *Cypripedium macranthos* are used to treat skin disease, promote dieresis, swelling, and pain and improve the flowing of blood; dried flowers are used to stop blood (Shimura et al. 2007). The rhizome of *Cypripedium parviflora* helps to treat insomnia, fever, headache, neuralgia, emotional tension, tumors, delirium, convulsions, anxiety, menstruate pain, and child birth (Moerman 1986; Grieve 1998; Kumar et al. 2005). The whole plant of *Cypripedium pubescens* is used as antispasmodic, diaphoretic, hypnotic, sedative, tonic, diabetes, diarrhea, dysentery, paralysis, and malnutrition, also in cases of nervous irritability, functions of the brain and promotes sleep. The dry powder roots are used as drugs for joint pains and treating stomach worms (Singh and Duggal 2009; Khory 1982).

The tubers of *Dactylorhiza hatagirea* are used as food and tonic and help in healing wound and fever and control burns and bleeding (Arditti 1992, 1967, 1968; Aggarwal and Zettler 2010; Arditti et al. 1982; Arditti and Ernst 1984). The leaves and pseudo bulb paste of *Dendrobium amoenum* are applied on skin diseases, burnt skin, and dislocated bones (Venkateswarlu et al. 2002). The leaf paste of *Dendrobium aphyllum* is applied on deformed abnormal head of a new born baby in order to form a normal shape (Pant 2013). The leaves of *Dendrobium candidum* are used to treat diabetes (Wu et al. 2004). The stem of *Dendrobium chrysanthum* is used as a tonic, enhances the immune system, and reduces fever. Leaves are used as antipyretic and mild skin diseases, which benefit the eyes (Bulpitt 2005; Jalal et al.

2008, 2010; Li et al. 2016). The whole plant of *Dendrobium chrysotoxum* possesses antitumorous and anticancerous properties, stem and flower extract is used as tonic and leaf extract as antipyretic (Bulpitt et al. 2007; Sood et al. 2006; Joshi et al. 2009). The pseudo bulb paste of *Dendrobium crepidatum* is used in fractured and dislocated bones. Stems are used as a tonic, in arthritis and rheumatism (Joshi et al. 2009; Reddy et al. 2001; Joshi and Joshi 2001). The leaves of *Dendrobium crumenatum* are used to cure boils and pimples (Joshi and Joshi 2001). The pseudo bulb pulps of *Dendrobium densiflorum* are used to cure boils, pimples, and various skin eruptions, leaf paste is applied upon fractures bones, sprains, and inflammations (Arditti 1992; Arditti et al. 1982; Arditti and Ernst 1984). The dried stems of *Dendrobium devonianum* is used as an enhancer for the immune system (Cakova et al. 2017). The stem of *Dendrobium draconis* are used in antipyretic and hematinic (Perfume workshop n.d.-b). The whole plant of *Dendrobium fimbriatum* is used during upset of the liver and severe anxiety; leaves are used in bone fracture and as a tonic, the pseudo bulbs are used in fever (Aggarwal and Zettler 2010; Arditti et al. 1982). The pseudo bulb paste of *Dendrobium heterocarpum* is used in treating fractured and bone dislocate (Arditti and Ernst 1984). The root, stem, and leaf of *Dendrobium lasianthera* act as anticancer (Utami et al. 2017).

The whole plant juice of *Dendrobium longicornu* is added to lukewarm water to bath for fever; roots are boiled to feed the livestock, to remove cough; stem juice is used to treat fever (Perfume workshop n.d.-b). The tender shoot tip juice of *Dendrobium macrostachyum* is used for earaches (Zhan et al. 2016). The pseudo bulb paste of *Dendrobium moschatum* is used for dislocated and fractured bone (Reddy et al. 2001). The pseudo bulb extracts of *Dendrobium nobile* are used in treating burns, and eye infections; the plant is used to cure pulmonary tuberculosis, fever, general debility, flatulence, dyspepsia, reduce salivation, parched, thirsty mouth, night sweats, antiphlogistic, and tonic. Seeds are used to heal wounds; stems to cure fever and tongue dryness; stems are used in longevity, aphrodisiac, stomachic, and analgesic (Aggarwal and Zettler 2010; Arditti et al. 1982; Arditti 1967). Whole plant juice of *Dendrobium ovatum* cures stomach aches, excites bile, and is a laxative for the intestines, curing constipation (Kirtikar and Basu 1981; Caius 1986). The dried stem of *Dendrobium primulinum* acts as an enhancer for the immune system (Pant and Thapa 2012). The pseudo bulb paste of *Dendrobium transparens* is used in treating fractures and dislocated bones (Arditti and Ernst 1984). The stem of *Dendrobium trigonopus* is used to cure fever and anemia (Perfume workshop n.d.-b). *Doritis pulcherrima* leaf is used to treat ear infections (Perfume workshop n.d.-c).

The whole plant of *Eria bambusifolia* is used in treating hyper acidity and various stomach aches (Zhan et al. 2016). The tubers of *Eulophia dabia* tubers are used as a tonic and aphrodisiac help to cure stomach aches, and stimulate blood flow, also used for consumption mixed with milk, sugar, and flavored species (Panwar et al. 2022). The tuber of *Eulophia epidendraea* is applied upon boils; controls pain in breast feeding mother; cures tumor and diarrhea; acts as an appetizer, anthelmintic, aphrodisiac, stomachic, worm infestation, stimulate appetite and purifies blood during heart troubles (Narkhede et al. 2016). The whole plant of *Eulophia nuda* is

used in stomachache and snake bites; the stems are used to stop bleeding and trauma pain; a thick paste of tuber is applied on the stomach to kill intestinal worms, cures rheumatoid arthritis, bronchitis, scrofulous glands, tumors, purifies blood, used as a tonic, acts as anti-aphrodisiac, demulcent, and anthelmintic. The leaf is used as a vermifuge (Hada et al. 2020). The tuber of *Gastrodia elata* is used to cure stroke, tetanus, migraine, headaches, backache, skin boils, ulcers, and pain in the lower extremities; for generalized dermatitis dizziness, sleepiness, insomnia, high blood pressure, blood circulation, rheumatism, numbness, and paralysis (Chen et al. 2014). The root paste of *Geodorum densiflorum* is applied on insect bites and wounds; the root paste by mixing with ghee and honey to correct menstrual disorders and the poultice made from pseudo bulbs is used as a disinfectant (Sheelavantmath et al. 2000). The stem of *Gymnadenia conopsea* helps the kidney, treats cough, lactation failure, sexual dysfunction, traumatic injuries, thrombosis, and chronic hepatitis (Gustafsson 2000).

The leaves and roots *of Habenaria edgeworthii* act as cooling and spermopiotic; the pseudo bulb of *Habenaria pectinata* is used during diathesis bleeding, burning sensation, fever, and phthisis (Singh and Duggal 2009). The root of *Herminium lanceum* is beneficial for the lungs and kidneys, strengthens muscles, bones, stops bleeding, and treats tuberculosis (Perfume workshop n.d.-d). The whole plant of *Liparis odorata* is soaked in wine for external use; tubers are used during stomach disorders (Perfume workshop n.d.-e). The pseudo bulb of *Malaxis acuminata* is used as a tonic, aphrodisiac, styptic, antidysentery, and febrifuge (Pushpa et al. 2011). The stem and leaves of *Papilionanthe teres* are used for improving blood flow and reducing swellings. The whole plant of *Pholidota articulata* is used to remove gas and reduce swelling, treat coughs, headaches, dizziness, ulcers, sores, traumatic injuries, uterine, and menses problems. The roots and pseudo bulb paste of *Pholidota pallida* are used to cure fever and induce sleep and juice to remove abdomen pain. The whole plant of *Platanthera chlorantha* is used to strengthen the kidneys and lungs, hernia, and sexual dysfunction (Perfume workshop n.d.-c).

The leaves and roots paste of *Rhynchostylis retusa* are used in rheumatism, leaf juice is used in constipation, gastritis, acidity, and as emollient; root juice is used to heal cuts and wounds; root is used to treat menstrual pain and arthritis; dry flower is used as emetic (Basu et al. 1971; Dakpa 2007; Bulpitt et al. 2007; Bhattacharjee 1998; Dash et al. 2008). Tubers of *Satyrium nepalense* are used to treat diarrhea, dysentery, and malaria, consumed as an aphrodisiac, and used as a children's growth supplement. Juice is used in cuts and wounds (Gutierrez 2010; Baral and Kurmi 2006; Basu et al. 1971; Behera et al. 2013; Bulpitt et al. 2007). The pseudo bulb of *Spathoglottis plicata* is used in rheumatic swelling; the hot fomentation is pressed on to draw out pus from the infected part, helps in proper blood flow and reduces pain (Friesen and Friesen 2012). The whole plant of *Thunia alba* is used in treating cough, pneumonia, bronchitis, bone break treatment, and injury (Mathew 2013).

The flower juice of *Vanda coerulea* is used in treating glaucoma, cataract, and blindness. The root of *Vanda roxburghii* is used to treat fever, dyspepsia, bronchitis, cough, piles, snake bites, rheumatism, allied disorders, and nervous system disease (Uprety et al. 2010). The dried flower powdered juice of *Vanda spathulata* are used

to treat asthma, depression, enhance memory, antioxidant activity, and alleviate chronic disease, and degenerative ailments such as cancer, autoimmune disorders, hypertension, delay in aging process, and atherosclerosis (Jeline et al. 2021). The leaf of *Vanda tessellata* is used in inflammation, rheumatism, dysentery, bronchitis, dyspepsia, and fever (Chowdhury et al. 2014). The leaf, root, and flower powdered extract of *Vanda testacea* is used in nervous disorders, piles, inflammations, rheumatism, bronchitis, and anti-cancerous drugs (Kaur and Bhutani 2009). The fruit of *Vanilla planifolia* is used to treat intestinal gas and fever, increase sexual desire, used as flavoring syrup and perfume fragrance (Rxlist n.d.).

The phytochemicals such as alkaloids, flavonoids, and glycosides made the orchids therapeutically important (Hossain 2011); they are, however, mainly used as nutraceuticals because the active principles responsible for their medicinal properties are yet to be identified with further accuracy.

7 Phytochemistry

Gas Chromatography and Mass Spectrometry (GC/MS) analyzed the essential oil and the oleoresins for various medicinal orchids. In our present study, we accessed and summarized the phytochemicals of 45 orchid species (Table 3).

Major phytochemicals reported in *Ansellia africana* namely n-Hexanal, Mesityl oxide, 4-Heptenoic acid, 3,3-dimethyl-6-oxo-methyl ester, Pentadecanoic acid, Succinic acid, 3,7-dimethyloct-6-en-1-yl pentyl ester, Linoleic acid, Linolenic acid, l-Ascorbyl 2,6-Dipalmitate, Toluene, Ethylbenzene, Mesitylene, Erythro-1-Phenylpropane-1,2-diol, Styrene, Hyacinthin, 2-Ethylbutyric acid, 3-methylbenzylester which possess cytotoxic effect against cancerous cell line (Saleh-E-In et al. 2021). Gramniphenol, a potent marker reported in *Arundina graminifolia* showed anti-tobacco mosaic virus activity (Gao et al. 2012). Phytochemicals of *B. striata* showed major biological activity in aiding hemostasis, cytotoxicity, antimicrobial, anti-inflammation, anti-oxidation, immunomodulation, anti-fibrosis, antiaging, and anti-allergy (He et al. 2017). Densiflorol B, the most active compound reported from *Bulbophyllum odoratissimum* exhibit cytotoxic activity against the five tested cell lines (Chen et al. 2008). Major stilbenoids, flaccidin, oxo flaccidin and isoflaccidin were reported in *Agrostophyllum callosum, Coelogyne flaccida (*Majumder and Maiti 1988, 1989, 1991*;* Majumder et al. 1995*)*. 5-hydroxy-3-methoxy-flavone-7-*O*-[β-D-apiosyl-(1 → 6)]-β-D-glucoside, an alpha-glucosidase inhibitor reported from *Dendrobium devonianum (*Sun et al. 2014*)*. Sesquiterpene such as alloaromadendrene, emmotin, and picrotoxane from *Dendrobium nobile* possesses immunomodulatory potential (Ye et al. 2002). Dendroparishiol a marker reported from *Dendrobium parishii* exhibited antioxidant and anti-inflammatory activity against RAW264.7 cells (Kongkatitham et al. 2018). 9, 10-dihydrophenanthrene, a novel marker reported from *Eria bambusifolia* showed anticancer activity against the human cell line (Rui et al. 2016). Major aromatic phytochemicals were reported in *Platanthera chlorantha* namely β-Ocimene, Lilac

Table 3 Screening of phytochemicals in some medicinal orchids

Sl. No.	Species	Phytochemicals	References
1	*Anacamptis pyramidalis*	Disaccharide, Citric acid, Parishin G isomer-1, Parishin G isomer-2, Gastrodin derivative, Parishin B, Gastrodin derivative, Parishin C, Dihydroxybenzoic acid derivative, Caffeic acid derivative, Acacetin derivative, Oxo-dihydroxy-octadecenoic acid, Trihydroxy-octadecenoic acid	Fawzi Mahomoodally et al. (2020)
2	*Ansellia africana*	2,4,4-Trimethyl-1-hexene, 2-Hexene, 2,5,5-trimethyl, 2,3-Dimethyl-2-heptene, Cyclopentane, 1,2,3,4,5-pentamethyl, pentane, 1,2,3,4,5-pen, Nonane 4,5 dimethyl, Octane 5-ethyl-2-methyl, n-Decane, 1-Undecane, 4-methyl, Dodecane, Cyclohexane, (1,2,2-trimethylbutyl), tetradecane, pentadecane, Hexadecane 4-methyl, heptadecane, Nonadecanol, Lignoceric alcohol, cis-4-Hexen-1-ol, n-Hexanal, Mesityl oxide, 4-Heptenoic acid, 3,3-dimethyl-6-oxo-methyl ester, Pentadecanoic acid, Succinic acid, 3,7-dimethyloct-6-en-1-yl pentyl ester, Linoleic acid, Linolenic acid, l-Ascorbyl 2,6-Dipalmitate, Toluene, Ethylbenzene, Mesitylene, Erythro-1-Phenylpropane-1,2-diol, Styrene, Hyacinthin, 2-Ethylbutyric acid, 3-methylbenzylester	Saleh-E-In et al. (2021)
3	*Arundina graminifolia*	graminibiben-zyls A, 5,12-dihydroxy-3-methoxybibenzyl-6-carboxylic acid, dihydropinosylvin, 2,5,2′,5′-tetrahydroxy-3-methoxybibenzyl, rhapontigen, pinosylvin, bauhiniastatin D, arundinaol, coelonin, cucapitoside, blestriarene A, isoshancidin, obovatin, kaempferol-β-3-O-glycos, dihydropinosylvin, 4′--methylpinosylvin, 3-(γ,-γ-dimethylallyl)resveratrol, 5-(γ,γ dimethylallyl)oxyresveratrol, 3-hydroxy-4,3′,5′-trimethoxy-trans-stilbene, gramniphenol, 9'-dehydroxy-vladinol, vladinol F,	Gao et al. (2012), Hu et al. (2013), Zhang et al. (2021)

(continued)

Table 3 (continued)

Sl. No.	Species	Phytochemicals	References
		9-O-β-D-xylopyranoside-vladinol F, 4,9-dihydroxy-4',7-epoxy-8',9'-dinor-8,5'-neolignan-7'-oic acid	
4	*Bletilla striata*	3,3′-dihydroxy-5-methoxybibenzy, gigantol, 5,4′-dimethoxybibenzyl-3,3′-diol, 3′-hydroxy-5-methoxybibenzyl-3-O-β-D-glucopyranoside, 5-hydroxy-4-(p-hydroxybenzyl)-3′,3-dimethoxybibenzyl, bulbocol, gymconopin D, bulbocodin D, blestritin B, 4,7-dihydroxy-2-methoxy-9,10-dihydrophenanthrene, 9,10-dihydro-4,7-dimethoxyphenanthrene-2,8-diol, blestriarene A, 2,4,7-trimethoxy-phenanthrene, 7-hydroxy-2-methoxyphenanthrene-3,4-dione, 3′,7′,7-trihydroxy-2,2′,4′--trimethoxy-[1,8′-biphenanthrene]-3,4-dione, cyclomargenone, β-sitosterol, stigmasterol, protocatechuic acid, cinnamic acid, p-hydroxybenzaldehyde, 3,7-dihydroxy-2,4,8-trimethoxyphenanthrene, 9,10-dihydro-4,7-dimethoxyphenanthrene-2,8-diol, 9,10-dihydro-1-(4'-hydroxybenzyl)-4,7-dimethoxyphenanthrene-2,8-diol, 3',4"-dihydroxy-5',3",5"-trimethoxybibenzyl, batatasin III	He et al. (2017), Woo et al. (2014)
5	*Bulbophyllum odoratissimum*	Moscatin, 7-hydroxy-2,3,4-trimethoxy-9,10-dihydrophenanthrene, coelonin, densiflorol B, gigantol, batatasin III, Tristin, vanillic acid, syringaldehyde, 3,7-Dihydroxy-2,4,6-trimethoxyphenanthrene, Bulbophyllanthrone	Chen et al. (2008), Sharifi-Rad et al. (2022)
6	*Coelogyne cristata*	Coelogin, coeloginin, 3,5,7-trihydroxy-1,2-dimethoxy-9,10-dihydrophenanthrene, 3,5,7-trihydroxy-1,2-dimethoxyphenanthrene	Majumder et al. (2001)

(continued)

Table 3 (continued)

Sl. No.	Species	Phytochemicals	References
7	*Coelogyne flaccida*	Callosin, flaccidin, oxoflaccidin, 2,7-dihydroxy-6-methoxy-5H-phenanthro [4,5-bcd] pyran-5-one	Majumder and Sen (1991), Majumder and Maiti (1988, 1989), Majumder et al. (1995)
8	*Cymbidium aloifolium*	1,2 diarylethanes, 9,10 dihydrophenanthrene, 6-0-methylcoelonin, batatasin III, coelonin, gigantol, 5-hydroxy-3-methoxy-1,4-phenanthraquinone, Friedelin, sitosterol, n-hexadecanoic acid, 9,12-octadecadienoic acid, 9,12,15-octadecatrienoic acid, octadecanoic acid, phytol; 2-butyne; 2-cyclopenten-1-one; and 1,4-benzenedicarboxylic acid	Juneja et al. (1987), Barua et al. (1990), Rampilla and Khasim (2020)
9	*Cymbidium ensifolium*	Cymensifins, cypripedin, and gigantol	Jimoh et al. (2022)
10	*Cymbidium finlaysonianum*	1-(4-Hydroxybenzyl)-4,6-dimethoxy-9,10-dihydrophenanthrene-2,7-diol, Cymbinodin-A	Lertnitikul et al. (2018)
11	*Cymbidium giganteum*	1,2-diarylethane, gigantol, 4ξ-(β-d-glucopyranosyloxymethyl)-14-α-methyl-22ξ, 24ξ, 25,28-tetrahydroxy-9,19-cyclo-5α,9-β-ergostan-3-one	Juneja et al. (1985), Dahmén and Leander (1978a)
12	*Cymbidium goeringii*	Gigantol	Won et al. (2006)
13	*Cymbidium kanran*	Vicenin-2, Schaftoside isomer, Schaftoside, Vicenin-3, Vitexin, Isovitexin	Jeong et al. (2017)
14	*Dendrobium amoenum*	3,4′-dihydroxy-5-methoxybibenzyl and 4,4′-dihydroxy-3,3′,5-trimethoxybibenzyl, 3,4,5-trimethoxybenzaldehyde, picrotoxinin, aduncin, 9,10-dihydro-5H-phenanthro-(4,5-*b*,*c*, *d*)-pyran, amoenumin, (E)-13-docosenoic acid; oleic acid; 11-octadecenoic acid, methyl ester; and hexadecanoic acid, 2,3-dihydroxypropyl ester, aphyllone B, (R)-3,4-dihydroxy-5,4′,α-trimethoxybibenzyl, 4-[2-[(2S,3S)-3-(4-hydroxy-3,5-dimethoxyphenyl)-2-hydroxymethyl-8-methoxy-2,3-dihydrobenzo (Stewart and Griffith	Venkateswarlu et al. (2002), Majumder et al. (1999), Dahmén and Leander (1978b), Veerraju et al. (1989), Paudel and Pant (2017)

(continued)

Table 3 (continued)

Sl. No.	Species	Phytochemicals	References
		1995; Kaushik 1983) dioxin-6-yl] ethyl]-1-methoxyl benzene, dendrocandin B, 4,4′-dihydroxy-3,5-dimethoxybibenzyl, 3,4-dihydroxy-5,4′--dimethoxybibenzyl, 3-O-methylgigantol, dendrophenol, gigantol, dendrocandin C, dendrocandin D, and 3,3′,4,4′--tetrahydroxy-5-methoxybibenzyl	
15	*Dendrobium candidum*	3,4′-dihydroxy-5-methoxybibenzyl, uridine, sucrose, adenosine	Li et al. (2008, 2009)
16	*Dendrobium chrysanthum*	Denchrysan B, dengibsin, moscatin, dendroflorin, denchrysan A, moscatilin, gigantol, batatasin III, Tristin, 4,9-dimethoxy-2,5-dihydroxyphenanthrene, 3,4-dihydroxybenzoic acid, dibutyl phthalate, stigmasterol, β-sitosterol, daucosterol	Li et al. (2016)
17	*Dendrobium chrysotoxum*	Chrysotoxols A and B, bibenzyls, phenanthrenes, fluorenones, coumarin, flavonoid, gigantol, 3-O-methylgigantol, moscatilin, 4-[2-(3-hydroxy-4-methoxyphenyl) ethyl]-2,6-dimethoxyphenol, crepidatin, chrysotoxine, erianin, isoamoenylin, batatasin III, tristin, nobilin C, moscatin, 2,5-dihydroxy-4,9-dimethoxyphenanthrene, confusarin, nudol, fimbriatone, 1,5,6,7-tetramethoxy-2-hydroxyphenanthrenol, 7-hydroxy-2,3,4-trimethoxyphenanthrene, 1,2,6,7-tetrahydroxy-4-methoxyphenanthrene, 2,4-dihydroxy-7-methoxy-9,10-dihydrophenanthrene, erianthridin, 2,5-dihydroxy-4-methoxy-9,10-dihydrophenanthrene, 1,4,7-trihydroxy-5-methoxy-9H-fluoren-9-one, nobilone, 6-methylesculetin, and homoeriodictyol	Hu et al. (2012), Liu et al. (2022)
18	*Dendrobium crepidatum*	Crepidatuols A, (±)-homocrepidine A, Crepidatin, crepidatumines A and B,	Li et al. (2013), Hu et al. (2016), Xu et al. (2020, 2019b), Ding et al. (2021)

(continued)

Table 3 (continued)

Sl. No.	Species	Phytochemicals	References
		dendrocrepidine B, crepidatumines C and D, crepidine, isocrepidamine, crepidamine, octahydroindolizine	
19	*Dendrobium densiflorum*	Densiflorol, Dendroflorin	Fan et al. (2001)
20	*Dendrobium devonianum*	Quercetin, Taxifolin, Rutin, Luteolin, Kaempferol, Myricetin, (−)-Epiafzelechin, 5-Hydroxyauranetin, 6-C-Hexosyl-hesperetin O-hexoside, 8-C-Hexosyl-apigenin O-feruloylhexoside, 8-C-Hexosyl-apigenin O-hexosyl-O-hexoside, 8-C-Hexosyl-chrysoeriol O-feruloylhexoside, Isorhamnetin hexose-malonate, Isorhamnetin O-acetyl-hexoside, Isorhamnetin-3-O-rutinoside, Isoschaftoside, Isovitexin, Isovitexin 7-O-glucoside, Jaceosidin, Kaempferide 3-O-β-D-glucuronide, Ladanein, Naringenin, Nepetin, Peonidin 3-O-glucoside chloride, Pinobanksin, Quercitrin, Rhoifolin, Schaftoside, Tamarixetin, Tangeretin, Tricin 7-O-hexoside, Tricin 7-O-hexosyl-O-hexoside, Tricin O-malonylhexoside, Tricin O-saccharic acid, Tricin O-sinapoylhexoside, Violanthin, Vitexin, Vitexin 2"-O-β-L-rhamnoside, Vitexin-2-O-D-glucopyranoside, 5-hydroxy-3-methoxy-flavone-7-O-[β-d-apiosyl-(1 → 6)]-β-d-glucoside	Zhao et al. (2021), Sun et al. (2014)
21	*Dendrobium draconis*	5-methoxy-7-hydroxy-9, 10-dihydro-1,4-phenanthrenequinone, hircinol, gigantol, batatasin, 7-methoxy-9,10-dihydrophenanthrene-2,4,5-triol	Sritularak et al. (2011)
22	*Dendrobium fimbriatum*	Plicatol B, hircinol, plicatol A, and plicatol C, 1 bibenzyl (3′,4-dihydroxy-3,5′--dimethoxybibenzyl), furostanol, protodioscin, Denfigenin, gigantol-5-O-β-d-glucopyranoside, 9,10-dihydro-aphyllone A-5-O-β-d-	Talapatra et al. (1992), Xu et al. (2017), Favre-Godal et al. (2022)

(continued)

Table 3 (continued)

Sl. No.	Species	Phytochemicals	References
		glucopyranoside, ficusal-4-O-β-d-glucopyranoside, botrydiol-15-O-β-d-glucopyranoside	
23	*Dendrobium heterocarpum*	Methyl 3-(4-hydroxyphenyl) propionate, 3,4-dihydroxy-5,4'--dimethoxybibenzyl, dendrocandin B, dendrofalconerol A, syringaresinol, batatasin III, 3-*O*-methylgigantol, gigantol, moscatilin, dendrocandin A, (S)-3,4,-α-trihydroxy-4′,5-dimethoxybibenzyl, densiflorol A, dendrocandin I, dendrocandin F, coelonin, carthamidin, 4-hydroxy-2-methoxy-3,6-dimethylbenzoic acid	Warinhomhoun et al. (2022), Xiao-bei et al. (2019)
24	*Dendrobium longicornu*	Longicornuol A, 4-[2-(3-hdroxyphenol)-1-methoxyethyl]-2,6-dimethoxyphenol, 5-hydroxy-7-methoxy-9,10-dihydrophenanthrene-1,4-dione, 7-methoxy-9,10-dihydrophenanthrene-2,4,5-triol, *erythro*-1-(4-*O*-*β*-*D*-glucopyranosyl-3-methoxyphenyl)-2-[4-(3-hydroxypropyl)-2,6-dimethoxyphenoxy]-1,3-propanediol, Longicornuol B	Hu et al. (2008b, 2010)
25	*Dendrobium nobile*	Vitamin A Aldehyde; Longifolene; 1-Heptatriacotanol; Z,Z6,28-Heptatriactontadien-2-One and Dendroban-12-One, alloaromadendrane, emmotin, picrotoxane, dendronobilate, 4-*O*-demethyl-nobilone, dendronobilate, 4-*O*-demethyl-nobilone	Ye et al. (2002), Cao et al. (2021), Meitei et al. (2019)
26	*Dendrobium ovatum*	Stilbenoid	Pujari et al. (2021)
27	*Dendrobium parishii*	(−)-Dendroparishiol	Kongkatitham et al. (2018)
28	*Dendrobium primulinum*	2,4,7-trihydroxy-9,10-dihydrophenanthrene, denthyrsinol, moscatin, moscatilin, gigantol, batatasin III, tristin, 3,4,5-trihydroxybibenzyl, 3,6,9-	Ye et al. (2016)

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Table 3 (continued)

Sl. No.	Species	Phytochemicals	References
		trihydroxy-3,4-dihydroanthracen-1 (2H)-one, -sitosterol, -daucosterol	
29	*Dendrobium thyrsiflorum*	Denthyrsin, denthyrsinol, denthyrsinone, 2,3,5-Trihydroxy-4-methoxyphenanthrene, 3,7-Dihydroxy-2,4-dimethoxyphenanthrene, 2,7-Dihydroxy-1,5,6-trimethoxyphenanthrene, Syringaresinol, Pinoresinol, Ayapin, Scopoletin, and 6,7-Dimethoxycoumarin, 4, 7-dihydroxy-2-methoxy-9, 10-dihydrophenan-threne, syringaldehyde, moscatin, gigantol, batatasin Ill, tristin, stigmasterol	Zhang et al. (2005), Wrigley (1960), Ruixuan et al. (2015)
30	*Dendrobium trigonopus*	Trigonopols A and B, gigantol, tristin, moscatin, hircinol, naringenin, 3-(4-hydroxy-3-methoxyphenyl)-2-propen-1-ol, (−)-syringaresinol	Hu et al. (2008a)
31	*Eria bambusifolia*	Erathrins A and B, bambusifolia, batatasin III, tristin, 3-hydroxy-5-methoxy bibenzyl, gigantol, 3′,5-dimethoxy-9,9′-diacetyl-4,7′-epoxy-3,8′-bilign-7-ene-4′--methol, and balanophonin	Rui et al. (2016)
32	*Eulophia epidendraea*	β-sitosterol, β-sitosterol glucoside, β-amyrin, lupeol	Maridass and Ramesh (2010)
33	*Eulophia nuda*	Eulophiol, Nudol, 2,3,4,7-tetramethoxyphenanthrene, 9,10-dihydro-4-methoxyphenanthrene-2,7-diol, l,5-dimethoxyphenanthrene-2,7-diol, 1,5,7-trimethoxyphenanthrene-2,6-diol, 5,7-dimethoxyphenanthrene-2,6-diol, 4,4,8,8-tetramethoxy-[1,1-biphenanthrene]-2,2,7,7-tetraol, 2,2,4,4,7,7,8,8-octamethoxy-1,1-biphenanthrene, Lupeol, 9,10-dihydro-2,5-dimethoxyphenanthrene-1,7-diol, 9,10-dihydro-4-methoxyphenanthrene-2,7-diol, 1,5-dimethoxyphenanthrene-2,7-diol, 1,5,7,-trimethoxyphenanthrene-2,6-diol,	Hada et al. (2020), Bhandari et al. (1985), Tuchinda et al. (1988)

(continued)

Table 3 (continued)

Sl. No.	Species	Phytochemicals	References
		5,7-dimethoxyphenanthrene-2,6-diol, and 4,4′,8,8′-tetramethoxy [1,1′-biphenanthrene]-2,2′,7,7′--tetrol. 4-Hydroxybenzaldehyde, 4-hydroxybenzyl alcohol, 2,7-dihydroxy-3,4-dimethoxyphenanthrene	
34	*Gastrodia elata*	Parishins B and C, gastrodin A, gastrol A	Lin et al. (1996), Li et al. (2007)
35	*Gymnadenia conopsea*	Gymnoside, loroglossin, dactylorhin, daucosterol, dioscin, gymconopin, blestriarene, 2,6-dimethoxy phenol, eugenol, 4-hydroxybenzene, 4-methoxy phenylpropanol, 4-ethoxy phenylpropanol, contra-hydroxybenzyl, dithioether, syringol, syringaldehyde, gastrodin, arabinose, xylose, lupenone, 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol, lupeol, cirsimarin, astragalin, kaempferol-7-O-glucoside, desmethylxanthohumol, isorhamnetin, naringenin chalcone, equol, galangin, 1-((4-hydroxyphenyl)methyl)-4-methoxy-2,7-phenanthrenediol, gymconopin A,9,10-dihydro-2-methoxy-4,5-phenanthrenediol, blestriarene A, gymconopin, blestriarene B	Gustafsson (2000), Shang et al. (2017)
36	*Liparis odorata*	Anodendrosin A, Liparisglycoside, Liparis alkaloid, 4-(O-β-D-Glucopyranosyl)-3,5-bis(3-methyl-2-butenyl) benzoic acid, Adenosine, D-α-2-Alanin, p-Hydroxybenzoic acid	Liang et al. (2019)
37	*Malaxis acuminata*	Catechin, phloridzin, rutin, Caffeic acid, chlorogenic acid, ellagic acid, 3-hydroxy benzoic, 4-hydroxy benzoic, protocatechuic acid, 3-hydroxy cinnamic acid, p-coumaric acid, Stigmasterol and β-sitosterol, Sibutramine, limonene, diethylene glycol, p-cymene, eugenol, benzene, piperitone, glycerol, ribitol, and myo-inositol, 6-octadecenoic acid,	Suyal et al. (2020)

(continued)

Table 3 (continued)

Sl. No.	Species	Phytochemicals	References
		8-octadecenoic acid, 9-octadecenal, batatasin III, bulbophythrin A, butyl oleate, cerasynt, cis-oleic acid, cyclopentadecanolide, diethyl phthalate, cyclopentanetridecanoic acid	
38	*Phalaenopsis cornucervi*	1,2-saturated pyrrolizidine mono-esters, T-phalaenopsine	Frölich et al. (2006)
39	*Pholidota pallida*	Oelonin, lusianthridin, flavanthrin, batatasin-III, 3′,5-dihydroxy-2-(4-hydroxybenzyl)-3-methoxybibenzyl, gigantol, 3-[2-(3-hydroxyphenyl) ethyl]-2,4-bis[(4-hydroxyphenyl) methyl]-5-methoxyphenol, hydroxytyrosyl butyrate, (24R)-ethylcholest-5-en-3-ol-7-one, taraxerone, friedelin, hydroxytyrosyl	Yu et al. (2021)
40	*Platanthera chlorantha*	β-Ocimene, Lilac aldehyde, β-Elemene, α-Bergamotene, Cedrene, Germacrene D, Pentadecane, b-Bisabolene, b-Sesquiphellandrene, 1,2,3-Trimethoxy-5-(2-propenyl) benzene, Tetradecanal, Benzophenone, Galaxolide, Docosane, Tetradecyl benzoate	D'Auria et al. (2020)
41	*Platanus acerifolia*	5,7,40-trihydroxy-8-(1, 1-dimethylallyl)-30-methoxyflavonol, 5,7,40-trihydroxy-60-prenyl-30-methoxyflavonol, Kaempferol-3-O-a-L-(300-E-p-coumaroyl)-rhamnoside, Quercetin-3-O-α-l-(2″-E-p-coumaroyl-3″-Z-p-coumaroyl)-rhamnopyranoside (E, Z-3′-hydroxyplatanoside, and quercetin-3-O-α-l-(2″-Z-p-coumaroyl-3″-E-p-coumaroyl)-rhamnopyranoside (Z,E-3′--hydroxyplatanoside, 8-methoxy-6-*C*-methyl-5,7-dihydroxyflavonol, 8-*C*-(1,1-dimethyl-2-propen-1-yl)-5,7-dihydroxyflavonol, and 8-*C*-(1,1-dimethyl-2-propen-1-yl)-4′--methoxy-5,7-dihydroxyflavonol	Wu et al. (2022), Kaouadji (1989), Thai et al. (2016)
42	*Thunia alba*	Batatasin-III, lusianthridin, 3,7-dihydroxy-2,4-	Majumder et al. (1998), Ya-ping et al. (2019), Yan et al. (2016)

(continued)

Table 3 (continued)

Sl. No.	Species	Phytochemicals	References
		dimethoxyphenanthrene, 3,7-dihydroxy-2,4,8-trimethoxyphenanthrene, cirrhopetalanthrin and flavanthrin, hircinol, scoparone, β-sitosterol, 3,7-dihydroxy-2,4-dimethoxyphenanthrene, lusianthridin, coelonin, thunalbene	
43	*Vanda coerulea*	Imbricatin, methoxycoelonin, gigantol, phenanthropyrans, bibenzyl, dihydrophenanthrenes	Simmler et al. (2009)
44	*Vanda tessellate*	Tessalatin, Oxo-tessallatin, 2,5-Dimethoxy-6,8-dihydroxy isoflavone, Gallic acid, 2.7.7-Trimethyl bicycle () heptanes, Octacosanol, Heptacosane	Khan et al. (2019)
45	*Vanda roxburghii*	Stigmasterol, γ-sitosterol, β-sitosterol, β-sitosterol-D-glucoside, tetracosylferulate	Khan et al. (2019)
46	*Vanilla planifolia*	Vanillin	Podstolski et al. (2002)

aldehyde, β-Elemene, α-Bergamotene, Cedrene, Germacrene D, Pentadecane, b-Bisabolene, b-Sesquiphellandrene, 1,2,3-Trimethoxy-5-(2-propenyl) benzene, Tetradecanal, Benzophenone, Galaxolide, Docosane, Tetradecyl benzoate (D'Auria et al. 2020). Quercetin-3-O-α-L-(2″-E-p-coumaroyl-3″-Z-p-coumaroyl)-rhamnopyranoside (E, Z-3′-hydroxyplatanoside and quercetin-3-O-α-L-(2″-Z-p-coumaroyl-3″-E-p-coumaroyl)-rhamnopyranoside (Z, E-3′-hydroxyplatanoside) markers reported from *Platanus acerifolia*. The leaves exhibit antimicrobial activity against *Staphylococcus aureus (*Wu et al. 2022*)*. Phytochemicals reported in genus *Vanda* possess major pharmacological activities, markers such as stigmasterol, γ-sitosterol, β-sitosterol, β-sitosterol-D-glucoside, tetracosylferulate possess anti-aging, antimicrobial, anti-inflammatory, antioxidant, neuroprotective, membrane stabilizing, and hepato-protective activities (Khan et al. 2019).

7.1 Secondary Metabolites

A wide range of secondary metabolites is present in Orchids, of which only a very slight portion was analyzed. Normally several phytochemicals viz., alkaloids, saponins, flavonoids, anthocyanins, carotenoids, polyphenols, sterols, etc. were produced and integrated into in vitro culture of orchids (Mulabagal and Tsay 2004; Yesil-Celiktas et al. 2007; Shinde et al. 2010). Among them, polyphenols were responsible

for their crucial role in curing many degenerative and age-linked ailments (Brewer 2011; Procházková et al. 2011). Likely, other bioactive compounds like flavonoids, tannins, and alkaloids were bestowed for the medication of several chronic diseases (Lu et al. 2004; Zhang et al. 2005; Harris and Brannan 2009).

7.1.1 Bioactive Compounds

Various plant parts (leaf, root, and pseudobulb) of orchids possess a group of important phenolic acids such as gentisic acid, gallic acid, salicylic acid, protocatechuic acid, syringic acid, caffeic acid, sinapic acid, ferulic acid as well as flavonoids viz., catechin, apigenin, myricetin, naringin, rutin, quercetin, kaempferol, and alkaloids viz., chysine, drobine, dendronine, grandifolin, crepidine, and vanilin in higher concentration. In in vitro raised plants, bioactive compounds were more dominant than in wild plants of medicinal orchids (Fig. 2).

The majority of bioactive compounds viz., ayapin, n-octastylferulate, crepidatin, confusarin, physcion, scopolin, rhein, fimbriatone, and β-sitosterol were reported in *Dendrobium fimbriatum* which were important for pharmacological point of view (Paul et al. 2017; Bi et al. 2003; Shailajan et al. 2015). However, studies on the phytochemical analysis of medicinal orchids raised in vitro are very few (Bhattacharyya et al. 2014, 2015, 2018, 2016a,b; Bhattacharyya and Staden 2016; Giri et al. 2012b; Bose et al. 2017). A bioactive compound such as bisbenzyl erianin was isolated from the callus culture of *Dendrobium chrysotoxum* which was the potential as an antioxidant, antitumor, and antiangiogenic agent (Zhan et al. 2016). The presence of polyphenols was reported in *Habenaria edgeworthii* culture (Giri et al. 2012a). Different biochemical constituents like total phenolic, flavonoid, alkaloids, and tannins contents were analyzed and comparisons were reported between the various parts of mother plants and micropropagated plants of *Dendrobium nobile* (Bhattacharyya et al. 2014). Compounds with higher concentrations are reported in micropropagated plants of *Herminium lanceum* (Singh and Babbar 2016) and *Habenaria edgeworthii* (Giri et al. 2012a) than in wild plants. The phytochemical evaluation of various parts of the mother plant and in vitro propagated plants of *Bulbophyllum odoratissimum* was performed by using HPLC (Prasad et al. 2021). Extracts of *Dendrobium crepidatum* contained bioactive compounds like tetracosane, hexadecanoic acid, triacontane, phenol derivatives, and tetradecanoic acids are responsible for antioxidant and cytotoxic activities (Paudel et al. 2019).

7.1.2 Biological Activity

Antioxidant Activity

Bioactive components exhibited vigorous antioxidant properties in divergent in vitro methods which showed high scavenging potentiality to various Reactive Oxygen

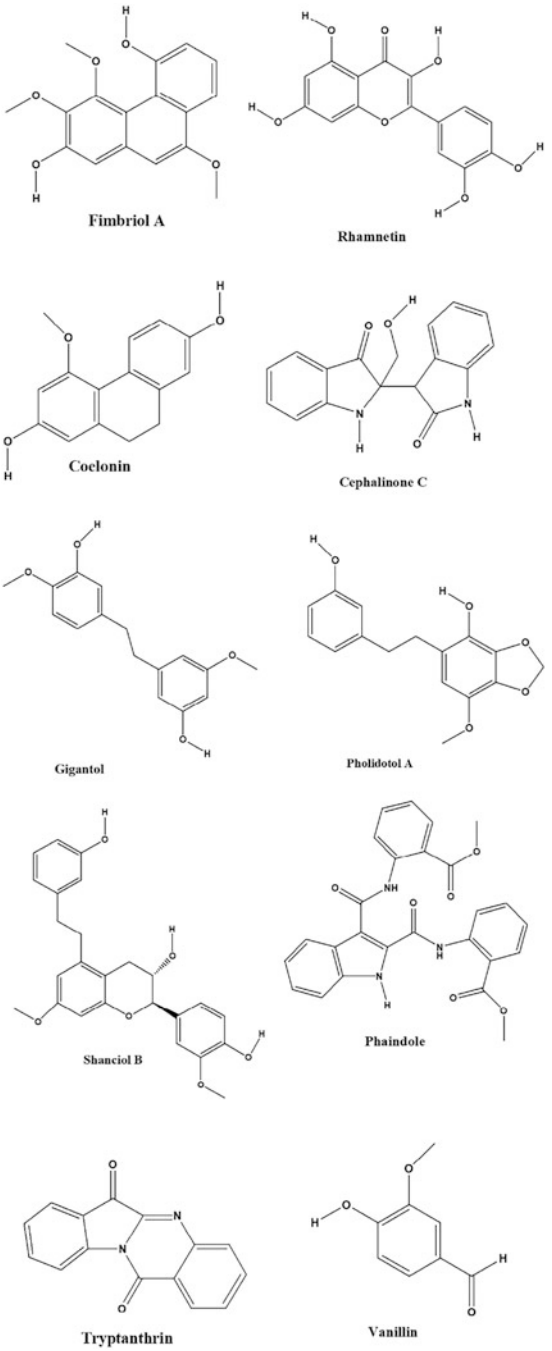

Fig. 2 Chemical structure of bioactive molecules of medicinal orchids (Drawn in Chemdraw 8.0)

Species (ROS) viz. hydroxyl radical, peroxynitrite, superoxide anion, and hypochlorous acid (Halliwell 2008). Unlike synthetic antioxidants, vigorous studies were conducted on antioxidants present in natural fruits, vegetables and medicinal plants, which are considered less toxic due to their effective free radical scavenging activity.

1,1- diphenyl-2-picrylhydrazyl (DPPH) and Ferric Reducing Antioxidant Power (FRAP) assay were used for the analysis of the antioxidant activity of the plant extracts of mother and micropropagated *Dendrobium nobile* plants (Cao et al. 2021). Both the assays describe the antioxidant response of *Dendrobium nobile* determining the high antioxidant potential in samples of leaf due to its high content of polyphenols, alkaloids, and flavonoids. Among the different solvents and plant parts of the tested species, the DPPH activity of the methanolic leaf extraction was the highest (89.8 ± 2.9%), but the activity of radical scavenging of the chloroform leaf extraction was the lowest (28 ± 2.9%) of the micropropagated plant. *D. nobile* plantlets grown through tissue culture reported higher levels of free radical scavenging activity than mother plants (Bhattacharyya et al. 2014). Total phenol content (TPC), radical scavenging activity DPPH and ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), Total Flavonoid Content (TFC) as well as total reducing power ability is being reported from all plant material extracts of mother plants and in vitro-cultured plants of *Bulbophyllum odoratissimum* (Prasad et al. 2021). DPPH radical scavenging activity was studied in some of the following orchid species viz. *Acampe papillosa, Aerides odorata, Bulbophyllum lilacinum, Arundina graminifolia, Cymbidium aloifolium, Dendrobium aphyllum, Papilionanthe teres, Luisia zeylanica, Dendrobium tortile, Rhynchostylis retusa* (Rahman and Huda 2021); *Rhynchostele rossii* (Gutiérrez-Sánchez et al. 2020); *Dendrobium candidum (*Wang et al. 2016*)*; *Dendrobium chrysanthum (*Aswandi and Kholibrina 2021*)*; *Dendrobium draconis (*Sritularak et al. 2011*)*; *Pholidota articulata* (Singh et al. 2016a); *Papilionanthe teres* (Mazumder et al. 2010); *Geodorum densiflorum* (Keerthiga and Anand 2014). DPPH assay measures the total phenolic, alkaloid and flavonoid content by using Folin-Ciocalteu, spectrophotometry and modified acid-alkalimetry methods in *Dendrobium crumenatum* (Topriyani 2013). DPPH radical, column chromatography Diaion HP-20 or reverse-phase silica gel column chromatography was studied in *Gymnadenia conopsea* (Shang et al. 2017). A DPPH radical, spectrophotometric method, Liquid Chromatography Mass Spectrometry (LC-MS) was studied in *Paphiopedilum villosum* (Khamchatra et al. 2016). DPPH and ABTS assay were studied in *Cymbidium kanran* (Axiotis et al. 2022); *Dactylorhiza hatagirea* (Kumari et al. 2022); *Dendrobium moschatum* (Robustelli della Cuna et al. 2018); *Geodorum densiflorum* (Keerthiga and Anand 2014); *Gastrodia elata* (Song et al. 2016). DPPH, ABTS radical scavenging assays and reducing capacity assays have been studied in *Dendrobium aphyllum* (Liu et al. 2017) and *Dendrobium macrostachyum* (Sukumaran and Yadav 2016). DPPH, ABTS, and metal chelating in *Malaxis acuminata* (Bose et al. 2017) and in *Dendrobium nobile* hydroxyl radicals scavenging assay was also studied (Luo et al. 2010). MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay in *Dendrobium aphyllum* (Liu et al. 2018) and

DPPH assay in *Dendrobium densiflorum* (Pant et al. 2022), and in *Dendrobium crepidatum* by using GC–MS (Gas Chromatography and Mass Spectrometry) was used to identify the compounds (Paudel et al. 2019). DPPH, ORAC, and deoxyribose assays in *Dendrobium parishii* (Raja 2017); DPPH scavenging activity, reducing power and chelating activity against iron ions (Fe^{2+}) in *Dendrobium candidum* (Ng et al. 2012). DPPH and FRAP assay were studied in *Dendrobium devonianum* (Wang et al. 2018) and *Dendrobium fimbriatum* (Paul and Kumaria 2020). Deoxyribose assays, non-site-specific scavenging assays, or antioxidants and iron ions, also known as site-specific scavenging assays have been studied in *Dendrobium chrysotoxum* (Zhao et al. 2007) (Table 4).

Antimicrobial Activity

Five different multidrug resistance (MDR) bacterial clinical isolates were used for testing the antibacterial activity of the epiphytic orchid *Pleione maculata* which includes *Escherichia coli* (2461), *Enterococcus* sp. (2449), *Staphylococcus aureus* (2413), *Serratia* sp. (2442), and *Acinetobacter* sp. (2457) along with antimycobacterial activity with *Mycobacterium tuberculosis* strain (H37Rv) (Bhatnagar and Ghosal 2018). Likely methanolic extracts of tubers of *Satyrium nepalense* were studied against both Gram-negative and -positive food pathogenic bacteria namely *Staphylococcus mutans, Pseudomonas aeruginosa, Staphylococcus aureus*, and *Klebsiella pneumonia* and 6 mg/100 μL concentration was responsible for the minimal effect against all the tested microorganisms (Mishra and Saklani 2012).

Ethanolic and hexane extracts of *Coelogyne cristata* and *Coelogyne fimbriata*, leaves and pseudobulbs were explored against human pathogens like Gram-positive *Bacillus cereus* (ATCC 14579), *Staphylococcus aureus* (ATCC 12600), and Gram-negative *Escherichia coli* (ATCC 10798), *Yersinia enterocolitica* (ATCC 9610), and *Klebsiella pneumonia* (ATCC BAA-3079) bacteria. Only 70% of ethanolic leaf extracts inhibited the growth of the investigated human pathogens (Pyakurel and Gurung 2008; Subedi 2002; Wati et al. 2021; Subedi et al. 2013). Methanolic and water extract of *Peristylus densus* showed better antimicrobial activity against bacterial and fungal strains with an inhibition zone of 8–10 mm when tested against *S. typhi, P. aeruginosa, S. aureus, E.coli, and Aspergillus niger* (Jagtap 2015). Methanolic and ethanolic extract of *Malaxis acuminata* revealed strong antimicrobial activity against *P. aeruginosa* and *S. aureus* strain in Minimum Inhibitory Concentration (MIC) assay and Butanol extract showed a strong inhibition zone of 32 mm compared to control 28 mm against *Candida albicans* (Suyal et al. 2020). Ethyl acetate extract showed significant antimicrobial activity against bacterial strains *K. pneumoniae, S. enteric* and *E. coli* with an inhibition zone of 14–18 mm in *Pholidota articulata (*Singh et al. 2016b*)*. Whereas ethanolic extract of the species showed antimicrobial activity against microbial strains *S. aureus, Vibrio cholerae, B. subtilis, E. coli,* and *K. pneumoniae* with inhibition zone ranges from 9 to 12 mm. No activity was observed in *V. cholerae (*Marasini and Joshi 2012*)*. Ethanolic extract

Table 4 Testing of antioxidant activity of some medicinal orchids

Sl No.	Species	Antioxidant activity	References
1	*Cymbidium kanran*	DPPH and ABTS assays	Axiotis et al. (2022)
2	*Dactylorhiza hatagirea*	DPPH and ABTS assays. Further, UPLC-DAD analysis	Kumari et al. (2022)
3	*Dendrobium aphyllum*	DPPH and ABTS-free radical scavenging assays and the reducing power assay. MTT assay	Liu et al. (2017)
4	*Dendrobium candidum*	DPPH scavenging activity, 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity, reducing power, and ferrous ion (Fe^{2+}) chelating activity	Wang et al. (2016), Ng et al. (2012)
5	*Dendrobium chrysanthum*	DPPH radical scavenging activity	Xiao-Ling et al. (2014)
6	*Dendrobium chrysotoxum*	Deoxyribose assay, non-site-specific scavenging assay) or antioxidants and iron ions (referred as a site-specific scavenging assay)	Zhao et al. (2007)
7	*Dendrobium crepidatum*	DPPH (2, 2-diphenyl-1-picrylhydrazyl) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays	Paudel et al. (2019)
8	*Dendrobium crumenatum*	1-1 Diphenyl-2-picrylhydrazyl (DPPH) method, measurement of total phenol, flavonoid, and alkaloid content using Folin-Ciocalteu method, spectrophotometry method, and modified acid-alkalimeter method	Topriyani (2013)
9	*Dendrobium draconis*	DPPH-free radical assay	Sritularak et al. (2011)
10	*Dendrobium densiflorum*	DPPH and MTT assays	Pant et al. (2022)
11	*Dendrobium devonianum*	DPPH Radical-Scavenging Assay, Ferric Reducing Antioxidant Power (FRAP) Assay	Wang et al. (2018)
12	*Dendrobium fimbriatum*	DPPH and FRAP assay	Paul and Kumaria (2020)
13	*Dendrobium macrostachyum*	DPPH, ABTS radical scavenging, and reducing power activity	Sukumaran and Yadav (2016)
14	*Dendrobium moschatum*	DPPH assay and ABTS assay	Robustelli della Cuna et al. (2018)
15	*Dendrobium nobile*	Free radical scavenging activity assay; ABTS assay; DPPH assay; hydroxyl radicals scavenging assay	Luo et al. (2010)
16	*Dendrobium parishii*	DPPH, ORAC, and deoxyribose assays	Kongkatitham et al. (2018)
17	*Gastrodia elata*	The DPPH and ABTS radical scavenging activities	Song et al. (2016)
18	*Geodorum densiflorum*	DPPH method (1,1-diphenyl-2-picrylhydrazine)	Keerthiga and Anand (2014)
19	*Gymnadenia conopsea*	Diaion HP-20 column chromatography (reverse-phase silica gel column chromatography, DPPH radical	Shang et al. (2017)

(continued)

Table 4 (continued)

Sl No.	Species	Antioxidant activity	References
20	*Malaxis acuminata*	DPPH, metal chelating, and ABTS	Bose et al. (2017)
21	*Paphiopedilum villosum*	Anti-free radical activity (DPPH), spectrophotometric methods, liquid chromatography coupled to mass spectrometry (LC-MS)	Khamchatra et al. (2016)
22	*Papilionanthe teres*	DPPH assay	Mazumder et al. (2010)
23	*Pholidota articulata*	DPPH radical scavenging	Singh et al. (2016a,b)

of *Pholidota imbricata* showed effectiveness against *S. aureus, V. cholerae, B. subtilis, E. coli,* and *K. pneumonia* microbial strains with inhibition zone ranges from 8 to 14 mm (Marasini and Joshi 2012). *Rhyzopus stolonifer, Candida albicans,* and *Mucor* sp. were tested with the different orchid species. No activity against fungal organisms reported in *Coelogyne stricta (*leaf), *Coelogyne stricta* (Pseudobulb), and *Dendrobium amoenum.* Whereas *Pholidota imbricata* and *P. articulata* extracts showed fine activity. *Dendrobium nobile, Eria spicata, Rynchostylis retusa, Bulbophyllum affine*, and *Vanda cristata* showed very weak to moderate activity against selected fungal pathogens (Marasini and Joshi 2012).

Cytotoxic Activity

The cytotoxic activity of crude extracts from *Dendrobium longiflorum* plants was determined by the Mean Transit Time (MTT) assay (Mosmann 1983; Sargent and Taylor 1989). This study tested tumor cells of the human brain (U251) and cervical cancer cells (HeLa). The cytotoxicity results of *D. longicornu* acetonic extract showed a significant cell growth inhibitory effect on the U251 cell line which may be due to high levels of flavonoids, while ethanolic extract had no significant cytotoxic activity on U251 cells. Similarly, the higher flavonoid levels in the ethanolic extract of *D. longicornu* showed significant results on the cytotoxic activity of the HeLa cell line. The cytotoxic activity of flavonoids has been described by previous researchers (Patel and Patel 2011; Awah et al. 2012; Jeune et al. 2005).

Methanolic extract of the whole plant of *Pleione maculata* was tested for cell cytotoxicity and found to be within permissible limit, i.e., 7% at MIC assay. This supports scientific evidence in favor of folk medicinal utilization of *Pleione maculata* for various ailment treatments (Bhatnagar and Ghosal 2018). However, no cytotoxic effect was observed at an extract dosage of 50–100 μg/mL in the methanolic extract of *Pholidota articulata*, whereas 200–400 μg/mL of the extract showed better activity in HeLa cells (IC50 673.04) compared to U251 cells (IC50 3170.55). The control showed a better cytotoxic effect (Joshi et al. 2020). Similarly in *Papilionanthe uniflora* no cytotoxic effect was observed at a methanolic extract

dosage of 50–100 μg/mL, whereas 200–400 μg/mL of the extract showed better activity in HeLa cells (IC50 781.85) compared to U251 cells (IC50 2585.88) and control showed better cytotoxic effect (Joshi et al. 2020).

The cytotoxic activity of *Dendrobium crepidatum* was determined against HeLa (Human Cervical Cancer) and U251 (Human Glioblastoma) cell lines. The extract contains bioactive compounds like tetracosane, tetradecanoic acid, triacontane, phenol derivatives, and hexadecanoic acid which cause cytotoxic activity. The percentage of growth inhibition of HeLa cells for extraction of hexane (DCH) at 100 g/mL and chloroform extract (DCC) at 800 g/mL was found to vary from 19.84 to 4.31% and 81.49–0.43%, respectively. Whereas higher growth inhibition percentage was recorded in DCC at 800 μg/mL and in the extraction of acetone (DCA) at 400 μg/mL (74.35–0.59%) of HeLa cell, which was significantly different compared to other extracts. Likewise, ethanol extracts (DCE) at 100 μg/mL and methanol extracts (DCM) at 200 μg/mL showed significantly higher growth inhibition percentages of HeLa cells (Paudel et al. 2019).

8 Economics

Orchids are popular due to their attractive and long-lasting flowers, with unique shapes and forms. This is a flowering plant consisting of diverse genera and species. Nowadays using the micropropagation technique it has become easy to multiply some of the rare medicinal orchids. Flowers have bagged a significant position in present-day contemporary society. Therefore, a potential pressure for flowers was created especially in terms of the orchid flower as they have a plethora of flower forms and colors. As Orchid reproduction is in a very germinal stage in India, different medicinal orchid varieties can be reproduced by adopting a well-planned Orchid augmentation strategy for the cut-flower trade.

Orchids were the first horticultural crop mass multiplied successfully through the micropropagation technique and the commercial aspects of this group were being increasing day by day for their medicinal importance. Commercial Tissue Culture laboratories around the globe have aided the orchid's mass multiplication and helped the orchid industry revolutionize in the form of cut flower business in several countries. The Indian flower market is expected to grow to INR 661 billion by 2026. North East India, along with Sikkim, Arunachal Pradesh, and Himachal Pradesh, is the orchid-rich state in the country. In southern India, Kerala and Tamil Nadu have high humidity, low temperatures, abundant rainfall, and a pleasant climate suitable for commercial orchid cultivation. The orchid industry in India is in its infancy in terms of in vitro micropropagation or commercial cultivation. This is due to inappropriate or unsuitable planting material for large-scale cultivation, a deficit of technology for commercial mass propagation techniques, a lack of post-harvest commercial techniques for the cut-flower market for international trade, export policies, inappropriate commercial planting methods, etc. However, in India, it can be possible to grow commercially viable orchid varieties such as

Cattleya, Cymbidium, Dendrobium, Oncidiums, Phalaenopsis, Paphiopedilum, and *Vandaceous* for cut flower production. Presently, the inward demand for orchid cut-flower is mainly refilled through imports from outside India. However, with the installation of in vitro propagation technology, cost-effective greenhouses, and post-harvest and storage technology, the orchid cut-flower industry can commence in other parts of India also.

According to the National Horticultural Database released by the National Horticultural Administration, in 2020–2021, the flower planting area in India is 322,000 hectares, producing 2152 thousand tones of scattered flowers and 828,000 tones of cut flowers. Growing orchids is more than just a pleasure these days. This is an international trade that accounts for about 8% of the world's horticultural business and has the potential to change a country's economic outlook.

According to Biotech Consortium India Limited (Biotechnology Division) and Agri-Business of Small Farmers' Consortium, Indian Tissue Culture Market Research, 2005, *Dendrobium* sp. as cut flowers and *Vanilla* as spice are the most important plants in India which are suitable for micropropagation. Growing orchids in India, different agro-climatic regions, low labor costs, and accelerating high-end customer markets create a successful impact on society (Singh et al. 2008). But, the orchid cut flower business is consistently retarded by the unruly condition in airports; large numbers of infected and deserted cut flowers; moreover chemically processed flowers are rejected in Indian cities for violation of bio-safety norms (De 2008).

Presently, worth millions of dollars industry of orchid cut flower are flourishing in different countries such as Malaysia, Australia, Thailand, and Singapore among the top ten cut flowers of the world, the cut flower grasps sixth position and 3% *Cymbidium* orchid alone contributes in this list (De and Debnath 2011).

9 Conclusion

Biotechnological interventions and plant tissue culture techniques are accelerating the large-scale reproduction of the delicate and rare medicinal orchid for its potential uses as therapeutics. Since orchids are exotic breeders, they propagate by seed to produce hybrid plants. Therefore, protocols that allow regeneration from different vegetative parts of the plant are needed to achieve suitable types of micropropagation of medicinal orchids, which have shown amazing developments in germplasm conservation in recent years. Hardening and acclimatization of in vitro-propagated orchids have maintained in different ratios of the organic medium before ex vitro survivability. In recent years, as a research tool addition to being used, plant tissue culture techniques have also been of great industrial significance in the plant propagation field, plant improvement, and secondary metabolites production.

Furthermore, testing of clonal fidelity of micropropagated medicinal orchids by using markers like RAPD, ISSR, and SCOT can be adequately utilized in the sustainable implementation of plant genetic resources by identifying and eliminating

the difficulties of somaclonal variations. However, from various parts of the in vitro-raised medicinal orchid many compounds have been isolated which are a good source of bioactive molecules as well as phytochemicals. Antioxidant activities and ethnomedicinal properties have been offering better possibilities for the occurrence of value-added products, for the treatment of diseases with herbal medicines to boost health benefits.

Similar to micropropagation technology, synthetic seed technology has attracted much attention in recent years due to its broader application of germplasm conservation in natural habitats. Although little progress has been made in proving the feasibility of synseeds, their successful implementation in the conservation of orchid ornamental/medicinal genetic resources is achievable.

Emphasis on eco-rehabilitation study provides a new gateway for ex situ conservation of in vitro-raised medicinal orchids in their natural habitats. The host tree and orchid species symbiosis still maintains a proper balance for further reintroduction and population enhancement for the practical conservation of important orchids. Orchids have both flower value and medicinal value and are more demanding in the international market. Endemic and rare orchids have a plethora of flower shapes and colors that require scientific attention for their use in the cut flower industry.

Comprehensive research is still necessary to extensively study the different orchid species for various ailments. However, due to limited understanding and knowledge about the therapeutic values of these locally available plants, the use of orchids in the traditional healing process is restricted. For commercial scale, very less effort has been made for medicinal orchid cultivation due to its small size population and restriction in distribution. Different precious orchid species that have reached either the threatened or extinct category can survive with biotechnological interventions and human support for their mass propagation. Therefore, to meet the current need for medicinal orchids and to reduce the pressure on its natural population, plant tissue culture can be an acceptable alternative for its sustainable utilization which is the need of the hour.

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Gene Expression Profiling in Orchid Mycorrhizae to Decipher the Molecular Mechanisms of Plant–Fungus Interactions

Silvia De Rose, Silvia Perotto, Raffaella Balestrini, and Fabiano Sillo

1 Introduction

The Orchidaceae family comprises over 27,000 plant species (WFO 2022 http://www.worldfloraonline.org/) adapted to live in diverse environments, ranging from soil (terrestrial orchids), rock surfaces (lithophytic orchids) and on other plant species (epiphytic orchids) (Zhao et al. 2013).

Like 90% of plant species (Bonfante and Genre 2010), orchids form symbiotic associations with mycorrhizal fungi. From an ecological point of view, the study of orchid mycorrhizal (OM) symbiosis is pivotal since many orchid species are rare or at risk of extinction due to habitat destruction and over-harvesting. The presence of compatible OM fungi, necessary for seed development and plant growth, is therefore extremely crucial for the survival of plants in nature, as well as for ex situ horticultural growth. Furthermore, symbiotic germination from seeds may favor genetic variability compared to monocultures created by asexual propagation (Fig. 1) (Dearnaley et al. 2016).

In most mycorrhizal symbioses, the fungus provides inorganic nutrients in exchange for fixed carbon (C) from the photosynthetic host plant, which achieves several beneficial effects from this association (Genre et al. 2020). However, the OM symbiosis appears to be an unusual association because the usual mechanism of mycorrhizal nutrient exchange is reversed, at least during early development (Dearnaley and Cameron 2016); under natural conditions, orchids are entirely dependent on their associated symbiotic fungi for the supply of carbon and other

S. De Rose · S. Perotto
Dipartimento di Scienze della Vita e Biologia dei Sistemi, Università di Torino, Torino, Italy

R. Balestrini (✉) · F. Sillo
Consiglio Nazionale delle Ricerche-Istituto per la Protezione Sostenibile delle Piante, Torino, Italy
e-mail: raffaella.balestrini@ipsp.cnr.it

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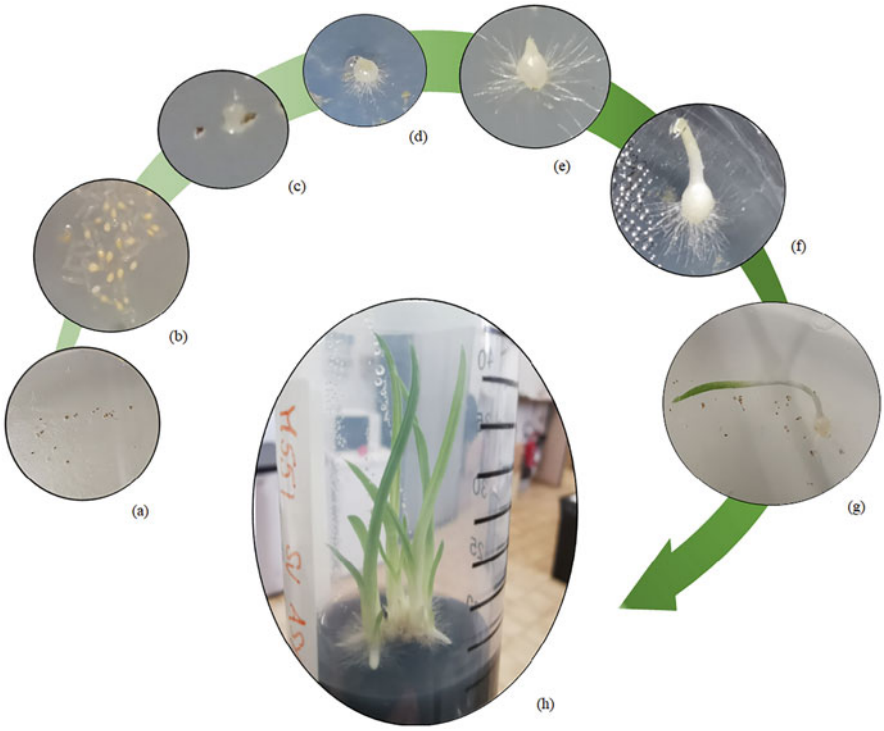

Fig. 1 In vitro development stages from seeds into the adult stage of *Serapias vomeracea*. (**a**, **b**) seeds seen under a stereomicroscope; (**c**) swollen seed; (**d**, **e**) protocorms; (**f**) protocorm with pre-leaf; (**g**) seedling; (**h**) adult plant

nutrients, particularly during seed germination and early plant development, apparently without getting anything in return (Selosse and Roy 2009). The plant dependency on the associated fungus is maintained throughout the orchid life cycle in achlorophyllous species. This peculiar trophic strategy, whereby orchids obtain carbon from their mycorrhizal fungi, instead of supplying carbon to their partner, is called mycoheterotrophy (Dearnaley et al. 2016).

The advent of *-omics* approaches, particularly transcriptomics, has contributed to elucidating the molecular mechanisms of this intriguing symbiosis and to providing insights into the nutrient exchanges between orchids and their associated fungi, thus helping to dissect this paradigm. By reflecting the gene expression changes during the development of organisms, as well as under the effects of biotic and abiotic factors, the transcriptome allows filling the gap between the genome and its phenotype at a particular time (Stark et al. 2019). Next-generation sequencing (NGS) approaches have now become widely available, thus providing the opportunity to explore differential gene expression at great resolution by the sequencing of whole transcriptomes, i.e., RNA-sequencing (RNA-seq; Marconi et al. 2014). RNA-seq approaches make it possible to investigate the extreme complexity of cellular life in

the round, redefining the fields of investigation that can be further explored by integrating with other *-omics* techniques (Lowe et al. 2017) or with target tools such as the use of laser microdissection (LM) technology (Balestrini et al. 2009).

In the next sections, the contributions that transcriptomics has made to the broad understanding of the mechanisms that drive this intriguing type of symbiosis, with reference to nutrient exchange, fungal genes and plant responses involved in the establishment of this association, are introduced and discussed.

2 Transcriptomics to Understand Nutrient Exchanges between Orchids and their Symbionts

As mentioned above, OM fungi play a key role during orchid seed germination and plant development during the early stages. Orchid seeds are often small (0.3–14 μg) and the embryos have limited nutrient reserves, which mainly consist of protein and lipids (Arditti and Ghani 2013; Zhao et al. 2013; Dearnaley et al. 2016). Germination of these tiny seeds requires the interaction with a compatible OM fungal species/isolate, which forms elaborate intracellular hyphal coils (Smith and Read 2008), called "*pelotons,*" responsible for nutrient exchanges between symbionts. The subsequent orchid developmental stages include the formation of tuber-like heterotrophic structures lacking chlorophyll, defined as protocorms (Smith and Read 2008). In the adult stage, orchids can develop different trophic strategies: most species become autotrophic, with green leaves and photosynthetic capacity (Dearnaley 2007), but around 100 species are reported as achlorophyllous and therefore completely dependent on the fungal symbiont for organic carbon (C), i.e., mycoheterotrophic (Selosse and Roy 2009; Hynson et al. 2013). Mixotrophy is an interesting evolutionary intermediate between the first two strategies, where the plant takes advantage of the fungal supply of organic C while retaining photosynthetic capacity (Julou et al. 2005; Gebauer et al. 2016). Mixotrophy allows strong adaptation under shady environments or in situations of reduced photosynthetic capacity (Girlanda et al. 2006; Lallemand et al. 2019).

Early studies on nutrient exchange based on experiments with isotope tracing showed that the OM fungus is responsible for the supply of C, phosphorus (P), and nitrogen (N) to protocorms and that the supply of P and N continues in seedlings and adulthood plants (Cameron et al. 2006, 2007, 2008). By performing a high-resolution secondary ion mass spectrometry, nutrient transfer through peloton lysis in the obligate mycoheterotrophic orchid *Rhizanthella gardneri* has been observed (Bougoure et al. 2014). In mycorrhizal protocorms of *Spiranthes sinensis*, the use of imaging of stable isotope tracers at the cellular level also demonstrated that C and N are translocated from the mycorrhizal fungus to the orchid cell either through intact pelotons or through the release of hyphal cytoplasm during peloton degradation (Kuga et al. 2014).

In the last years, several nutrient transporter genes have been detected and characterized in OM by transcriptomics (Perotto et al. 2014; Zhao et al. 2014; Fochi et al. 2017a, b), thus supporting the hypothesis that an active nutrient exchange takes place at the plant–fungal interface. Investigations have been mainly focused on symbiotic protocorms obtained in vitro, but recent work also considered the symbiosis in the roots of adult plants (Valadares et al. 2020, 2021). Before the advent of RNA-seq, methods relying on PCR-based amplification of cDNA fragments that differ from the control, like suppression subtractive hybridization (SSH), led to the identification of various genes involved in transport processes in the orchid *Dendrobium officinale* colonized by a *Sebacina* sp. fungus, including a cation transporter of the plant and an inorganic phosphate transporter of the fungus (Zhao et al. 2013).

By using RNA-seq technology, the transcriptomic responses of *Cymbidium hybridum* plantlets co-cultivated with two different beneficial fungi, one of them non-mycorrhizal, were investigated (Zhao et al. 2014). Among the different genes involved in nutrient transport, two plant phosphate transporters, co-regulated during interactions with both fungal species, were identified (Zhao et al. 2014). Two genes coding for phosphate transporters expressed in mycorrhizal roots of the adult green orchid *Oececlades maculata* collected in natural conditions were also recently identified (Valadares et al. 2020). These results provide evidence that the acquisition of inorganic phosphorus in adult plants is mediated by the associated fungus, as suggested for the terrestrial orchid *Goodyera repens* based on isotope studies (Cameron et al. 2007). In soils with limited P availability, naturally occurring orchids have been found to acquire significant amounts of inorganic P from the symbiotic fungal partners; plant–fungus combinations, which may be more or less efficient, strongly influence P acquisition, with plant-mediated niche differentiation (Davis et al. 2022).

Bidirectional transfer of C between a green adult orchid and its fungal symbiont has been demonstrated by isotope tracing experiments, thus allowing a more complete view of C fluxes in OM symbiosis (Cameron et al. 2006). Interestingly, an up-regulated putative bidirectional sugar plant transporter belonging to the SWEET family has been identified by high-throughput transcriptomics of a normalized cDNA library by 454 GS-FLX Titanium pyrosequencing in *Serapias vomeracea* protocorms colonized in vitro by the fungus *Tulasnella calospora* (Perotto et al. 2014). Pathogenic microorganisms and beneficial symbionts are both known to target plant SWEET transporters for nutritional gain (Chen et al. 2010). SWEET transporters were also identified in mycorrhizal roots of albino variants of *Epipactis helleborine*, a mixotrophic orchid (Suetsugu et al. 2017); in symbiotic protocorms of *Bletilla striata* (Miura et al. 2018) and mycorrhizal roots of adult orchids *O. maculata* and *Limodorum abortivum* (Valadares et al. 2020, 2021) both collected in nature. Intriguingly, a sucrose transporter that may allow sucrose import at the symbiotic interface in mycoheterotrophic *Gastrodia elata* associated with the fungus *Armillaria* has been found (Ho et al. 2021). Moreover, invertase, an enzyme cleaving sucrose into glucose and fructose, has been identified, through proteomic

approaches, in mycorrhizal protocorms of the orchid *Oncidium sphacelatum* (Valadares et al. 2014).

Most orchid tissues are highly N-enriched (Hynson et al. 2013,) and the fungus has been demonstrated to provide N to protocorms and adult green orchids by exploiting inorganic and organic N sources in the substrate (Kuga et al. 2014; Cameron et al. 2006). The exchange of N in OM has been clarified thanks to transcriptomics approaches. RNA-seq analysis of the plant and fungal N uptake pathways in the model system *S. vomeracea-T. calospora* identified several up-regulated plant and fungal genes associated with N metabolism (Fochi et al. 2017a). To understand the preferential N form taken up by the fungus and transferred to the orchid protocorm, the fungal mycelium was grown on two different N sources. Based on transcriptomic and genomic data, it has been hypothesized that the fungus can obtain N from organic and inorganic sources, excluding nitrate, and two ammonium fungal transporters were identified, one of which was up-regulated in symbiosis. Plant transporters for N compounds resulted to be up-regulated in symbiosis, such as ammonium and oligopeptide transporters, as well as amino acid transporters, including a plant lysine histidine transporter (LHT1). A homologous LHT1 gene was previously reported in mycorrhizal roots of *Cymbidium hybridum* (Zhao et al. 2014) and recently detected in symbiotic protocorms of *B. striata* (Miura et al. 2018) and symbiotic roots of adult orchid plants (Valadares et al. 2021). This repertoire of fungal and plant genes was further investigated through the use of laser microdissection (Balestrini et al. 2018; Fochi et al. 2017b). By combining a microscope and a computer-assisted laser beam to separate various cellular components from sections placed under a microscope slide, laser microdissection enables the quick separation of specific cells from a piece of heterogeneous tissue making it possible to extract a variety of cellular compounds, including RNA (Balestrini et al. 2009, Balestrini and Fiorilli 2020). The analysis of gene expression in RNA samples originating from orchid cells harboring fungal coils at diverse developmental stages, as well as cells non-colonized by the fungus, demonstrated that plant genes coding for transporters of N compounds are differentially expressed in symbiosis (Fochi et al. 2017b). Based on these findings, it has been hypothesized that N-rich amino acids may be transferred from the fungus to the host plant, also contributing to the C requirement. Moreover, in addition to active transport, recovery of organic N forms from peloton lysis may occur (Kuga et al. 2014). Recent transcriptomic and proteomic studies focused on orchid species collected in nature support this hypothesis (Valadares et al. 2021). For example, in the mycorrhizal roots of the orchid *L. abortivum*, genes encoding a lysine histidine transporter 1 (LHT1) and an amino acid permease, as well as several NRT1/PTR family members putatively associated with N transfer from the fungus to the host plant, were found to be up-regulated (Valadares et al. 2021). Similarly, it has been discovered that the mycorrhizal roots of the terrestrial orchid *O. maculata* regulates genes for LHT1 and NRT1/PTR family members, which are amino acid transporters (Valadares et al. 2021). Interestingly, members of this protein group have also been discovered to transport peptides, but also chloride, nitrite, glucosinolates, and several phytohormones such as auxin, abscisic acid, gibberellins, and jasmonate

(Corratgé-Faillie and Lacombe 2017). However, their roles in mycorrhizal symbiosis are still under debate (Corratgé-Faillie and Lacombe 2017).

A recent metabolomic study using the model system *S. vomeracea—T. calospora* integrated previous transcriptomic data (Fochi et al. 2017a, b) and showed that the external mycelium of the mycorrhizal fungus freely growing close to the host protocorms affected several metabolic pathways. The interaction between plant and fungus increased compounds associated with structural, signaling, and energy, mostly lipids, particularly glycerolipids (GP) and sphingolipids (SP) (Ghirardo et al. 2020). Lipids are known to be the main structural components of cell membranes but also provide other important biological functions, ranging from signaling, C storage, plant–microbe interactions, and even response to environmental stresses (Ghirardo et al. 2020). Notably, a percentage decrement of N- and S-containing compounds in the mycorrhizal fungus growing close to the host protocorms, led the authors to hypothesize that this depletion may mirror a transfer of N compounds to the host plant. However, among the identified S-containing compounds, the amount of S-adenosylmethionine (SAM) increased in the mycelium surrounding protocorms (Ghirardo et al. 2020). This molecule is used by methyltransferases as a methyl group donor for a variety of target substrates (Mato et al. 1997; Ghirardo et al. 2020). Currently, scarce information on S transfer from fungi to the host plant in OM is available. This nutrient is essential for plant growth and development, as a constituent of amino acids such as cysteine and methionine and sulfated peptides (i.e., glutathione or phytosulfokines) (Kopriva et al. 2019). Very recently, experiments with labeled S, N, and C showed that these elements could be translocated from the substrate to the protocorm cells via the fungal hyphae (De Rose et al., submitted), corroborated by target transcriptomic data which showed up-regulation of several plants and fungal transporter genes, as well as genes related to S assimilation enzymes involved in movement and redistribution of S in the cell. Overall, these findings support the hypothesis of transfer during symbiosis of S in a reduced organic form that also contains N, such as S-amino acids or small peptides, including glutathione (De Rose et al., submitted).

Based on the reports so far available, the model originally proposed for nutrient transport in OM (Dearnaley and Cameron 2016) can be strengthened and integrated with transcriptomic data. In non-photosynthetic stages, orchids may receive inorganic P, C, N, and S from the symbiotic fungal partner in the form of amino acids, and export ammonium in exchange (Cameron et al. 2006, 2007; Kuga et al. 2014; Fochi et al. 2017a, b). This exchange takes place across intact membranes (Kuga et al. 2014; Fochi et al. 2017b), but the lysis of senescent fungal pelotons may also release C, N, and P (Bougoure et al. 2014). Significant metabolic (particularly lipid-related) changes in the mycelium outside the plant could also participate in the nutrient supply to the host plant (Ghirardo et al. 2020). In photosynthetic orchids, C as sugars could be exported from the plant to the fungus, while inorganic P continues to be received by the mycorrhizal partner (Cameron et al. 2006; Valadares et al. 2020, 2021) in amounts that can vary, even greater, depending on plant-fungus compatibility (Davis et al. 2022). The flow of nutrients in the orchid mycorrhizal symbiosis may be even more complex than presented when considering the hyphal

interconnections that may exist in natural ecosystems, well investigated for mycoheterotrophic orchids, and the fungal diversity in orchid roots (extensively reviewed by Yeh et al. 2019). An example is the flow of C between the tree species *Salix repens* and *Betula pendula* with the orchid *Corallorhiza trifida* by hyphal network shared by the plants (McKendrick et al. 2000), or the coexistence of different endosymbionts in *Cymbidium hybridum* (Zhao et al. 2014).

3 Transcriptomics to Decipher the Mechanisms Involved in the Establishment of Symbiosis

Fungi that form mycorrhizal associations with orchids exhibit great phylogenetical and ecological diversity (McCormick et al. 2018). Orchids are known to associate with a plethora of fungi, including ectomycorrhizal basidiomycetes and ascomycetes wood degraders and other saprotrophic basidiomycetes (Bidartondo et al. 2004; Selosse et al. 2004; Dearnaley 2007; Ogura-Tsujita and Yukawa 2008; Martos et al. 2009; Kottke et al. 2010; Lee et al. 2015; Kinoshita et al. 2016). The most common taxa in photosynthetic orchid species include *Tulasnella*, *Ceratobasidium*, and *Serendipita* (Dearnaley et al. 2012).

The diversity of OM fungi in the soil may be a crucial element in determining the distribution and future of orchids (McCormick et al. 2018) because orchids in nature are entirely dependent on OM fungi at least for seed germination and early stages of development. After initial contact and seed germination**,** protocorms are colonized by OM fungi; the hyphae penetrate parenchyma cells, branch and fold to create thick hyphal coils (the pelotons) that at last degrade (Miura et al. 2018). The symbiotic germination of orchid seeds requires the coordinated expression of numerous functional genes as well as a crosstalk between genes associated with the mycorrhizal establishment and the germination process (Liu et al. 2015; Chen et al. 2020). According to Evangelisti et al. (2014), plant hormones may act as a node in the crosstalk between plant development and plant–microbe interactions. Strigolactones (SLs), for instance, are signaling molecules produced by plant roots either constitutively (Akiyama et al. 2005) or in response to low phosphorus levels (Kretzschmar et al. 2012) capable of recruiting arbuscular mycorrhizal (AM) fungi and promoting hyphal branching. In orchids, a carotenoid cleavage dioxygenase, involved in the strigolactones biosynthetic pathway, has been identified in a proteomic study in *O. sphacelatum* mycorrhizal protocorms by Valadares et al. (2014). Since the enzyme was more expressed in earlier stages of mycorrhizal protocorm development, it has been hypothesized that SLs may have essential early functions in luring compatible fungal symbionts to aid orchid seeds germination (Valadares et al. 2014).

The crosstalk of jasmonic acid (JA), abscisic acid (ABA), and SLs were investigated in *D. officinale* seeds colonized by *Tulasnella sp.* during the germination phase (Wang et al. 2018). The transcriptomic and RT-qPCR data, combined with the quantification of endogenous phytohormones, suggested that the OM fungus had a

role in hormone production (Wang et al. 2018). Additionally, endogenous JA, ABA, or SLs levels were maintained low to promote the formation of the *D. officinale-Tulasnella* protocorm-like structures (Wang et al. 2018). However, the great phylogenetic and ecological diversity of OM fungi might suggest that other signals are involved. A comparative analysis of gene expression in asymbiotic and symbiotic *Anoectochilus roxburghii* seeds through Illumina HiSeq 4000 transcriptome sequencing allowed focus on the regulatory module GA-GID1-DELLA (Liu et al. 2015). Gibberellins (GAs) are plant hormones that play key roles in growth and development (Wang and Deng 2014). The GID1 receptor and the DELLA repressor were found to be critical in the regulation of seed germination (Wang and Deng 2014). ABA, another important phytohormone, was found instead to inhibit the process through a finely tuned crosstalk (Liu et al. 2015). Among the differentially expressed plant transcripts identified in symbiotic and asymbiotic seeds, two transcripts coding for gibberellin 20 oxidase (GA20ox), two transcripts coding for gibberellin 2-oxidase (GA2ox) involved in GAs biosynthesis, and two transcripts coding for DELLA proteins, members of GRAS superfamily (Hernández-García et al. 2021), were established as common elements of the mycorrhizal signaling pathway (Jin et al. 2016). This study suggested that OM fungi could modulate the expression of these plant genes, possibly affecting the entire GA-GID1-DELLA regulatory module.

Investigations focused on the impact of GAs on symbiotic seed germination in the model system represented by *D. officinale* and *Tulasnella sp*. were also performed (Chen et al. 2020). Levels of endogenous gibberellic acid (GA3) using liquid chromatography-mass spectrometry (LC-MS/MS) were determined during symbiotic and asymbiotic germination of orchid seeds, and a significantly higher ratio between GA3 and ABA was found in symbiosis (Chen et al. 2020). Phenotypic and target gene expression investigations were conducted on the germination of seeds treated with various concentrations of exogenous GA3, showing a negative effect of high concentrations of GA3 on fungal colonization. These findings were combined with data obtained from RNA-seq and proteomic analyses that highlighted a significantly higher expression of an ABA receptor protein, PYR1, during the early stages of symbiotic germination in *D. officinale* (Chen et al. 2017). The expression profile of genes involved in GAs and ABA biosynthesis, identified in the transcriptomic data, and of genes reported to be part of the recognized common symbiotic pathway (including a calcium-binding protein and a calcium-dependent protein kinase), showed a fine-tuned regulation under the different GA treatments (Chen et al. 2020). These results suggest that an interplay between GAs metabolism and the establishment of symbiosis may occur in orchids (Chen et al. 2017, 2020).

A group of common symbiosis genes (CSG) have been identified in angiosperms forming arbuscular mycorrhizal (AM) symbiosis, reported to act in the signaling pathway for recognizing and transducing microbial signals that are diffuse throughout root colonization and nutrient exchange (Stougaard 2001; Kistner et al. 2005; Genre and Russo 2016). In contrast, angiosperms unable to form mycorrhizal associations with AM fungi, such as members of Brassicaceae, showed no functional or only a few members of CSG (Delaux et al. 2014). Members of the Pinaceae in the

gymnosperms, forming ectomycorrhiza, were also lacking CSG in their genomes (Garcia et al. 2015).

To explore the possible presence of CSG in orchids, protocorms of *B. striata* colonized by the mycorrhizal fungal taxa *Tulasnella sp.* were analyzed at different stages by transcriptomics (Miura et al. 2018). Notably, all of the CSG characterized in other plant species were found in the *B. striata* transcriptome (Miura et al. 2018). In vivo assay by functional complementation of L. *japonicus* CCaMK mutant through *B. striata CCaMK* gene showed that this gene complemented the function of LjCCaMK (Miura et al. 2018). Moreover, eight genes were strongly induced during symbiosis (Miura et al. 2018). The high similarity of these genes to the AM marker genes in rice (Gutjahr et al. 2008), as well as their strong induction during the plant–fungus interaction, allow considering this set of eight genes as marker genes also for OM (Miura et al. 2018).

A large-scale analysis of more than 250 transcriptomes and about 100 plant genomes, encompassing the whole land-plant diversity, demonstrated that a shared symbiosis signaling pathway occurred in all plants forming intracellular endosymbioses (Radhakrishnan et al. 2020). It is worth noting that co-evolution between plants and fungi began approximately 400 million years ago and that four mycorrhizal types evolved at different times: arbuscular mycorrhiza, orchid mycorrhiza, ericoid mycorrhiza, and ectomycorrhiza (Genre et al. 2020). The first three types are endosymbioses, i.e., fungal symbionts are harbored intracellularly inside plant cells. In particular, AM symbiosis is probably the most ancient plant–fungus symbiosis (Delaux et al. 2013) from which OM symbiosis appeared to be derived after plant diversification (Radhakrishnan et al. 2020). Comparative transcriptomic studies allowed us to identify six genes lost in non-mutualistic plant taxa: CcaMK, calcium- and calmodulin-dependent protein kinase, SymRK, a receptor-like kinase, CYCLOPS and RAD1, two transcription factors, and two transporters STR and STR2 showing a half-ATP-binding cassette (ABC). In particular, the first three genes were conserved in all plants forming intracellular endosymbiosis, while *STR, STR2,* and *RAD1* were supposed to be specific to AM (Radhakrishnan et al. 2020).

Starting from the earliest observations (Burgeff 1932; Burges 1939), OM has been often argued to represent a balanced antagonism between plant and fungus, since it has been documented that fungi occasionally might destroy the protocorm (Adamo et al. 2020) and because plant receives C from the fungus lacking a clear reward. However, large-scale transcriptomic data integrated with target gene expression analysis with RT-qPCR allowed to demonstrate the absence of a defense activation in *S. vomeracea* mycorrhizal protocorms (Perotto et al. 2014), as previously observed (Zhao et al. 2013). The OM symbiosis was suggested as "a friendly plant–fungus relationship" and the lack of a strong defense response was successively confirmed by other transcriptomic studies in other orchid species (Suetsugu et al. 2017; Miura et al. 2018). Using 454 pyrosequencing, Perotto et al. (2014) proposed a nodulin-like gene called *SVNod1* as a marker of OM symbiosis (Perotto et al. 2014). In this transcriptomic study, a set of plant and fungal genes expressed in *S. vomeracea* protocorms colonized by OM fungus *T. calospor*a was identified

(Perotto et al. 2014). In-depth gene expression analysis using an extremely efficient target approach, laser microdissection, tested the expression of several genes identified in the work by Perotto et al. (2014), confirming the results and suggesting *SvNod1* as a marker gene of orchid symbiosis, since its transcript was detected in the fungal colonized cells only (Perotto et al. 2014; Balestrini and Bonfante 2014).

An important process for endosymbiosis is clathrin-mediated endocytosis (Leborgne-Castel et al. 2010). The plant plasma membrane plays a pivotal role in the management of microbial interactions, as it senses and possibly allows the entry of endocellular symbionts or microbial substances, and endocytosis can regulate the entry of extracellular particles or cargoes into the cell (Zeng et al. 2017). The molecular components of this process have been investigated by transcriptomics in the peculiar OM system represented by the orchid *Gastrodia elata,* a fully mycoheterotrophic orchid able to establish a symbiosis with two genera of fungal partners, i.e., *Mycena* and *Armillaria*. *Mycena* species are known to interact with *Gastrodia* as symbionts during the early stages of plant development, including protocorm formation (Kim et al. 2006; Zeng et al. 2017). The transcriptomes of *G. elata* symbiotic seeds and protocorms were analyzed by RNA-seq and among the differentially expressed genes identified in the study, genes putatively linked to energy metabolism, plant defense, molecular signaling, and secondary metabolism were detected (Zeng et al. 2017). Genes coding for clathrin, an adaptor protein, dynamin, and HSC70, were found to be constitutively expressed in seeds but strongly expressed in protocorms, indicating that endocytosis mediated by clathrin may be crucial in *G. elata* during interactions with *Mycena* fungi (Zeng et al. 2017). Comparative transcriptome analysis was also used to unravel molecular mechanisms underlying gastrodin biosynthesis since this compound has been reported to have several positive effects on human health (Tsai et al. 2016). Two putative monooxygenases and one glycosyltransferase key enzymes involved in gastrodin biosynthesis were identified, which could be a target of genetic editing to improve gastrodin production (Tsai et al. 2016).

In OM symbiosis, the fungal hyphae must enter the cell walls of the epidermal cells to reach the internal parenchyma cells during the colonization of orchid seeds, protocorms, or roots (Chen et al. 2014; Favre-Godal et al. 2020). The plant cell wall is therefore the first physical structure where interactions between the host plants and the associated symbiotic partners take place (Balestrini and Bonfante 2014). Recently, Chen et al. (2022) compared transcriptomic profiles during seed germination, protocorm formation, and seedling development under symbiotic and asymbiotic conditions to further investigate changes in the expression of plant genes related to plant cell wall biosynthesis, structure modification, and the expression pattern of fungal genes related to plant/fungal cell wall degradation (i.e., CAZymes) (Chen et al. 2022). After being inoculated with two symbiotic fungi (*Tulasnella* sp. and *Serendipita* sp.), *D. officinale* seeds showed significantly increased expression of genes coding for secreted glycoproteins specifically associated with the epidermis, proline-rich receptor-like proteins, leucine-rich repeat (LRR) extensin-like proteins, and extensin-like proteins during the symbiotic stage. Extensins are a large class of hydroxyproline-rich glycoproteins that play a

variety of roles in plant defense, such as reinforcing the cell wall to preclude invasion by pathogens or to facilitate the interaction with symbiotic organisms (Chen et al. 2022). They were also probably essential for preventing fungal colonization of basal cells and spreading inside the whole protocorms (Li et al. 2018). The observed up-regulation of a microtubule-associated protein gene during the symbiotic stage suggested that cytoskeletal remodeling took place during the fungal colonization of orchid seeds (Chen et al. 2022). In addition, some genes involved in plant cell wall biosynthesis, including genes coding for cellulose synthase and pectin esterase, were significantly up-regulated in seeds of *D. officinale* inoculated with both fungal species, suggesting that the interaction between seeds and mycorrhizal fungi was particularly active in terms of modification in plant cell wall biosynthesis pathways (Chen et al. 2022). In the symbiotic fungal mycelium, several differentially expressed CAZymes were found, representing approximately 24.8% and 36.7% of the total number of CAZymes identified in *Serendipita* sp. and *Tulasnella* sp. genomes, respectively (Chen et al. 2022). These genes were hypothesized to play a role in the suppression of the plant defense responses, the identification of mycorrhizal fungi during the germination of orchid seeds, and also in the successful fungal colonization (Chen et al. 2022).

As mentioned earlier, OM fungi sometimes display a saprotrophic behavior that leads to disruption of the mycorrhizal association, and recent research by Adamo et al. (2020) focused on possible changes, during mycorrhizal symbiosis and saprotrophic growth, in the expression of fungal genes encoding CAZymes capable of breaking down the plant cell wall (PCW). They found that PCW-degrading enzymes are finely regulated in *T. calospora* during mycorrhizal and saprotrophic growth in the host and hypothesized that the expression of several important CAZymes was connected to OM fungal transitions from symbiotic to saprotrophic growth (Adamo et al. 2020).

Although most studies on OM have been focused on germinating seeds and protocorms, *omics* approaches are also revealing molecular mechanisms and actors involved in the adult stages of orchids. In comparison to protocorms, the interaction in adult plants is expected to be different because of it involves the root, an anatomically and metabolically complex plant organ (Valadares et al. 2020). Two recent studies have shed light on the mechanisms driving plant–fungus interactions in adult orchids in natural conditions (Valadares et al. 2020, 2021). The interaction between adult plants of the terrestrial orchid *O. maculata* and its mycorrhizal fungus *Psathyrella candolleana* (Basidiomycota) was analyzed using transcriptomic and proteomic analyses (Valadares et al. 2020).

In nature, the same individual of *O. maculata* can form older roots completely colonized by OM fungi as well as younger non-colonized roots (Valadares et al. 2020). For this reason, *O. maculata* is an excellent experimental system allowing the study of the molecular changes related to OM formation and functioning since it can be used to analyze both mycorrhizal and non-mycorrhizal roots from the same plant. Integration of transcriptomic and proteomic data showed that a chitinase and a mannose-binding lectin, two proteins involved in plant defense responses, decreased in mycorrhizal roots, suggesting that the proximity of the fungal symbiont might

cause a local decrease of plant defense responses in the orchid tissues (Valadares et al. 2020). Results also highlighted that allene oxide synthase transcripts, precursors involved in the biosynthesis of JA, were only found in non-mycorrhizal roots of *O. maculata,* while genes annotated as 9-lipoxygenase and allene oxide synthase were negatively regulated in mycorrhizal roots (Valadares et al. 2020). Based on these findings, the authors suggested that inhibition of JA production is probably needed to promote fungal colonization and OM formation (Valadares et al. 2020). In addition, the up-regulation of three ethylene-induced calmodulin genes and 15 ethylene-responsive transcription factors in the transcriptome of *O. maculata* mycorrhizal roots suggested that the activation of the ethylene pathways plays a role in OM (Valadares et al. 2020). Similar outcomes were also reported in *C. hybridum* mycorrhizal roots (Zhao et al. 2014).

To further explore the mycorrhizal interactions in adult orchids in natural conditions at the molecular level, a transcriptomic approach has been used to examine gene expression in roots of the mixotrophic orchid *Limodorum abortivum*, able to associate with ectomycorrhizal fungi of the taxon *Russula* (Valadares et al. 2021). This study, which focused on how the plant responds to the mycorrhizal symbiont (s) and used non-sterile non-mycorrhizal roots collected in nature as references, addressed for the first time OM interactions in an orchid species interacting with an ectomycorrhizal fungus. The comparison between non-sterile mycorrhizal and non-mycorrhizal roots made it simpler to distinguish between the general orchid responses to microbes and the mycorrhiza-specific plant responses. A shared core of plant genes engaged in endomycorrhizal symbioses already identified in arbuscular mycorrhiza was identified in *L. abortivum* and mirrored by the overexpression of several molecular marker genes for symbiosis in mycorrhizal roots. Further studies and gene characterization are needed to determine whether the unique characteristics of OM depend on the precise regulation of these elements, or if additional genes are involved in the process (Valadares et al. 2021). Among the genes differentially expressed *in planta*, pectin methyl esterase (PME) genes were detected to be significantly down-regulated in *L. abortivum*, while PME inhibitor genes were up-regulated in mycorrhizal roots, demonstrating that the main proportion of pectin is in a highly methylated state in the outer cell wall and/or symbiotic interface (Valadares et al. 2021). The high expression of two expansin coding genes in mycorrhizal roots further supports the hypothesis that a loosening of the cell wall during symbiosis may occur (Valadares et al. 2021).

The transcriptome of *L. abortivum* mycorrhizal roots also showed a significant up-regulation of subtilisin-like serine protease-coding genes. The major part of subtilisin-like serine proteases is primarily directed to the plant cell wall, in which they can play a role in the regulation of the structural remodeling of the cell wall (Schaller et al. 2018). Additionally, two genes in mycorrhizal *L. abortivum* roots coding for syntaxin-132 (SYP132) proteins were found to be up-regulated, and thus it has also been documented in mycorrhizal roots of other orchid species (Zhao et al. 2014; Valadares et al. 2020). Syntaxins have been characterized in the AM symbiosis and the SYP132α isoform has been demonstrated by knockdown mutant experiments to be needed for arbuscule formation in the model plant *Medicago*

truncatula (Huisman et al. 2020). It has been hypothesized that SYP132, which is localized on the perisymbiotic plant membrane surrounding functional arbuscules, is essential for the development of a functional plant-fungus interface (Huisman et al. 2020). Even though it is currently unknown where the SYP132 proteins are located in OM roots, it is intriguing to hypothesize that the AM and OM symbioses share a similar exocytotic route (Valadares et al. 2021).

4 Conclusion

In the last decades, thanks to advances in technology applied to transcriptomics, the understanding of how orchids interact with their symbiotic partners has been strongly improved. The application of RNA-seq in several orchid species has allowed in-depth analyses of the molecular bases of nutrient transfer between OM fungi and their orchid hosts, as well as the identification of fungal and orchid genes involved in the establishment of the symbiotic association. The current availability of annotated orchid and fungal transcriptomes will help to fill the gap between the genomic data and the phenotypic observations, also in natural conditions. From the plant side, a broad transcriptomics resource for orchid species has been developed, named Orchidstra 2.0 database (http://orchidstra2.abrc.sinica.edu.tw), including data from EST libraries and RNA-seq of 18 species from the five major subfamilies of the Orchidaceae (Chao et al. 2017). This tool has already proven to be useful for the comparison of whole transcriptomes across different orchid species. In addition, international sequencing efforts, including the "1000 Fungal Genomes" project of the U.S. Department of Energy (DOE) Joint Genome Institute (JGI) (Grigoriev et al. 2014), boosted knowledge on the fungal genomes and transcriptomes of several fungal taxa, including OM fungi. The integration of this data with outcomes of other *-omics* approaches, such as metabolomics, will improve our knowledge of the orchids and their fungal "friends," and allow a better understanding of this fascinating and complex symbiosis.

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Exploring the Potential of In Vitro Cultures as an Aid to the Production of Secondary Metabolites in Medicinal Orchids

Arshpreet Kaur, Jagdeep Verma, Vikramaditya G. Yadav, Sandip V. Pawar, and Jaspreet K. Sembi

Abbreviations

BA	6-benzyladenine
CA	Caffeic acid
CW	Coconut water
CITES	Convention on international trade in endangered species of wild fauna and flora
CHI	Chalcone isomerase
FA	Ferulic acid
KC	Knudson C
Kn	Kinetin
MeJA	Methyl jasmonate
MemTR	6, 3-methoxybenzylamino-9-b-D-ribofuranosylpurine
M	Mitra
MS	Murashige and Skoog
mTR	Meta-topolin riboside
NAA	α-Naphthaleneacetic acid

A. Kaur · J. K. Sembi (✉)
Department of Botany, Panjab University, Chandigarh, India
e-mail: jaspreet.sembi@pu.ac.in

J. Verma
Department of Botany, Sardar Patel University, Mandi, Himachal Pradesh, India

V. G. Yadav
Department of Chemical and Biological Engineering, University of British Columbia, Vancouver, BC, Canada

School of Biomedical Engineering, University of British Columbia, Vancouver, BC, Canada

S. V. Pawar
University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh, India

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pCA	*p*-Coumaric acid
PAL	Phenylalanine ammonia lyase
PLBs	Protocorm-like bodies
SA	Salicylic acid
STS	Stilbene synthase
TDZ	Thidiazuron
VW	Vacin and Went

1 Introduction

Orchids belong to one of the largest and most diverse plant families in flowering plants (Christenhusz and Byng 2016). This diversity is due to their ability to acclimatize to almost every type of habitat. Their exquisitely beautiful flowers confer these plants high ornamental and economic value in the global commercial market. Apart from the immense ornamental significance of orchids, these plants are also medicinally important and have been utilized as therapeutics as acknowledged in the traditional pharmacopeias worldwide (Hossain 2011; Sut et al. 2017). *Habenaria edgeworthii, Habenaria intermedia, Malaxis acuminata,* and *Malaxis muscifera* are components of *Astavarga*, which is a popular rejuvenating herbal formulation in Ayurveda (Dhyani et al. 2010). In the traditional Chinese medicine system, *Anoectochilus roxburghii* and *A. formosanus* have been used for preventing cancer, protecting the liver, and treating diabetes and cardiovascular diseases (Han et al. 2008; Zhang et al. 2013). Shi-Hu, an orchid-based Chinese therapeutic formulation derived from *Dendrobium nobile*, has been effectively used in treating lung, kidney, and stomach diseases (Teoh 2016). Over the time, there have been numerous reports on the wide usage of different plant parts of orchids in the treatment of a myriad of diseases and ailments. The tubers of *Bletilla striata* are used in the treatment of tuberculosis and gastric and duodenal ulcers (Ming et al. 2003). *Dendrobium candidum* extracts maintain the tonicity of the stomach and have a body fluid-promoting effect (Ng et al. 2012). Pseudobulbs of *Malaxis acuminata* are used as a curative for burning sensations, fever, and tuberculosis and as a nutritive tonic (Hossain 2011). Whole plants of *Ansellia africana* are used for their aphrodisiac properties (Chinsamy et al. 2011). Direct application of seeds of *Acampe praemorsa* on wounds serves as a substitute for antibiotics (Shanavaskhan et al. 2012). Dried powder of whole plants of *Bulbophyllum odoratissimum* is used to treat fractures, chronic inflammations, and tuberculosis (Mohanty et al. 2015). There have been wide ethnomedicinal evidence on the use of leaves of numerous species of *Dendrobium* for treating musculoskeletal and nervous system problems (Wang 2021). The roots, rhizomes, pseudobulbs, stems, flowers, and whole plant of species belonging to the genus *Calanthe* are used in curing toothaches, rheumatism, jaundice, typhoid, stomach-ache, ulcers, asthma, sore throat, etc. (Nanjala et al. 2022). Additionally, there are a plethora of reports on the traditional medicinal usage of

Bulbophyllum species in various countries such as Nepal, India, China, Japan, Bangladesh, Thailand, and Malaysia (Sharifi-Rad et al. 2022). It can therefore be concluded that orchids have an immense therapeutic potential which is indicated by the ethnobotanical reports and the presence of the wide variety of secondary metabolites (Gantait et al. 2021).

Plant secondary metabolites are a rich source of compounds having potent biological activity. These metabolites are classified into numerous categories such as alkaloids, flavonoids, anthocyanins, and terpenoids (Sut et al. 2017; Ghai et al. 2021). The biosynthesis of these metabolites is regulated by pathways such as phenylpropanoid pathway, mevalonate (MVA) pathway, and methyl-d-erythritol 4-phosphate (MEP) pathway. (Ghai et al. 2022). Some of the genes involved in these pathways like *Phenylalanine Ammonia Lyase* (*PAL*), *Chalcone synthase (CHS), Chalcone Isomerase (CHI)*, *Flavonol Synthase* (*FLS*), and *Stilbene Synthase* (*STS*) encode the key rate-limiting enzymes in specific secondary metabolites biosynthetic pathways, and hence regulate their biosynthesis (Ghai et al. 2022; Halder et al. 2019; Kaur et al. 2022).

In orchids, pharmaceutically important biomolecules such as polysaccharides, bibenzyl derivatives, phenylpropanoids, phenanthrene derivatives, alkaloids, and flavonoids are widely present (Hossain 2011; Sut et al. 2017). Numerous studies on the evaluation of the biological activity of the phytochemicals extracted from orchids have been reported (Table 1). There have been reports on disease amelioration using orchid phytochemicals. Antidiabetic properties of extracts of *Aphyllorchis montana* and *Anoectochilus roxburghii* have been reported (Thalla et al. 2013; Cui et al. 2013). Immunomodulatory effects of polysaccharides derived from orchids have been evaluated in *Bletilla striata* where the polysaccharides derived from it improved the spleen and thymus indices (Chen et al. 2020). Additionally, a Type II arabinogalactan polysaccharide extracted from *Anoectochilus formosanus* stimulated the maturation of dendritic cells to induce immune responses against pathogens, thus attributing to their immune-enhancing potential (Lai et al. 2015). The compounds extracted from orchids also exhibit antimicrobial properties. For instance, bibenzyl derivatives of *Dendrobium nobile* displayed broad-spectrum antifungal activity against several phytopathogenic fungi (Zhou et al. 2016). Retusiusines B, a phenylpropanoid compound extracted from *Bulbophyllum retusiusculum* showed effective antifungal activity against *Candida albicans* (Fang et al. 2018). The significant antioxidant potential has also been evaluated through DPPH (2, 2-Diphenyl-1-picrylhydrazyl) radical scavenging activity, for a flavonoid compound, rutin, isolated from *Dendrobium officinale* (Zhang et al. 2017). The compounds derived from orchids have also been reported to possess anticancer properties. Bulbophythrins, the phenanthrene derivatives isolated from *Bulbophyllum odoratissimum* displayed cytotoxic potential against human hepatoma, leukemia, adenocarcinoma, and stomach cancer cell lines (Xu et al. 2009). Similarly, in *Dendrobium nobile,* 'nudol', a phenanthrene derivative, inhibited the osteosarcoma cell growth (Zhang et al. 2019). High cytotoxicity against the growth of Hela human cervical cancer cell line has been observed for the bibenzyl compounds derived from *Dendrobium officinale (*Ren et al. 2020*)*. Different researches

Table 1 Compounds isolated from orchid species and their biological activity

Biological activity	Plant name	Compound	Reference
Antibacterial activity	*Bletilla ochracea*	Blestriarene A, Blestriarene B, Blestriarene C	Yang et al. (2012)
	Bletilla striata	Bletistrin F, Bletistrin G, Bletistrin J, Bulbocol, Shanciguol, and Shancigusin B	Jiang et al. (2019b)
	Bulbophyllum retusiusculum	Retusiusines B	Fang et al. (2018)
	Liparis regnieri	Erianthridin, Gigantol, Hircinol, Nudol, Coelonin, Moscatin	Ren et al. (2016)
Antidiabetic property	*Aerides multiflora*	Aerimultin C	Thant et al. (2021)
	Dendrobium crepidatum	Dendrocrepine	Xu et al. (2020)
	Dendrobium formosum	Confusarin	Inthongkaew et al. (2017)
	Dendrobium loddigesii	Loddigesiinols G–J	Lu et al. (2014)
	Dendrobium scabrilingue	Dendroscabrol B	Sarakulwattana et al. (2020)
Antioxidant activity	*Cremastra appendiculata*	Coelonin, Orchinol	Tu et al. (2018)
	Dendrobium officinale	Rutin	Zhang et al. (2017)
	Dendrobium palpebre	Dendroflorin	Kyokong et al. (2019)
	Dendrobium parishii	Dendroparishiol	Kongkatitham et al. (2018)
	Gastrodia elata	Gastrodin	Jiang et al. (2020)
Anticancer activity	*Bulbophyllum odoratissimum*	Bulbophythrins A and B	Xu et al. (2009)
	Cattleya tigrina	Triterpene 24-methylenecycloartanol, gigantol, phocantone	Ferreira et al. (2021)
	Dendrobium brymerianum	Moscatilin, gigantol, lusianthridin, and dendroflorin	Klongkumnuankarn et al. (2015)
	Dendrobium draconis	Gigantol	Bhummaphan and Chanvorachote (2015)
	Dendrobium falconeri	Dendrofalconerol A	Pengpaeng et al. (2015)
	Dendrobium nobile	Nudol	Zhang et al. (2019)
	Dendrobium williamsonii	Aloifol I, moscatilin, moniliformine, balanophonin	Yang et al. (2018)
	Goodyera schlechtendaliana	Goodyschle A	Dai et al. (2021)

(continued)

Table 1 (continued)

Biological activity	Plant name	Compound	Reference
	Spiranthes sinensis	Spiranthes phenanthrene A	Liu et al. (2019)
Anti-inflammatory activity	*Dendrobium chrysanthum*	Dendrochrysanene	Yang et al. (2006)
	Dendrobium crepidatum	(+)-Dendrocrepidamine A, Dendrocrepidamine B, (+)-Homocrepidine A	Hu et al. (2020)

have been conducted to test the anti-inflammatory effects of the compounds derived from different orchid species. The ethanolic extract of *Bletilla striata* yielded a dihydrophenanthrene, coelonin, which possessed the potential to decrease inflammation (Jiang et al. 2019a). The alkaloids and phenanthrene isolated from *Dendrobium crepidatum* and *Dendrobium chrysanthum*, respectively, exhibited anti-inflammatory activity (Hu et al. 2020; Yang et al. 2006). To summarise, it can be inferred that the secondary metabolites of orchids have the potential to be used as leads for therapeutic cures in pharmaceutical industries after systematised preclinical and clinical studies.

The therapeutic properties of orchids have aroused curiosity amongst people all over the world and have in turn led to unscrupulous collections from their natural habitats for trade and consumption. As a result, these plants face threats due to their habitat destruction and indiscriminate exploitation. Resultantly, the family Orchidaceae is included in the Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), and the international trade in orchids is austerely governed (Hinsley et al. 2018). Hence, there is a dire need to develop approaches for developing alternative methods for propagation and protection of these high-value plants. In vitro culture methods play a pivotal role in ameliorating the pressures and restoring the decimated natural populations. Besides this, it facilitates the production of biomass and amassing of metabolites in plant tissues and culture media. In vitro cultures also serve as a tool for extensive investigation of the controls and mechanisms of metabolic pathways.

2 In Vitro Propagation and Production of Secondary Metabolites

There are several limitations associated with the extraction of secondary metabolites from the wild plants cultivated in the field such as fluctuations in the yield due to variability in the geographic location, seasonal variation, and environment of the plant, therefore, in vitro propagation has emerged as a better substitute (Murthy et al. 2014). In vitro cultures are grown on defined media under controlled conditions. In orchids, there are several nutrient media which are generally employed for tissue

culture such as media proposed by as Murashige and Skoog (MS) (Murashige and Skoog 1962), Knudson C (KC) (Knudson 1946), Vacin and Went (VW) (Vacin and Went 1949), Mitra (M) (Mitra et al. 1976), etc.

A general trend of decline in secondary metabolite production under normal in vitro growing circumstances in comparison to wild plants has been observed. However, culture media conditions, concentrations of plant growth regulators, nitrogen source, carbon source, and other organic/inorganic additives have been optimised for enhanced production of secondary metabolites (Chandran et al. 2020). In *Habenaria edgeworthii*, three times more phenolic content was found in callus grown on half strength Murashige and Skoog (MS) and 3 μM 6-benzyladenine (BA), as compared to the wild tubers. These also showed enhanced antioxidant activity evaluated by the standard in vitro assays (Giri et al. 2012). In *Dendrobium candidum*, the supplementation of the MS basal medium with 0.5 mg L^{-1} NAA, ratio 5:25 (mM) of NH_4:NO_3, 2.5% (w/v) sucrose, and 1% (v/v) banana homogenate were favourable for the production of polysaccharides, polyphenolics, and flavonoids (Cui et al. 2015). In *D. huoshanense*, phosphate at 0.312 mmol L^{-1} concentration was optimum in the medium for maximum accumulation and production of polysaccharides (Jiang et al. 2006). Similarly, the supplementation with 50 g L^{-1} sucrose in protocorm-like bodies (PLBs) cultures of *D. huoshanense* resulted in a 109-fold increase in the polysaccharide content compared to the media which lacked sucrose feeding (Zha et al. 2007).

In vitro cultures with specific additives and growth conditions have been reported to favour secondary metabolite content and resultant antioxidant potential in comparison to the mother plant as in *Dendrobium nobile* (Bhattacharyya et al. 2014), *Dendrobium thyrsiflorum (*Bhattacharyya et al. 2015*)*, *Aphyllorchis montana* (Mahendran and Bai 2016), *Dendrobium crepidatum* (Bhattacharyya et al. 2016), *Malaxis acuminata* (Bose et al. 2017), *Coelogyne ovalis* (Singh and Kumaria 2020), *Cymbidium aloifolium* (Kumar et al. 2022). Additionally, antimicrobial activity was also found to be higher in in vitro propagated plants of *Aphyllorchis montana* as compared to the wild plants (Mahendran and Bai 2016). In *Dendrobium longicornu*, the in vitro protocorms have higher anticancer, antioxidant, and antimicrobial potential (Paudel et al. 2020). Similarly, the protocorms of *Dendrobium chryseum* were reported to significantly inhibit the growth of human cervical carcinoma cell lines (Pant et al. 2021). Thus, besides mass multiplication for the production of greater biomass, in vitro propagation protocols can also be used for the heightened phytochemical production and significant biological activities. Additionally, the use of in vitro cultures offers a sustainable strategy for conservation and utilization of these endangered medicinal plants.

3 Elicitation stimulates Secondary Metabolism

Several reports suggest the use of specific conditions/compounds as elicitors to enhance secondary metabolism. Elicitor-specific receptors are present on the cell membrane of plants which get activated in the presence of an elicitor which subsequently induces a cascade of downstream signalling events in plant cells involving changes in the expression of genes encoding rate-limiting enzymes of the secondary metabolites biosynthetic pathways (Halder et al. 2019). Thus, stimulation by an elicitor can increase the content of metabolites and/or also generate new compounds, mimicking the inherent strategy of the plant to protect and adapt itself to the abiotic and biotic stresses such as drought, salinity, UV-irradiation, and pathogenesis (Khare et al. 2020). There are various types of elicitors that affect secondary metabolite production in orchids. Abiotic elicitors are derived from non-living sources and include light and plant hormones, etc., while biotic elicitors have a biological origin like chitosan and microorganisms (Table 2). This biotechnological technique of elicitation offers a beneficial approach to exploit the therapeutic potential of medicinal plants and has unfolded a hot topic for research which has tremendous potential for the therapeutic industry (Fig. 1).

3.1 Variable Light Exposure alters Phytochemical Profile

The growth and metabolism of the plants is influenced by light. A number of studies have highlighted the effect of intensity and quality of light on the phytochemical profile in orchids. In *Anoectochilus roxburghii*, the plants grown under light filtered through blue and red film for 8 months displayed enhancement in the content of active compounds such as polysaccharides and flavones and greater antioxidant enzyme activities; the highest phenolic content was observed in the plants with red film treatment (Ye et al. 2017). In another report on the same plant, a noteworthy increase in the total polyphenols and total flavonoids upon supplementation of blue light has been reported while yellow light treatment produced higher soluble sugar and polysaccharide content (Wang et al. 2018). Some research studies have also focussed on using combinations of lights of different wavelengths to promote the production of active compounds. A treatment using a combination of blue-red light in the ratio 1:4 in *Anoectochilus roxburghii* plants enhanced the total flavonoid content. An increase in the expression of genes such as *CHI (Chalcone Isomerase)* and *FLS (Flavonol Synthase)* involved in the flavonoid biosynthetic pathway was also observed (Gam et al. 2020). In *Dendrobium* Enopi x *Dendrobium* Pink Lady hybrid orchid, the in vitro PLB cultures showed the highest flavonoid content upon treating the PLBs pre-illuminated with cool-white LED with blue-red (1:1) LED irradiation. In addition, the PLBs precultured with red fluorescent light for two subculture cycles upon exposure to blue LED light displayed the highest antioxidant activity (Yeow et al. 2020). Thus, the technique of altering the light source during

Table 2 Use of elicitors in in vitro cultures to boost the production of secondary metabolites

Plant species	Elicitor	Type of culture	Secondary metabolite	Reference
Anoectochilus roxburghii	Methyl jasmonate (MeJA) and salicylic acid (SA)	Rhizome suspension cultures	Kinsenoside and polysaccharide content	Luo et al. (2018)
	Mycorrhizal fungi	Symbiotic cultures of tissue cultured plantlets	Flavonol-glycosides (narcissin, rutin, isorhamnetin-3-O-β-d-glucoside, quercetin-7-O-glucoside, and kaempferol-3-O-glucoside), two flavonols (quercetin and isorhamnetin), and two flavones (nobiletin and tangeretin)	Zhang et al. (2020a)
Ansellia africana	Meta-topolin riboside (mTR) and 6, 3-methoxybenzylamino-9-b-D-ribofuranosylpurine (MemTR)	Protocorm-like bodies (PLBs) culture	Phenolic compounds (benzoates and cinnamates)	Bhattacharyya et al. (2019)
Dendrobium candidum	Methyl jasmonate (MeJA)	Protocorm-like bodies (PLBs) culture	Alkaloids, polysaccharides, and flavonoid and phenolic content	Wang et al. (2016)
Dendrobium fimbriatum	Caffeic acid (CA), p-coumaric acid (pCA), and ferulic acid (FA)	Cell suspension cultures established from Protocorm-like bodies (PLBs)	Flavonoid, phenolic, alkaloid, and tannin content	Paul and Kumaria (2020)
Dendrobium ovatum	L-phenylalanine	Callus-derived plantlets	Bibenzyl derivative (moscatilin)	Pujari et al. (2021)
Dendrobium Sabin blue	Thidiazuron (TDZ)	Protocorm-like bodies (PLBs) culture	Anthocyanin content	Chin et al. (2021)
Habenaria edgeworthii	6-Benzyladenine (BA)	Callus suspension culture	Phenolic content	Giri et al. (2012)

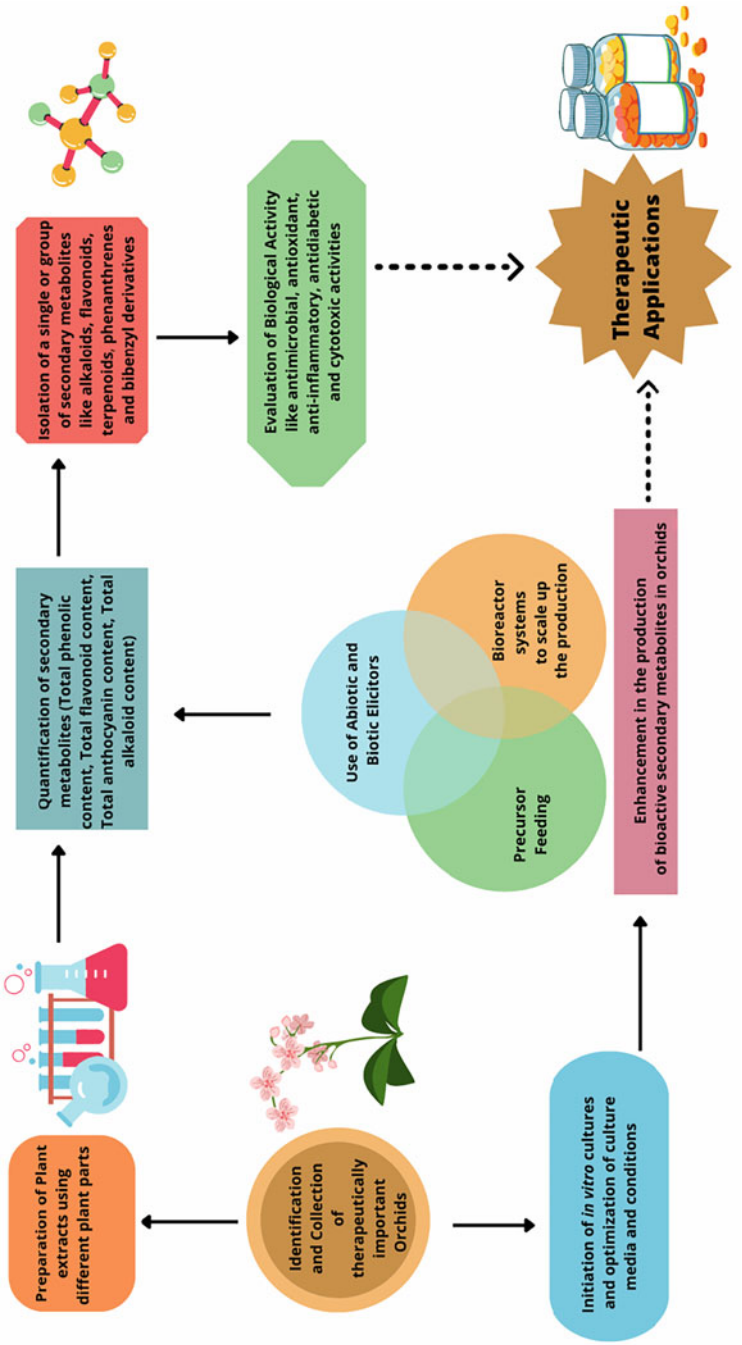

Fig. 1 A schematic flowchart depicting the use of plant tissue culture and pharmacological studies in medicinal orchids

the propagation of orchids is an effective choice to increase the production of bioactive compounds.

3.2 Chemical Abiotic Elicitors trigger Stress Responses

There are various plant growth regulators which act as elicitors and play a key role in modifying the secondary metabolism in plants. The exogenous applications of these chemicals have frequently been used in cell or organ culture to accentuate secondary metabolite biosynthesis (Thakur et al. 2019).

3.2.1 Methyl jasmonate

Methyl jasmonate (MeJA), a derivative of jasmonic acid is one such hormone that functions as a signalling molecule and strongly activates secondary metabolism in medicinal orchids as it induces defence response against pathogens and wounding (Nabi et al. 2021). Also, it triggers the expression of pivotal genes involved in flavonoid and anthocyanins biosynthesis such as *Phenylalanine Ammonia Lyase (PAL)*, *Stilbene Synthase (STS)*, and *Chalcone Isomerase (CHI)* which play a pivotal role in the production of flavonoids and anthocyanins (Nabi et al. 2021). There have been reports on the role of MeJA in the accumulation of alkaloids in *Dendrobium officinale* (Chen et al. 2019). Besides alkaloids, elicitation with MeJA in the root tissue of *D. officinale* has been observed to induce the production of bibenzyl compounds such as erianin and gigantol (Adejobi et al. 2021). The treatment of 75 μM MeJA to the protocorm-like bodies (PLBs) of *Dendrobium candidum* showed augmentation in the production of alkaloids, polysaccharides, and flavonoids while the increase in phenolic content was observed under 100 μM MeJA treatment (Wang et al. 2016). Thus, optimization of elicitation is required for the production of a particular group of active compounds. Besides chemical concentration, the duration of exposure also matters. The rhizome suspension cultures of *Anoectochilus roxburghii* upon treatment with 550 μM MeJA for a period of 14 and 16 days, resulted in the maximum production of kinsenoside and polysaccharide, respectively (Luo et al. 2018). In addition, the increase in the concentration of MeJa beyond the optimum concentration may result in the reduction of the metabolite content. For instance, in *Habenaria edgeworthii*, the total phenolic content decreased beyond 10 μM MeJA (Giri et al. 2012). Similarly, using MeJA beyond 50 μM in the protocorm-like bodies (PLBs) of *Dendrobium* Sabin Blue (a hybrid species between *Dendrobium* Blue Angel and *Dendrobium* Sanan Blue) orchid resulted in the reduction in anthocyanin content (Abd Malik et al. 2021).

3.2.2 Salicylic acid

Salicylic acid (SA), a phenylpropanoid compound, is another common elicitor involved in signalling in plants. Besides its significant role in the physiological processes of plants such as seed germination, photosynthesis, uptake of nutrients, nodulation in legumes, and induction of flowering, it also regulates the expression of genes associated with the enzymes of secondary metabolism (Ali 2021). The effect of this important signal molecule may differ in different plant tissues. In *Coelogyne ovalis*, the leaf tissues treated with SA yielded the highest content of flavonoids and anthocyanins while the SA-treated pseudobulbs showed the highest phenolic content. In addition, the SA-treated plantlets exhibited significantly higher antioxidant activity (Singh and Kumaria 2021). SA, like MeJA, regulates the metabolite content in a concentration and time-dependent manner. Alkaloids and polysaccharides accumulated in high amounts in the protocorm-like bodies (PLBs) of *Dendrobium candidum* upon elicitation with 75 μM SA while 100 μM SA led to high production of flavonoids (Wang et al. 2016). *Anoectochilus roxburghii* rhizomes exhibited maximum kinsenoside and polysaccharide contents upon treatment with 500 μM SA for 12 days (Luo et al. 2018). However, some sporadic studies also report the inhibitory role of SA (Chin et al. 2021).

3.2.3 Cytokinins

Apart from playing a vital role in the growth and development of plants, cytokinins, and their derivatives also influence the production of active compounds in plants. In *Habenaria edgeworthii*, the callus grown on 3 μM BA showed a significant improvement in phenolic content and antioxidant activity (Giri et al. 2012). Similarly, another cytokinin, topolin, and its derivatives like meta-topolin riboside (mTR) and 6, 3-methoxybenzylamino-9-b-D-ribofuranosylpurine (MemTR) showed a positive impact in *Ansellia africana*. The PLBs showed an increase in the production of phenolic compounds like benzoates and cinnamates along with an increase in antioxidant activity (Bhattacharyya et al. 2019). The PLBs of *Dendrobium* Sabin Blue supplemented with 4 mgL^{-1} thidiazuron (TDZ) depicted an increase in the anthocyanin content (Chin et al. 2021).

Thus, chemical or hormonal elicitors are promising for increasing the production of the metabolites and their utilization is considered an advantageous strategy. However, it is important to formulate the optimum concentration and duration of exposure to the elicitor being used.

3.3 Biotic Elicitors alter Secondary Mechanism as a Defence Mechanism

3.3.1 Fungal elicitors

In orchids, mycorrhizal fungi play a key role in the germination of seeds and development as the fungi supplement organic and inorganic nutrients for the growing entity (Dearnaley et al. 2012). Thus, orchid-mycorrhizal symbiosis constitutes a pivotal part in the life cycle of orchids. Fungal elicitation also leads to the activation of specific genes related to secondary metabolite biosynthetic pathways. Moreover, fungal elicitors have been reported to be more promising in the biosynthesis of metabolites in comparison to the chemical elicitors in a plethora of studies (Favre-Godal et al. 2020). A significant increase in the production of active metabolites in the host plant upon inoculation with different types of mycorrhizal fungi has been demonstrated in a few orchids. F-23 fungus (*Mycena* sp.) improved the production of kinsenosides and flavonoids of *Anoectochilus formosanus (*Zhang et al. 2013*)*. Similarly, *Dendrobium nobile* upon inoculation with the same fungus (F-23) showed an increase in dendrobine level in the stem thus suggesting the role of mycorrhizal fungi in dendrobine synthesis (Li et al. 2017). Another fungus, *Ceratobasidium* sp. AR2 stimulated the accumulation of flavonol-glycosides (narcissin, rutin, isorhamnetin-3-O-β-d-glucoside, quercetin-7-O-glucoside, and kaempferol-3-O-glucoside), flavonols (quercetin and isorhamnetin), and flavones (nobiletin and tangeretin) in *Anoectochilus roxburghii* (Zhang et al. 2020a). Similar results showing the promoting role of AR2 on flavonoid production in *Anoectochilus roxburghii* have been reported in another study (Zhang et al. 2020b). An enhancement in the antioxidant and the hepatoprotective activity upon inoculation of *Rhizoctonia* mycorrhizal fungi had also been observed in *Anoectochilus formosanus* (Cheng and Chang 2011).

3.3.2 Chitosan

Another biotic elicitor, chitosan, a polysaccharide, derived from the exoskeletons of insects and fungi showed a promoting role in the accretion of secondary metabolites by augmenting the production of the enzymes involved in the biosynthetic pathways of secondary metabolites (Zhao et al. 2005). Chitosan is a non-toxic natural biopolymer consisting of glucosamine and *N*-acetylglucosamine subunits (Sanford and Hutchings 1987). It basically mimics the fungal pathogen and gets recognised at the plant membrane through the mechanism of cell surface recognition which induces a series of downstream events activating the defence response in plants (Singh and Kumaria 2021). The micropropagated plantlets of *Vanda coerulea* when treated with chitosan displayed an improvement in the phytochemical contents and antioxidant potential. A positive correlation between the phytochemical content and Phenylalanine ammonia lyase (PAL) enzyme activity in *Vanda coerulea* upon treatment with

chitosan was also observed (Nag and Kumaria 2018), thus suggesting that chitosan triggered the genes involved in secondary metabolism.

4 Precursors Feeding in Cultures accentuates Secondary Metabolite Production

The use of precursor molecules as elicitors has also been elucidated in some orchids. Precursors are intermediates in the pathway of secondary metabolite biosynthesis which upon adding to the culture media tend to increase the amount of the related secondary metabolites. This strategy of precursor feeding is quite useful when the precursor compound is available at a low cost compared to the final desired product (Namdeo et al. 2007).The highest phenolic and flavonoid content in the cultures of *Dendrobium fimbriatum* was observed upon application of caffeic acid (CA) while 2 mM ferulic acid (FA) and 4 mM *p*-coumaric acid led to the highest alkaloid and tannin content, respectively. Also, the cultures treated with caffeic acid exhibited the highest antioxidant activity (Paul and Kumaria 2020). In a similar manner, in *Dendrobium ovatum*, the use of L-Phenylalanine as a precursor ensured high content of moscatilin, a bibenzyl derivative compound that possesses anticancer properties (Pujari et al. 2021).

5 Bioreactors as Mini Factories for Scale-up

A bioreactor is an instrument for large scale in vitro propagation. It consists of a closed and sterile culture vessel in which the internal environmental conditions can be monitored and controlled (Mamun et al. 2015). The application of bioreactor systems offers an alternative strategy for the production of bioactive compounds at the industrial level. The method of bioreactor systems consumes less time and is cost-effective compared to the use of gelled or semi-solid medium which requires the transfer of the plant material into a fresh expensive media at periodic intervals of time (Murthy et al. 2018). Moreover, in solid media all the plant parts are not in direct contact with the medium and resultantly, growth occurs slowly (Zhang et al. 2018). Thus, to overcome these problems, different plant parts and culture media under bioreactor systems have been utilised in orchids (Table 3). Several factors affect the plant biomass and phytochemical production in a bioreactor that needs to be optimised for desired results. The selection of a suitable type of bioreactor is vital for the growth and metabolism of plant cultures. Temporary and continuous immersion bioreactor systems are usually used for plant cultures. In the continuous immersion system, the plant cultures are continuously immersed in the liquid medium whereas the temporary immersion system works on the principle of temporarily submerging the cultures in the medium at specific intervals of time (De Carlo

Table 3 Use of bioreactor cultures for mass production of active ingredients in orchids

Plant name	Plant part used	Culture media and PGRs	Type of bioreactor	Active ingredient	Reference
Anoectochilus roxburghii	Rhizomes	3/4 MS + 2 mg L^{-1} BA + 0.2 mg L^{-1} Kn + 0.5 mg L^{-1} NAA	CIB (continuous immersion bioreactor)	Polysaccharide and Kinsenoside	Jin et al. (2017)
Dendrobium candidum	Protocorms	MS + 0.5 mg L^{-1} NAA	CIB (continuous immersion bioreactor)	Polysaccharides, coumarins, polyphenolics, flavonoids, vitamin C and vitamin E	Cui et al. (2014)
Dendrobium nobile	Seedlings	1/2 MS + 0.5 mg L^{-1} + NAA + 80 g L^{-1} CW	TIB (temporary immersion bioreactor)	Alkaloids	Zhang et al. (2022)
Bletilla striata	Pseudobulbs	1/2 MS + 60 g L^{-1} potato lixivium + 0.5 mg L^{-1} NAA	TIB (temporary immersion bioreactor) system	Polysaccharides	Zhang et al. (2018)

et al. 2021). In *Anoectochilus roxburghii,* the continuous immersion bioreactor having a net at the bottom of the sphere of the bioreactor was found apt for the mass production of rhizomes; nearly 2980.5 mg L^{-1} of kinsenoside and 5672.9 mg L^{-1} of polysaccharides were produced (Jin et al. 2017). In 2014, a research group cultured the protocorms of *Dendrobium candidum* in different bioreactor systems and found that the continuous immersion bioreactor system was the most appropriate for the production of polyphenolics, flavonoids, vitamin C, and vitamin E, coumarins, and polysaccharides (Cui et al. 2014). In *Epipactis flava,* maximum in vitro micropropagation efficiency was obtained by using the temporary immersion system in comparison to the continuous immersion bioreactor system (Kunakhonnuruk et al. 2019). RITA® bioreactor based on a temporary immersion system has been employed to cater to the demand of *Cattleya forbesii* in the commercial market (Ekmekçigil et al. 2019). Similarly, in *Vanda tricolor*, a temporary immersion bioreactor system has been established to be efficient for its commercial production (Esyanti et al. 2016).

For optimisation of the bioreactor system, different aspects associated with bioreactors such as inoculation density, air volume, immersion frequency, and light intensity hold significant importance. Inoculation density affects the number of nutrients that are available for each explant and aeration volume affects the mixing of the constituents and oxygenation. Inoculation density of 50 g L^{-1} and an aeration volume of 0.1 vvm (air volume per culture volume per minute) were found beneficial in protocorm immersion culture of *Dendrobium candidum* (Cui et al. 2014). In *Anoectochilus roxburghii,* an inoculation density of 12.5 g L^{-1}, air volume lower than 500 mL L^{-1} and 45 μmol m^{-2} s^{-1} light intensity was favourable (Jin et al. 2017). The temporary immersion frequency of 5 min every 6 h maximised the biomass and total alkaloid content in the plantlets of *Dendrobium nobile* (Zhang et al. 2022).

The secondary metabolite content reaches its peak value after a specific number of days of initiation of bioreactor culture. For instance, for the rhizome immersion bioreactor culture in *Anoectochilus roxburghii*, 30 days period was the optimum for maximum polysaccharide (4251.2 mg L^{-1}) and kinsenoside (1724.0 mg L^{-1}) production (Jin et al. 2017). However, for *Dendrobium nobile*, 20 days culture period was most advantageous for alkaloids production (Zhang et al. 2022). Further, the concentration of carbon source used in the culture medium also has a role in PLB bioreactor cultures of *Dendrobium candidum*, the optimal concentration of sucrose in the culture medium was found to be 30 g L^{-1} for improved polysaccharide and alkaloid yields (Yang et al. 2015). Additionally, the elicitors have also been tested in bioreactor cultures in a few orchids. In *Dendrobium candidum*, the MeJA treatment to 30 days bioreactor cultured PLBs for 4 and 10 days has been observed to induce mass production of alkaloids or polysaccharides and phenolics or flavonoids, respectively (Wang et al. 2016). The content of polysaccharide and kinsenoside and antioxidant activity showed improvement upon elicitation with MeJA or salicylic acid in rhizome immersion bioreactor cultures of *Anoectochilus roxburghii* in comparison to the plants grown in the field (Luo et al. 2018). In *Dendrobium nobile,* 10 μM MeJA improved the accumulation of alkaloids in the bioreactor

culture of plantlets (Zhang et al. 2022). Similarly, elicitation with 0.25 mmole L^{-1} of MeJA enhanced the biosynthesis of polysaccharides and enlarged the pseudobulbs of *Bletilla striata* (Zhang et al. 2018). Hence, the use of elicitors in bioreactor cultures is considered suitable for orchids to serve as mini-factories of important metabolites by scaling up the secondary metabolites production.

6 Conclusions

In vitro cultures serve as a consistent source of valuable plant-specific metabolites in orchids. Thus, this technique could be utilised for the scale-up process resulting in the mass production of orchid-specific bioactive compounds to cater to the demands of the pharmaceutical, cosmetic, and nutraceutical industries. Additionally, it reduces overexploitation and unscrupulous collections pressures on the natural populations of orchids. Hence, resulting in sustainable utilization, commercial propagation, and conservation of high-value therapeutically important orchid species. This review offers insights into the strategies for improvement of phytochemical production in orchids and provides a baseline data for future research.

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Declarations: Conflicts of Interest/Competing Interests

The authors have no conflicts of interest to declare that are relevant to the content of this article.
Author's Contributions JKS conceived the original idea and outline. AK and JV prepared the original draft. SVP and VGY gave significant inputs. All the authors critically revised the work and approved the final manuscript.

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Ethnomedicinal Uses, Phytochemistry, Medicinal Potential, and Biotechnology Strategies for the Conservation of Orchids from the *Catasetum* Genus

Luis J. Castillo-Pérez, Daniel Torres-Rico, Angel Josabad Alonso-Castro, Javier Fortanelli-Martínez, Hugo Magdaleno Ramírez-Tobias, and Candy Carranza-Álvarez

L. J. Castillo-Pérez
Programa Multidisciplinario de Posgrado en Ciencias Ambientales, Universidad Autónoma de San Luis Potosí, San Luis Potosí, San Luis Potosí, Mexico
e-mail: jesus.perez@uaslp.mx

D. Torres-Rico
Facultad de Ciencias Químicas, Universidad Autónoma de San Luis Potosí, San Luis Potosí, San Luis Potosí, Mexico

A. J. Alonso-Castro
División de Ciencias Naturales y Exactas, Departamento de Farmacia, Universidad de Guanajuato, Guanajuato, Mexico

J. Fortanelli-Martínez
Instituto de Investigación de Zonas Desérticas, Universidad Autónoma de San Luis Potosí, San Luis Potosí, San Luis Potosí, Mexico
e-mail: fortanel@uaslp.mx

H. M. Ramírez-Tobias
Facultad de Agronomía y Veterinaria, Universidad Autónoma de San Luis Potosí, San Luis Potosí, San Luis Potosí, Mexico
e-mail: hugo.ramirez@uaslp.mx

C. Carranza-Álvarez (✉)
Programa Multidisciplinario de Posgrado en Ciencias Ambientales, Universidad Autónoma de San Luis Potosí, San Luis Potosí, San Luis Potosí, Mexico

Facultad de Ciencias Químicas, Universidad Autónoma de San Luis Potosí, San Luis Potosí, San Luis Potosí, Mexico

Facultad de Estudios Profesionales Zona Huasteca, Universidad Autónoma de San Luis Potosí, Ciudad Valles, San Luis Potosí, Mexico
e-mail: candy.carranza@uaslp.mx

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1 Introduction

The obtention of new drugs from plant secondary metabolites plays an important interest in the pharmaceutical industry (Radice et al. 2020). Many medicinal plants from the Solanaceae, Asteraceae, and Fabaceae families have been studied, whereas other plant families like Apiaceae, Ranunculaceae, and Orchidaceae lack scientific information validating their medicinal properties (Marrelli 2021).

The Orchidaceae family is the most diverse in the plant kingdom and represents an important part of the biodiversity in the Neotropics. This family has a wide distribution in this area. Some of the genera, including *Laelia*, *Stanhopea*, *Cyrtopodium*, and *Epidendrum* belonging to this family have reports of medicinal properties (Castillo-Pérez et al. 2019). These orchid genera have shown biological activities as antihypertensive, antipyretic, anti-inflammatory, antinociceptive, and antidysentery, among others (Vergara-Galicia et al. 2013; Morales-Sánchez et al. 2014; Emeterio-Lara et al. 2016; Arora et al. 2017).

A particular and low-studied genus within the Orchidaceae family is the *Catasetum* genus, which possesses approximately 170 species and is widely distributed in the neotropical region of America (Milet-Pinheiro and Gerlach 2017). However, some *Catasetum* hybrids have been successfully cultivated and adapted to other regions, being grown in Europe, Asia, and America (Cantuaria et al. 2021). They have diverse growth habits, most species are epiphytic (Fig. 1a), but some species present terrestrial, lithophyte, or saprophyte development (Milet-Pinheiro and Gerlach 2017). Moreover, they are sexually dimorphic and exhibit male and female flowers (Fig. 1b, c) (Gerlach 2013). Another important piece of data is these orchids present mycorrhizal and myrmecophile ecological interactions for their

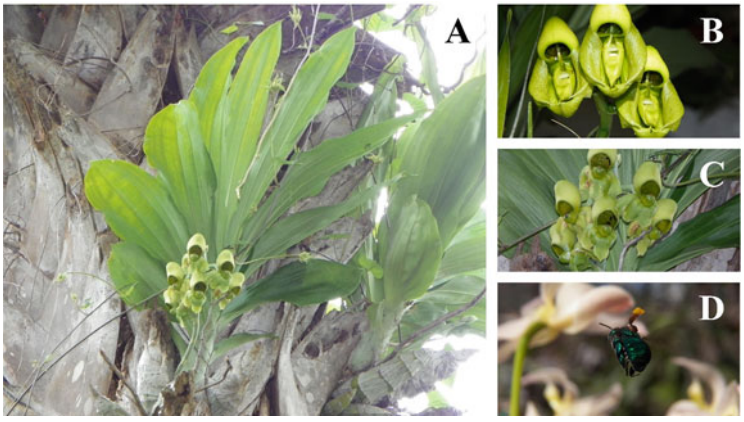

Fig. 1 *Catasetum integerrimum* (Orchidaceae) is a common example of the genus *Catasetum*. (**a**) Whole plant in situ with epiphytic growth; (**b**) Male flowers; (**c**) Female flowers; (**d**) Interaction with the bee of the Euglossini tribe, the main pollinator of the genus *Catasetum*

growth and defense, and the male bees of the Euglossini tribe are the main pollinating agent (Fig. 1d) (Gerlach 2013; Bonilla-Morales et al. 2016).

Currently, species of the *Catasetum* genus are used mainly as ornamental plants in several parts of the world. Some species of this genus have medicinal properties attributed to different population groups around the world. The objective of this chapter is to summarize all the research findings available on various aspects, such as botanical description and distribution, ethnopharmacology, phytochemistry, and conservation of the *Catasetum* genus.

The information search was based on the following groups of keywords: *Catasetum* orchids, Medicinal *Catasetum*, Phytochemical *Catasetum*, Biotechnology *Catasetum*, and Ecology of *Catasetum*. We search the most relevant data in "PubMed", "ScienceDirect", "Scopus", "Web of Science", and "Google Scholar", in addition, physical and digital books were consulted. The current taxonomy of the species was validated using the website of The World Flora Online (http://www.worldfloraonline.org/). The article search was carried out from 15 March 2022 to 15 August 2022. Based on all the compiled information, the research gap has also been discussed. This chapter provides the basis for further studies on the conservation and development of identifying better therapeutic agents and health products from the *Catasetum* orchids.

2 Botanical Description of the Species

This section describes the general characteristics shared by *Catasetum* orchids. We suggest consulting the taxonomic keys provided in the botanical bibliography for the specific description of any species from this genus. Most *Catasetum* orchids are epiphytic, perennial, medium-sized with a height of 30–70 cm, composed of pseudobulbs ovoid to fusiform and fibrous roots at the base. The plants have leaves that can be oblong-lanceolate to elliptic and deciduous. The flowers are terminal racemes, and some species develop non-resupinate, unisexual, dimorphic and fragrant flowers. The column in the *Catasetum* species is short and truncated and has a pollinial vestigial. After pollination, these species develop ellipsoid and glaucous capsules with seeds minute and powdery. Most of the pseudobulbs are fleshy, smooth, shining, greenish, covered with membranous sheath, and slightly mucilaginous (Salazar et al. 1990). The flowering of these species varies throughout the year and some species can develop flowers more than once a year. Anatomical and histochemical studies revealed the presence of endophytic mycorrhizal fungus in the root and protocorm (Silva et al. 2015). The anatomical similarity between rhizomes and pseudobulbs indicates that species can be propagated from its rhizomes as well as pseudobulbs.

3 Habitat, Distribution, and Ecology

Catasetum orchids have different development forms, some species are epiphytic, others are terrestrial or lithophyte and some species have even been described with saprophyte growth. The species of the *Catasetum* genus usually develop mycorrhizal and myrmecophile interactions for their growth, plant development, and defense. Another interesting ecological aspect is that the wide majority of *Catasetum* species share their main pollinating agent, the male bees of the Euglossini tribe, also known as orchid bees (Milet-Pinheiro and Gerlach 2017; Gerlach 2013; Bonilla-Morales et al. 2016).

The *Catasetum* genus is present only in the neotropical region of the American continent and has approximately 170 species. Brazil encompasses the largest number of *Catasetum* orchids (Romero-Gonzales 2012; Ramos et al. 2012; Chase et al. 2015). In the case of Mexico, two of the most important orchids of the *Catasetum* genus, *C. integerrimum*, and *C. laminatum* are distributed in the states of Tamaulipas, San Luis Potosí, Hidalgo, Veracruz, Puebla, Querétaro, Oaxaca, Chiapas, Tabasco, Campeche, Yucatan, and Quintana Roo (Salazar et al. 1990).

Table 1 shows the few available studies published about the habitat and ecology of the Catasetum orchids. Most of these species are distributed in tropical forests, which is not surprising since many of these orchids are epiphytes. Interestingly, *C. discolor* grows in more arid ecosystems in countries like Bolivia, Brazil, and Venezuela (Milet-Pinheiro and Gerlach 2017; Dodson 1978).

Catasetum orchids have their pollinating species. Nevertheless, there are few records about the pollinating organisms of these orchids, including insects of the Hymenoptera order, Apidae family, and Euglossini tribe, specifically two genera, *Eufriesea*, *Euglossa*, and *Eulaema* (Table 1). However, this work found records of 16 Catasetum species, which represents a gap in the ecological knowledge of these species.

Another ecological aspect with limited information is the time of flowering in these orchids. For example, of the 16 species presented in this work, this data is only known in eight of the 16 species. Interestingly, some species such as *C. integerrimum* and *C. viridiflavum* flower for most of the year (Table 1) (Milet-Pinheiro and Gerlach 2017; Hernández-Ramírez 2021).

4 Ethnomedicinal Uses

The medicinal uses conferred on the Catasetum orchids have been documented in several reports (Table 2). Firstly, it was recorded in 1958 that the ashes of pseudobulbs from *C. maculatum* were used for the treatment of inflammations, abscesses, sores, and warts (Kunow 1958). Afterward, Arenas and Moreno-Azorero (1977) documented using *C. gardneri* pseudobulbs as a sterilant. The application of this orchid was recommended in conjunction with the rhizomes of another plant

Table 1 Some ecological aspects of *Catasetum* orchids

Species	Habitat	Main growth type	Pollinator species	Flowering season	References
Catasetum arietinum F.E.L. Miranda and K.G. Lacerda	Neotropical cloud forests	Epiphyte	*Euglossa nanomelanotricha*, *Euglossa securigera*	February–July	Brandt et al. (2020)
Catasetum integerrimum Hook	Tropical deciduous and semi-deciduous forests, warm oak and palm forests, neotropical cloud forests, and montane forests	Epiphyte	*Eulaema cingulata, Eulaema polichroma, Eulaema cingulate, Eulaema meriana, Exaerete frontalis*	April–November	Salazar et al. (1990), Hernández-Ramírez (2021)
Catasetum uncatum Rolfe	The short palm stems in dry forest	Epiphyte	*Euglossa nanomelanotricha, Euglossa carolina*	March–May	Milet-Pinheiro et al. (2015)
Catasetum pusillum C. Schweinf	Semi-humid forests	Lithophyte and terrestrial	*Euglossa sp.*	February–May	Huatangare-Córdova (2000)
Catasetum saccatum Lindl.	Primary forests	Epiphyte	*Eufriesea violacens, Euglossa augaspis, Euglossa chalybeata, Euglossa cordata, Euglossa ignita, Euglossa imperialis, Eulaema cingulata*	NM[a]	Milet-Pinheiro and Gerlach (2017), Huatangare-Córdova (2000)
Catasetum peruvianum Dodson and D.E. Benn	Primary and secondary forests	Epiphyte	NM	NM	Huatangare-Córdova (2000)
Catasetum cernuum (Lindl.) Rchb.f.	Tropical and riparian forests	Epiphyte	*Eufriesea violacea*	NM	Nunes et al. (2017)
Catasetum ochraceum Lindl.	Tropical dry forests	Terrestrial	*Euglossa modestior, Euglossa gaiani, Euglossa deceptrix, Euglossia liopoda*	NM	Romero and Nelson (1986), Zapata-Hoyos et al. (2021)

(continued)

Table 1 (continued)

Species	Habitat	Main growth type	Pollinator species	Flowering season	References
Catasetum macrocarpum Rich. ex Kunth	Tropical forests bordering rivers	Epiphyte and terrestrial	*Eulaema bombiformis, Eulaema nigrita*	Starts in January	Dodson (1978), Carvalho and Machado (2002), Ferreira et al. (2018)
Catasetum discolor (Lindl.) Lindl	Savannah, in sand	Terrestrial	*Eulaema bombiformis, Eulaema bomboides Eulaema cingulata, Eulaema nigrita, Eulaema meriana, Euglossa ignita*	May–November	Milet-Pinheiro and Gerlach (2017), Dodson (1978)
Catasetum longifolium Lindl.	NM	Epiphyte	*Eulaema bombiformis Eulaema meriana*	NM	Dodson (1978)
Catasetum maculatum Kunth	NM	Epiphyte	*Eulaema polychroma, Eulaema meriana, Eulaema cingulata*	Twice a year, March and July	Janzen (1981)
Catasetum viridiflavum Hook	Tropical cloud forest	Epiphyte	*Eulaema cingulata, Eulaema nigrita, Eulaema marcii, Exaerete frontalis*	April–December	Milet-Pinheiro and Gerlach (2017), Zimerman (1991)
Catasetum galeritum Rchb. f.	Tropical cloud forest	Epiphyte	*Eufriesea superba*	NM	Milet-Pinheiro et al. (2018)
Catasetum gardneri Schltr.	NM	Epiphyte	*Eufriesea auriceps, Eufriesea violacens, Eufriesea combinata, Eulaema cingulata, Euglossa sp.*	NM	Milet-Pinheiro et al. (2018), Coelho-Ferreira (2005)
Catasetum barbatum (Lindl.) Lindl	NM	Epiphyte	*Euglossa augaspis, Euglossa cognata, Euglossa cordata, Euglossa mixta, Eulaema cingulata*	NM	Milet-Pinheiro et al. (2018)
Catasetum macroglossum Rchb. f.	NM	Epiphyte	*Eulaema cingulata, Eulaema tropica, Eulaema bomboides, Eulaema speciosa, Eulaema polychroma*	NM	Milet-Pinheiro et al. (2018), Vogel (1963)

[a]*NM* Not mentioned

Table 2 Ethnopharmacological uses of the genus *Catasetum*

Species	Plant section used	Preparation way	Ethnopharmacological uses	Country where it is used	References
Catasetum maculatum Kunth	Pseudobulb	Plaster	Treatment of inflammations, abscesses, sores, and warts	Mexico	Kunow (1958), Cervantes-Reyes (2008)
Catasetum gardneri Schltr.	Pseudobulb	Infusion with rhizome of *Typha latifolia*	Sterilization	Paraguay	Arenas and Moreno-Azorero (1977)
		Infusion	Contraceptive	Paraguay and Brazil	Teoh (2019)
Catasetum barbatum (Lindl.) Lindl.	Aerial parts	NM	Asthma and lumbago	Paraguay	Shimizu et al. (1988)
Catasetum integerrimum Hook	Leaf	NM	Treatment of pimples	Mexico	Ankli et al. (1999)
	All plant	NM	Dermatological diseases		Alonso-Castro et al. (2011)
	Pseudobulb	Liquefied with water	Supplement against kidney and urinary infections		Castillo-Pérez et al. (2021)
	NM	Infusion with leaf of *Laelia autumnalis*	Cough treatment		Cervantes-Reyes (2008)
		NM	Snake bite		Téllez-Valdés et al. (1989)
			Cure of tumors and in the treatment of abscesses and wounds		Cox-Tamay (2013)
			Burns and wounds		Cruz-Garcia et al. (2014)
			Antidiarrheal		Teoh (2019)
	Pseudobulb, leaf, root, capsule	Infusion or liquefied with water	Treatment of colitis, diabetes, high blood pressure, kidney conditions, and cancer		Galicia-Mendieta (2017), Hernández-Bautista and Martínez-Espinoza (2019)

(continued)

Table 2 (continued)

Species	Plant section used	Preparation way	Ethnopharmacological uses	Country where it is used	References
Catasetum expansum Rchb. f.	Stem	Plaster or poultice	-Treatment of broken bones and bone fractures	Ecuador	Zambrano-Intriago et al. (2015)
Catasetum macroglossum Rchb. f.	Pseudobulb	Plaster	-Treatment of broken bones and bone fractures—anti-inflammatory and anti-rheumatic	Ecuador	Ramos et al. (2012), Ramos-Corrales et al. (2011)

NM Not mentioned

Fig. 2 Pseudobulbs on sale of *C. integerrimum* in a local market in the municipality of Matlapa, Huasteca Potosina, Mexico

denominate *Typha latifolia*. To obtain the sterilizing effect, both parts of the plants should be boiled in water and consumed at the morning. The consumption of the pseudobulbs of *C. gardneri* as an infusion is registered as a contraceptive method by residents of indigenous regions from Paraguay and Brazil.

C. barbatum is another species of the *Catasetum* genus documented as medicinal. This species is used in traditional medicine from Paraguay for the treatment of asthma and lumbago. However, there is no information on the preparation of this plant for the treatment of these diseases (Shimizu et al. 1988).

One of the *Catasetum* species with the most records of medicinal properties is *C. integerrimum* (Table 2). In the late 1980s, this species was reported to be useful for treating viper bites (Téllez-Valdés et al. 1989). Subsequently, it was reported that the leaves of this species were used by Mayan communities in the state of Yucatan, Mexico for the treatment of "large grains", which possibly may allude to tumors (Ankli et al. 1999). Another investigation carried out by Alonso-Castro et al. (2011) mentioned the entire use of the orchid for the treatment of dermatological conditions, and Cox-Tamay (2013), documented its application in the treatment of tumors, abscesses, and wounds by communities of Yucatan, Mexico. Another use that has been conferred to *C. integerrimum* is in the treatment of burns and wounds (Cruz-Garcia et al. 2014), and recently in the state of Veracruz, Mexico, its application is used for treating diarrhea (Teoh 2019). However, the information on which plant part should be used for the medicinal purpose, the way of administration, and the way of preparation are frequently omitted in the scientific literature.

In the Huasteca Potosina region, pseudobulbs of *C. integerrimum* are traded in local markets with other medicinal plants and fruits (Fig. 2). The inhabitants of this region comment that the pseudobulbs should be prepared as an infusion with water

and orally consumed for treating kidney, gastrointestinal, urinary tract infections, and against diabetes mellitus (Castillo-Pérez et al. 2021).

Other *Catasetum* species documented with medicinal properties are *C. expansum* and *C. macroglossum*, used in communities in Provincias del Rio, Ecuador. According to Zambrano-Intriago et al. (2015), *C. expansum* is used in the treatment of broken bones and bone fractures, through the preparation of plaster or poultice, made from the scape floral, which is then applied to the affected area. On the other hand, Ramos-Corrales et al. (2011) mention that *C. macroglossum* is used in the treatment of inflammation, pain and broken bones, also applied by making a poultice from the pseudobulbs. Likewise, Ramos et al. (2012) reported the topical use of the pseudobulbs of *C. macroglossum* as anti-inflammatory and anti-rheumatic in the middle lands and forests of Ecuador.

Finally, some reports documented the consumption of the stem floral wand of some *Catasetum* species for the reduction of headaches in Shuar communities (Ecuador). However, the *Catasetum* species was not reported (Bennett 1992). Likewise, Kunow (1958) and Teoh (2019) reported the use of *Catasetum maculatum* used in traditional Mayan medicine for treating external tumors and abscesses. The *Catasetum* genus has a wide variety of medicinal applications. However, the studies that support these properties are scarce.

5 Phytochemicals Isolated and Pharmacological Activities

As shown in Table 3, the studies on the secondary metabolites isolated *Catasetum* species and their pharmacological actions are limited to three species. Four compounds, including the phenanthrene 2,7-dihydroxy-3,4,8-trimethoxyphenanthrene were isolated from an ethanolic extract of the aerial parts of *C. barbatum* and tested on their anti-inflammatory and antinociceptive activity through the carrageenan-induced plantar edema test and the histamine-induced contortion tests in rats (Shimizu et al. 1988).

Currently, *C. integerrimum* is one of the orchids most studied under different approaches. There are two studies carried out to verify its pharmacological activities. First, 25 μg/mL ethyl acetate extract of leaves, roots, and pseudobulbs of *C. integerrimum* showed cytotoxic activity by 79.47% and 97.79% on breast cancer cell lines MCF-7 and MDA-MB231, respectively. The compounds identified included phenolic acids (ferulic, gallic, p-coumaric, p-hydroxybenzoic, syringic, and vanillin) and flavonoids (phloretin, galangin, naringenin, quercetin, and rutin) (Cruz-Garcia et al. 2014).

The antioxidant activity of a root extract from *C. integerrimum* and its metabolites showed antioxidant activity in the ABTS and DPPH assays. The phytochemical qualitative test revealed the presence of sterols, unsaturations, flavonoids, and coumarins in wild plants and vitroplants (Table 3). This is one of the first studies reporting the phytochemical profile of Catasetum vitroplants (Torres-Rico 2021).

Table 3 Biological activities and phytochemicals isolated from *Catasetum*

Species	Plant part analyzed	Biological activity studies	Tested extracts	Isolated chemical compounds	References
Catasetum barbatum (Lindl.) Lindl	Aerial parts	Anti-inflammatory activity by carrageenan-induced plantar edema	Ethanolic extract	1. 2,7-dihydroxy-3,4,8-trimethoxyphenanthrene 2. 2,7-dihydroxy-3,4-dimethoxyphenanthrene 3. 2,7-dihydroxy-3,4-dimethoxy-9,10-dihydrophenanthrene 4. 2,7-diacetoxy-3,4-dimethoxy-9,10-dihydrophenanthrene	Shimizu et al. (1988)
Catasetum integerrimum Hook	Leaf, root, and pseudobulb	Cytotoxic activity by breast cancer cell lines (MCF-7 and MDA-MB231)	Ethyl acetate extract	1. Ferulic acid 2. Gallic acid 3. p-coumaric acid 4. p-hydroxybenzoic acid 5. Syringic acid 6. Vanillin 7. Phloretin 8. Galangin 9. Naringenin 10. Quercetin 11. Rutin	Cruz-Garcia et al. (2014)
	Root	Antioxidant activity by DPPH and ABTS assays	Ethanolic extract	1. Flavonoids 2. Phenols 3. Reducing sugars 4. Alkaloids 5. Tannins 6. Saponins 7. Sterols 8. Terpenes	Torres-Rico (2021)

(continued)

Table 3 (continued)

Species	Plant part analyzed	Biological activity studies	Tested extracts	Isolated chemical compounds	References
Catasetum macroglossum Rchb. f.	Pseudobulb	Anti-inflammatory activity by carrageenan-induced plantar edema	Aqueous extract	1. Reducing sugars 2. Flavonoids 3. Glucomannan 4. Phenanthrene 5. Stilbene	Ramos et al. (2012)
		Antioxidant activity by DPPH assay	Ethanolic extract	1. Phenols 2. Flavonoids 3. 1,5-anhydro-D-sorbitol 4. Xylitol 5. Octanedioic acid 6. Fructose 7. Linoleic acid	Molina-Sandoval (2020), Buenaño-Morales and Santillán-Chávez (2021)

Aqueous extract prepared from *C. macroglossum* pseudobulbs showed anti-inflammatory activity on the carrageenan-induced plantar edema test in Wistar rats. These properties were attributed to the presence of flavonoids. An HPLC-DAD analysis determined the presence of phenanthrenic and stilbenic dihydroderivatives (Ramos et al. 2012). Recent current works on *C. macroglossum* suggested the presence of phenols, flavonoids, various sugars, and some fatty acids in this plant species (Table 3). Some of these compounds have antioxidant effects, which can confer add value to many of these edible orchids. The presence of biological activity in *Catasetum* species confirms the traditional use of these orchids, demonstrating the need for more ethnobotanical studies.

6 Propagation and Cultivation Effort

Current biotechnological efforts in plants are an integral part of the works associated with in vitro and ex vitro conservation and propagation, genetic transformation, acclimatization, and product development from several plant genera and species (López-Puc and Herrera-Cool 2022). Several works were published on biotechnological studies about the *Catasetum* genus (Table 4), focusing on the propagation and in vitro conservation of these species from various types of explants, denoting a preference for the conservation of the genus, but with little research focused on the acclimatization and development of products from species with phytochemicals of pharmacological potential.

The conservation protocols of six different Catasetum species were published. Seeds and protocorms are the most widely used explants, although pseudobulbs, roots, and in vitro plants have also been used. Seed germination and micropropagation for mass propagation studies are available for these species. Fernandes et al. (2015) used seeds from an immature capsule of *Catasetum boyi* and obtained up to 90% germination. The percentage of seed germination is low, approximately 5% of all seeds, under natural conditions (Arditti 1967). Micropropagation work was also carried out for *C. gardneri* (Silva-Maia and Pedroso-deMoraes 2017), *C. macrocarpum* (Ferreira et al. 2018), and *C. schmidtianum* (Leles-Gaudêncio et al. 2014) using seeds as explants.

There are micropropagation protocols using roots as explants in two species of *Catasetum* orchids (*C. gardneri* and *C. integerrimum*) have worked micropropagation protocols using roots as an explant. In the case of *C. gardneri*, in vitro plants were obtained with a developed of 3.75 cm root growth per explant (Peres et al. 2009). Indirect organogenesis was tested and observed in the production of *C. integerrimum* in vitro plants by adding kinetin as a plant growth regulator (López-Puc and Herrera-Cool 2022). No *Catasetum* orchid micropropagation protocol has reported leaves as an efficient explant to generate in vitro plants. Castillo-Pérez et al. (2021) tested this type of explant in *C. integerrimum*, obtaining a null response to regenerate seedlings (Fig. 3).

Table 4 Propagation effort by plant tissue culture techniques in *Catasetum* species

Species	Explant type used	Composition of the culture media	Response obtained	References
Catasetum boyi Mansf.	Seed	30 mg L^{-1} sucrose 2 g L^{-1} fertilizer B and G 100 mg L^{-1} coconut water 2 g L^{-1} activated carbon 4 g L^{-1} agar	90% seed germination was obtained	Fernandes et al. (2015)
Catasetum gardneri Schltr.	Protocorm	MS basal medium modified with 1/2 macronutrients	Vitroplants were obtained by direct organogenesis way with growth of 6 cm per explant, 3 roots developed per explant and pseudobulbs with 3 cm in diameter	Rego-Oliveira and de Faira (2005)
		Commercial formulation N.P.K (10–5-5) 2 mL L^{-1}	Vitroplants were obtained by direct organogenesis way with a growth of 8.04 cm per explant	
	Seed	MS basal medium 1 g L^{-1} activated carbon 30 g L^{-1} sucrose 7 g L^{-1} agar Jasmonic acid (concentration not mentioned)	Vitroplants were obtained by direct organogenesis way with the development of 2.4 roots per explant, 1 leaf per explant and approximately 1.75 cm leaf and root growth per explant	Silva-Maia and Pedroso-deMoraes (2017)
	Roots	Vacin and Went medium modified by substituting $Fe_2(C_4H_4O_6)_3$ by 27.8 mg dm^{-3} Fe-EDTA MS micronutrients Sucrose	Vitroplants were obtained with a developed of 3.75 cm root growth per explant	Peres et al. (2009)
	Vitroplants	Vacin and Went medium Micronutrients of MS 0.01% thiamine 0.1% soy peptone 2% sucrose 0.2% phytagel	In this work ethylene production showed a decreasing trend in the first 4 months, presenting an initial and a final concentration of 66.11 ± 10.68 and 21.92 ± 6.67 µL g^{-1} FW h^{-1}, respectively. Likewise, an increase in ethylene production was observed at the end of the 8 months	Rodrigues et al. (2013)

(continued)

Table 4 (continued)

Species	Explant type used	Composition of the culture media	Response obtained	References
			(198.64 ± 5.17), coinciding with the termination of a growth cycle	
	Nodal explants	Vacin and Went medium Micronutrients of MS 0.1% activated charcoal 2% sucrose 0.7% agar Ethylene 1-MCP	The chronic exposure to exogenous ethylene-induced severe growth deterioration in young plants during the 5 weeks of treatment, on the contrary, the supply of 1-MPC, induced morphological effects opposite to those induced by ethylene	
Catasetum integerrimum hook	Vitroplants	4.46 g L^{-1} MS medium 8 g L^{-1} agar plant 30 g L^{-1} sucrose 3 g L^{-1} activated carbon IAA BAP	Vitroplants were obtained with 5.73 ± 0.45 shoots per explant and 5.84 ± 0.48 leaves per shoot. Moreover, vitroplants developed 11.20 ± 0.28 roots per explant and 13.20 ± 0.28 cm root growth	Castillo-Pérez et al. (2021)
	Pseudobulb	4.46 g L^{-1} MS basal medium 8 g L^{-1} agar plant 30 g L^{-1} sucrose 3 g L^{-1} activated carbon 1 mg L^{-1} IAA 1 mg L^{-1} BAP	By direct organogenesis in vitro plants were obtained with 1.00 ± 00 shoots per explant, 5.50 ± 0.18 leaves per shoot, 4.37 ± 0.37 roots per explant with a growth rate of 4.88 ± 0.20 cm and a plant growth of 7.96 ± 0.12 cm	
	Plantlet	MS basal medium (half-strength) Sorbitol Carbon	The treatment added with 3% carbon, and 2% sorbitol presented the lowest value of growth in plantlet length (17.70 ± 5.8). In the same way, showed the lowest shoot formation (1 ± 00)	López-Puc and Herrera-Cool (2022)
	Root and node	MS basal medium 3% sucrose 2.2 g L^{-1} Gelrite 2 g L^{-1} activated carbon BAP Kinetin	Direct shoot organogenesis was observed in node explant in BAP-supplemented MS and kinetin-supplemented MS at all concentrations tested.	

(continued)

Table 4 (continued)

Species	Explant type used	Composition of the culture media	Response obtained	References
			Indirect shoot organogenesis was observed in root explant in MS supplemented with 4.64 or 9.29 μM kinetin	
Catasetum macrocarpum Rich. ex Kunth	Seed	½ MS basal medium 0.4 mg L^{-1} tiamin 100 mg L^{-1} myo-inositol 2% sucrose BA NAA	Vitroplants were obtained with 4.1 shoots per explant and 6.1 roots per explant	Ferreira et al. (2018)
	Vitroplant	First phase: Bioplant Prata® with sphagnum (1:1) Second phase: Bioplant Prata with Ouro Negro substrate (1:2)	The survival rates observed in the acclimatization process were 93.3% for the first phase and 96.6% for the second phase	
Catasetum pileatum Rchb. f.	Protocorm	MS basal medium 3% sucrose 0.8% agar-agar Kinetin IBA	8.63 regenerated PLB were obtained per explant with 12.70 leaves and 7.40 average roots	Zakizadeh et al. (2019)
	Protocorm	MS basal medium 3% sucrose 0.8% agar 1.00 mg L^{-1} BA 0.50 mg L^{-1} NAA Colchicine	For the polyploid induction, treatment with 4.00 mg l^{-1} colchicine for 72 h was the only treatment to result in a mixoploid seedling. Moreover, developed 4.16 and 4.12 cm root growth per explant, 7.00 roots per explant, 4.58 cm leaf growth per explant, and 6.66 cm leaf per explant	Kazemi and Kaviani (2020)
Catasetum schmidtianum F.E.L. Miranda and K.G. Lacerda	Protocorm	30 mg L^{-1} sucrose 2 g L^{-1} fertilizer B and G 200 mg L^{-1} coconut water 2 g L^{-1} activated carbon 4 g L^{-1} agar 1 mg L^{-1} extract pyroligneous	By direct organogenesis in vitro plants were obtained with 27.6 cm leaf growth per explant and 4.1 roots per explant	Florestino-Silva (2021)

(continued)

Table 4 (continued)

Species	Explant type used	Composition of the culture media	Response obtained	References
	Seed	10 mL L^{-1} Kudson C medium 30 g L^{-1} sucrose 24 g L^{-1} natural gelatin	By direct organogenesis in vitro plants were obtained with 3 mm Protocorm growth per explant	Leles-Gaudêncio et al. (2014)
	Vitroplants	Fertilizers B and G Coconut water Activated carbon Agar Sucrose Water Sphagnum Moss Vermiculite Carbonized rice straw Charcoal	The acclimatization treatment consists of Chile Moss + vermiculite + carbonized Rice straw + charcoal (1:1:1:1 v/v) presented the most suitable conditions for the development of the species	Arenas-deSouza and Vera-Karsburg (2016)

Fig. 3 Null in vitro response of leaves after 16 weeks of culture in an experiment with different concentrations and types of plant growth regulators for induction of direct organogenesis in *C. integerrimum*

Finally, the most used culture media for the micropropagation of Catasetum orchids are the MS medium and the Vacin and Went medium. Furthermore, activated carbon is commonly used for the micropropagation of *Catasetum* orchids and the most frequently used carbon source is sucrose. Plant growth regulators and additives (vitamins or natural extracts) vary depending on the objective of each study (Table 4).

7 Future Prospective and Conclusions

Some *Catasetum* species showed in vitro anti-inflammatory, cytotoxic, and antioxidant activities. The ethnomedicinal information of these plant species was validated. However, in vivo assays and their molecular mechanism of action remains to be elucidated. Most of the secondary metabolites isolated from the *Catasetum* orchids correspond to polyphenols, and many of these compounds have previously reported anti-inflammatory and antioxidant actions. Nevertheless, some *Catasetum* orchids lack of chemical composition of their metabolites. The isolation and elucidation of the structure of new compounds obtained from the *Catasetum* genus should be carried out.

There is limited information about the obtention of new compounds from the Orchidaceae family. It is also necessary to work on the biological and ecological aspects of the *Catasetum* orchids, such as growth and climatic conditions, seasons, exposure to sunlight, altitude, and genetic composition, due to these abiotic factors influence the chemical composition and the pharmacological effects of these plant species.

Biotechnological plant tissue culture techniques, including symbiotic and asymbiotic germination, clonal propagation, and direct organogenesis are available in this orchid genus. The use of biotechnological techniques can prevent and control the reduction of the pressure on wild species of this genus. In our laboratory (Environmental Science Research Laboratory—Autonomous University of San Luis Potosí, Mexico), we have worked with an efficient propagation protocol for *C. integerrimum* from pseudobulb sections and using the direct organogenesis technique (Castillo-Pérez et al. 2021). Moreover, we have begun to study the production of phytochemicals produced by in vitro orchids, inducing different types of stress in vitro, and comparing them with the homologous produced by wild plants for establishing a biotechnological technique for the production of bioactive compounds. Overall, *Catasetum* orchids remain to be studied for their pharmacological, ecological, botanical, chemical, and toxicological aspects.

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Diversity and Antimicrobial Potential of Orchidaceae-Associated Fungal Endophytes

Muhammad Adil, Pragya Tiwari, Jen-Tsung Chen, Rabia Naeem Khan, and Shamsa Kanwal

1 Orchidaceae-Associated Fungal Endophytes: Introduction and Significance

As a major and diverse family of flowering plants, Orchidaceae represents almost 750–850 genera and 25,000–35,000 species (Hossain 2011; Sarsaiya et al. 2019). Subtropical and tropical regions are blessed with the highest diversity of these ubiquitous plants, whereas, orchids are not found in hot deserts and Antarctica (Hossain 2011). Orchids are capable of occupying a wide range of habitats including forest floors, sandy dunes, and tree barks as epiphytes, lithophytes, saprophytes, and terrestrial plants (Ma et al. 2015). Apart from the photosynthesis process, mycoheterotrophism is also employed by the adult orchid plants for carbon acquisition (Zhang et al. 2018). Orchids are characterized by their remarkable capability of deceiving pollinators using several mechanisms such as rendezvous attraction, shelter imitation, generalized food deception, sexual deception, brood-site imitation, food-deceptive floral mimicry, and pseudo antagonism (Jersáková et al. 2006;

M. Adil (✉)
Pharmacology and Toxicology Section, University of Veterinary and Animal Sciences, Lahore, Jhang Campus, Jhang, Punjab, Pakistan
e-mail: muhammad.adil@uvas.edu.pk

P. Tiwari
Department of Biotechnology, Yeungnam University, Gyeongsan, Gyeongsangbuk-do, Republic of Korea
e-mail: pragyatiwari@ynu.ac.kr

J.-T. Chen
Department of Life Sciences, National University of Kaohsiung, Kaohsiung, Taiwan

R. N. Khan · S. Kanwal
Microbiology Section, University of Veterinary and Animal Sciences, Lahore, Jhang Campus, Jhang, Punjab, Pakistan

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Shrestha et al. 2020). Several orchid species have been associated with the production of storage organs in terms of pseudo-bulbs or bulbs (Śliwiński et al. 2022). Monopodial as well as sympodial growth patterns have been documented in orchids.

Although, predominantly grown for ornamental purpose, orchids also exhibit culinary and medicinal values, on account of different bioactive compounds including flavonoids, carotenoids, alkaloids, xanthones, and saponins (Hossain 2011; Cheamuangphan et al. 2013). The ethnomedicinal significance of orchids is considerably exploited in Ayurvedic and Chinese medicines (Bulpitt et al. 2008; Kobayashi 2020). *Calanthe*, *Ephemerantha*, *Coelogyne*, *Dendrobium*, *Galeola*, *Cymbidium*, *Eria*, *Ludisia*, *Gastrodia*, *Cypipedium*, *Habenaria*, *Nevilia*, *Thunia*, *Luisia*, and *Gymnadenia* represent the major genera of medicinal orchids (Bungtongdee et al. 2019).

Fungal microorganisms, known for internally colonizing and inhabiting the leaves, stems, roots, seeds, and flowers of plants, without inflicting any damage or infection, are referred to as endophytic fungi (Dhayanithy et al. 2019; Zhang et al. 2019). Therefore, the association of fungal endophytes with host plants is primarily meant for mutual benefits and described as mutualism or symbiosis (Khare et al. 2018). Endophytic fungi are mainly harbored by flowering plants, ferns, and grasses (Sudheep et al. 2017). Plants may be invaded by a single or multiple species of endophytes. These beneficial and non-pathogenic fungi are dependent upon their host for shelter and nourishment, and improve the uptake of nutrients, growth as well as tolerance of plants to abiotic and biotic stress in exchange (Velma et al. 2018; Rana et al. 2019; Devi et al. 2020). Orchids are completely dependent on endophytic fungi for the germination of seed and subsequent growth, due to lack of endosperm (Shah et al. 2019). Certain secondary metabolites are secreted by the endophytes for counteracting the plant defense mechanisms and thereby enhancing their viability within the host tissues (Tiwari and Bae 2022). Besides, endophytes may potentially modify or enhance the synthesis of phytometabolites (Ludwig-Müller 2015).

Orchidaceae-associated fungal endophytes can be cultured to harvest their bioactive metabolites for agricultural, industrial, and pharmaceutical applications (Bungtongdee et al. 2019). Several industrially-important extracellular enzymes including cellulase, lipase, laccase, pectinase, and amylase have been isolated from Orchidaceae-associated fungal endophytes (Paramanantham et al. 2019). Orchidaceae-associated *Penicillium* isolates have shown tolerance to copper and lead and can be potentially used for bioremediation purpose (Khan and Lee 2013; Idris et al. 2019). Some beneficial metabolites obtained from fungal endophytes have been linked with the conferral of plant protection against pathogenic fungi and pests (Duan et al. 2019; Yadav et al. 2020). The causal role of fungal endophytes in orchid–endophyte interaction has been explicated in terms of bioprotection, bioregulation, and biofertilization (Pant et al. 2017).

2 Diversity of Orchidaceae-Associated Fungal Endophytes

Despite the one million globally recorded species of fungal endophytes, less than 30% of the entire orchids genera have been screened for the isolation and identification of fungal endophytes (Sarsaiya et al. 2019). Since the conventional fungal classification is based on their spore-bearing structures and spores, the identification of some fungi becomes difficult due to failure of in vitro sporulation. Endophytic fungi belonging to *Fusarium*, *Aspergillus*, *Trichoderma*, *Verticillium*, *Colletotrichum, Xylaria,* and *Phomopsis* genera have been frequently recovered from orchids (Chen et al. 2013a; Ma et al. 2015). *Fusarium*, *Penicillium,* and *Aspergillus* are most common, whereas *Nigrospora*, *Guignardia,* and *Gliocladium* are relatively infrequent endophytes of *Bulbophyllum* orchids (Sudheep and Sridhar 2012; Sawmya et al. 2013). *Cattleya* orchids predominantly harbor *Epulorhiza* and *Colletotrichum* genera, while, *Tetracladium*, *Monilliopsis* and *Botrytis* are their minor endophytic fungi (Ovando et al. 2005; Da Silva et al. 2018). Apart from *Fusarium* and *Tulasnella* as the most frequent endophytic fungi, *Cylindrocarpon*, *Cryptosporiopsis*, and *Cyperus* have also been sporadically associated with *Cymbidium* orchids (Yu et al. 2015; Shubha and Srinivas 2017). Table 1 enlists the important Orchidaceae-associated fungal endophytes.

Aspergillus, *Fusarium*, *Trichoderma*, *Acremonium*, *Rhizoctonia*, *Xylaria*, *Alternaria*, *Colletotrichum*, and *Phomopsis* are major endophytes of *Dendrobium* orchids, whereas, *Aureobasidium*, *Curvularia*, *Thielavia*, *Westerdykella*, *Chaetomium*, and *Scolecobasidium* have been rarely isolated (Yuan et al. 2009; Mangunwardoyo et al. 2012; Sour et al. 2015; Jin et al. 2017; Shrestha et al. 2018). In addition to *Rhizoctonia* as the most frequent endophyte, *Oncidium* orchids are less commonly invaded by *Pestalotia* and *Aspergillus* (Otero et al. 2002; Mohamed and Joseph 2016). *Tulasnella* is the most widespread endophyte of *Paphiopedilum* orchids along with *Valsa*, *Penicillifer*, *Lasiodiplodia*, and *Rigidoporus* as the uncommon inhabitants (Khamchatra et al. 2016; Rajulu et al. 2016; Parthibhan and Ramasubbu 2020). *Rhizoctonia* and *Tulasnella* have been widely isolated from *Phalaenopsis* orchids than *Cochliobolus* and *Trichoderma* (Saha and Rao 2006; Rachanarin et al. 2018). *Vanda* orchids have been commonly linked with *Ceratobasidium*, *Alternaria*, and *Fusarium*, while, *Agaricus*, *Mycena*, *Armillaria*, *Russulaceae*, and *Moniliopsis* are their infrequent endophytes (Sudheep et al. 2012; Chand et al. 2020).

3 Antimicrobial Screening of Orchidaceae-Fungal Endophytes

The association of orchid plant species with endophytes is attributed to the plant-endophyte dynamics and microbial development via plant host association (Chutulo and Chalannavar 2018). The screening of endophytic fungi for antimicrobial

Table 1 Diversity of Orchidaceae-associated fungal endophytes

Host orchid plants	Endophytic fungi	References
Bulbophyllum kaitiense	*Aspergillus*, *Penicillium*	Kasmir et al. (2011)
Bulbophyllum neilgherrense	*Aspergillus*, *Colletotrichum*, *Fusarium*, *Gliocladium*, *Guignardia*, *Nigrospora*, *Penicillium*, *Pestalotiopsis*, *Trichoderma*, *Xylaria* species	Sudheep et al. (2012); Sawmya et al. (2013)
Cattleya jongheana	*Colletotrichum*	Da Silva et al. (2018)
Cattleya skinneri	*Epulorhiza, Penicillifer, Trichoderma, Fusarium, Aspergillus, Tetracladium, Verticillium, Pestalotiopsis, Monilliopsis, Botrytis*	Ovando et al. (2005)
Cymbidium aloifolium	*Cyperus, Fusarium, Trichoderma, Alternaria, Penicillium, Colletotrichum, Aspergillus*	Shubha and Srinivas (2017)
Cymbidium dayanum	*Corynascus*, *Fusarium, Xylaria*, *Phoma, Pestalotiopsis, Chaetomium, Colletotrichum*	Sour et al. (2015)
Dendrobium friedericksianum	*Fusarium*, *Pestalotiopsis*, *Xylaria*	
Dendrobium hercoglossum	*Chaetomium cochliodes, Xylaria Colletotrichum*, *Nigrospora* species	
Cymbidium faberi	*Umbelopsis, Tulasnella, Fusarium, Trichoderma*	Yu et al. (2015)
Cymbidium goeringii	*Cylindrocarpon, Cryptosporiopsis, Nigrospora, Fusarium, Exophiala, Tulasnella*	
Dendrobium crumenatum	*Cladosporium*, *Scolecobasidium*, *Colletotrichum*, *Guignardia*, *Curvularia*, *Fusarium*, *Westerdykella*, *Xylohypha*, *Pestalotiopsis*, *Xylaria* species	Mangunwardoyo et al. (2012); Sour et al. (2015)
Dendrobium loddigesii	*Acremonium, Cladosporium, Fusarium, Colletotrichum, Sirodesmium, Chaetomella, Pyrenochaeta, Nigrospora, Thielavia*	Chen et al. (2010)
Dendrobium nobile	*Colletotrichum, Hypoxylon, Clonostachys, Guignardia, Penicillium, Trichoderma, Phomopsis, Fusarium, Pestalotiopsis, Rhizoctonia, Xylaria*	Yuan et al. (2009)
Dendrobium officinale	*Alternaria, Aspergillus, Aureobasidium, Cochliobolus, Colletotrichum, Cystobasidium, Epicoccum, Fusarium, Pestalotiopsis, Trichoderma, Xylaria*	Jin et al. (2017)
Dendrobium speciosum	*Epicoccum nigrum, Fusarium, Trichoderma, Nigrospora, Phialophora, Tulasnella*	Boddington and Dearnaley (2008)
Dendrobium aphyllum	*Colletotrichum*, *Fusarium*, *Phomopsis*, *Xylariaceae* species	Chen et al. (2013a, b)
Dendrobium chrysanthum		
Dendrobium chrysotoxum		

(continued)

Table 1 (continued)

Host orchid plants	Endophytic fungi	References
Dendrobium crystallinum		
Dendrobium falconeri		
Dendrobium fimbriatum		
Dendrobium monoliforme	*Aspergillus*, *Fusarium*, *Cladosporium*, *Hypoxylon*, *Colletotrichum*, *Trichoderma*, *Helminthosporium*, *Leptosphaerulina*	Shrestha et al. (2018)
Dendrobium transparens		
Oncidium altissimum	*Rhizoctonia, Colletotrichum, Pestalotia, Xylaria*	Otero et al. (2002)
Oncidium species	*Rhizoctonia, Cercospora, Aspergillus*	Mohamed and Joseph (2016)
Paphiopedilum druryi	*Colletotrichum, Penicillifer, Tulasnella*	Parthibhan and Ramasubbu (2020)
Paphiopedilum fairrieanum	*Xylaria, Penicillium, Lasiodiplodia, Fusarium, Cladosporium*	Rajulu et al. (2016)
Paphiopedilum villosum	*Valsa, Coriolopsis, Nigroporus, Flavodon, Ceratobasidium, Rigidoporus, Tulasnella*	Khamchatra et al. (2016)
Phalaenopsis manni	*Cochliobolus, Trichoderma, Rhizoctonia*	Saha and Rao (2006)
Phalaenopsis pulcherrima	*Rhizoctonia, Epulorhiza, Tulasnella*	Rachanarin et al. (2018)
Vanda cristata	*Mycoleptodiscus, Agaricus, Fusarium, Paraconiothyrium, Alternaria, Pseudochaetospaeronema*	Chand et al. (2020)
Cymbidium sinense	*Epulorhiza, Tulasnella*	Nontachaiyapoom et al. (2010)
Paphiopedilum sukhakulii		
Vanda testacea	*Ceratobasidium, Fusarium, Xylaria, Rhizoctonia, Tulasnella, Thanatephorus, Serendipita, Russulaceae, Mycena, Moniliopsis, Erythromyces, Ceratobasidium, Armillaria*	Behera et al. (2013)

potential is defined by literature, suggesting that endophytes modulate the host defense mechanisms in accordance with the spectrum of pathogens. Orchid species are being widely explored for endophytic associations and their potential to produce promising antimicrobial compounds. With recent advances in scientific technologies/assay systems, research initiatives are undertaken to screen the potential endophytic fungi from various species of orchids. Different fermentation conditions and types are employed for the synthesis of bioactive products using the endophytic fungi (Tiwari et al. 2021a) and include potato dextrose medium (liquid culture),

23 days culture, 25 °C for mullein production from *Penicillium janczewskii* (Patil et al. 2016), fermentation medium, for 8 days at 30 °C for Taxol production from *Aspergillus aculeatinus* (Qiao et al. 2017) mineral medium (liquid), 3 days at 25 °C for Vincristine production from *Fusarium oxysporum* (Patil et al. 2016), submerged culture, 30 days at 25 °C for pyrrocidine A and B production from *Acremonium zeae* (Patil et al. 2016), grain–bran–yeast medium for 40 days at 28 °C for rhizoctonic acid production from *Rhizoctonia* species (Patil et al. 2016), among other techniques.

The fungal endophytes from orchids are cultured via solid-state fermentation or submerged fermentation and the conditions, namely temperature, pH, media composition, partial pressures of carbon dioxide and oxygen (pCO_2 and pO_2), aeration, etc. are optimized for maximum product recovery. These media parameters are crucial to metabolite production and differ accordingly. The different fungal endophyte strains are screened via antimicrobial (antibacterial, antifungal, antiviral) assays for validation of their antimicrobial properties. Moreover, the culture broth of endophytes is screened for bioactive properties via common methods, namely mycelial radial growth test, disk diffusion technique, and agar dilution assay (Songrong et al. 2005; Aly et al. 2008; Hoffman et al. 2008; Pongcharoen et al. 2008). The increased bio-prospection of endophytes colonizing different plants has demonstrated significant antimicrobial potential, particularly from medicinal plants including *Paris polyphylla var. yunnanensis*, fungal endophytes from *Garcinia*, *Ophiopogon*, and *Cyrtomium* species (Jiang et al. 2006; Phongpaichit et al. 2006; Li et al. 2008; Zhao et al. 2010).

4 Antimicrobial Potential of Orchidaceae-Associated Fungal Endophytes

Antimicrobial compounds are gaining popularity on account of their therapeutic potential in combating the pathogenic microorganisms. Alternative biological resources are extensively screened and employed to produce novel antimicrobials (Tiwari et al. 2021a, 2022b). The endophyte species are documented to be prolific producers of bioactive metabolites (Tiwari et al. 2022a), exhibiting potent pharmacological activities and are commercialized as marketed drugs. For instance, taxol, a multi-billion-dollar drug is synthesized by endophytic fungus, *Taxomyces andreanae* that was isolated from *Taxus brevifolia* (Tiwari et al. 2021b, 2022a). Orchid-associated endophytes have been implicated in the synthesis of diverse antimicrobial metabolites. Fungal endophyte species, namely *Xylaria*, *Phoma,* and *Fusarium*, isolated from *Dendrobium devonianum*, *Dendrobium officinale*, *Acianthera teres*, and *Acianthera setaceus* have been investigated for their antimicrobial potential. *Fusarium oxysporum* has been extensively screened for antimicrobial effects against various pathogenic microorganisms including *Saccharomyces cerevisiae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Candida albicans*, *Candida krusei*, *Sarcina lutea*, and *Escherichia coli* (Vaz et al. 2009; Jin et al. 2017;

Table 2 The antimicrobial potential of fungal endophytes from diverse orchid species

Fungal species	Orchid species	Test microorganisms	Reference
Fusarium nivale	*Dendrobium crumenatum*	*Candida tropicalis*, *Candida albicans*	Mangunwardoyo et al. (2012)
Streptomyces strains DR5–1, DR7–3, DR8–5, DR8–8	*Dendrobium* species	*Alternaria alternate, Fusarium oxysporium, Curvularia oryzae, Colletotrichum gloeosporioides*	Tedsree et al. (2022)
Xylaria species	*Anoectochilus setaceus*	*Methicillin-resistant, Staphylococcus aureus, Bacillus subtilis*	Ratnaweera et al. (2014)
Aureobasidium pullulan, fusarium oxysporum	*Dendrobium officinale*	*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginoa, Candida albicans*	Jin et al. (2017)
Fusarium oxysporum	*Acianthera teres*	*Staphylococcus aureus, Escherichia coli, Candida albicans, Candida krusei*	Vaz et al. (2009)
Fungal endophyte DO14	*Dendrobium officinale*	*Candida albicans, Cryptococcus neoformans, Trichophyton rubrum, Aspergillus fumigatus*	Wu et al. (2015)
Alternaria species	*Oncidium warmingii*	*Staphylococcus aureus, Bacillus cereus*	Vaz et al. (2009)
Phoma species	*Dendrobium devonianum, Dendrobium thyrsiflorum*	*Escherichia coli, Staphylococcus aureus, Bacillus subtilis*	Xing et al. (2011)

Bungtongdee et al. 2019). Table 2 enlists the antimicrobial activities of endophytic fungi recovered from diverse orchid species.

Bioactive products with promising antimicrobial activity have been derived from different endophyte species. A triterpenoid, helvolic acid, isolated from the organic endophyte extract of a Sri-Lankan orchid (*Anoectochilus setaceus*), displayed potent antimicrobial effect against *Bacillus subtilis* and methicillin-resistant *Staphylococcus aureus* (Ratnaweera et al. 2014). The antimicrobial properties of metabolites from fungal endophytes, found in Thai orchids were examined and out of the 97 isolates, 13 endophyte strains demonstrated antifungal activity against *Colletotrichum* species, *Fusarium* species and *Curvularia* species. In addition, endophyte CK F05–5 showed potent antifungal activity against *Fusarium* species (Bungtongdee et al. 2019). A fungal endophyte was isolated, characterized from *Dendrobium moniliforme*, and the presence of phenolics in the organic extract contributed to the antimicrobial properties of the host plant (Shah et al. 2019). Endophytic *Pyrenochaeta* species, recovered from *Dendrobium loddigesii*, revealed antimicrobial activity against *Bacillus subtilis* and *Aspergillus fumigatus* (Chen et al. 2010). *Phoma* species of endophytic fungi also demonstrated significant antimicrobial effects against *Staphylococcus aureus, Bacillus,* and *Escherichia coli* (Xing et al. 2011). Surprisingly, the antibacterial efficacy of Orchidaceae-derived fungal endophytes was superior to that of some existing antimicrobial drugs such as ampicillin (Xing et al. 2011).

5 Conclusion and Future Perspectives

In addition to their ornamental and culinary values, orchids also possess a wide range of phytochemical ingredients including terpenoids, phenanthrenes, steroids, and flavonoids (Zhang et al. 2015). Accordingly, the antimicrobial, anticancer, neuroprotective, antioxidant, hypoglycemic, hepatoprotective, and immunomodulatory actions of these valuable plants are traditionally being exploited in several forms of ethnomedicine for the treatment of various diseases (Kong et al. 2003; Pant 2013; Biswas et al. 2016). Besides, Orchidaceae-associated fungal endophytes also synthesize diverse bioactive metabolites such as alkaloids, peptides, quinones and phenolics, exhibiting anti-inflammatory, antineoplastic and antimicrobial properties (Jin et al. 2017; Pant et al. 2017).

Several species of Orchidaceae-fungal endophytes have been linked with antimicrobial effects (Singh et al. 2012). So far, the antimicrobial potential of *Phoma*, *Xylaria,* and *Fusarium* species of fungal endophytes associated with different orchids including *Anoectochilus setaceus*, *Acianthera teres*, *Dendrobium thyrsiforum*, *Dendrobium Officinale*, *Dendrobium lindleyi*, *Dendrobium devonianum*, and *Dendrobium crumenatum* have been analyzed. Consequently, the metabolic products of Orchidaceae-associated fungal endophytes can serve as lead compounds for potential development of new antimicrobial agents against the drug-resistant microbial pathogens (Cui et al. 2012). Recent advances in omics, medicinal chemistry and computer-aided drug development are projected to expedite the translation of complicated orchid–endophyte interaction into more prolific and ecofriendly therapeutic products. Nevertheless, challenges in terms of scarce taxonomic data, lack of biotechnologically based in vitro reproduction and rapid deterioration of orchid diversity necessitate adequate and long-term solutions. Moreover, bioactive metabolites of Orchidaceae-associated fungal endophytes with proven antimicrobial efficacy during in vitro assays should be further evaluated through appropriate in silico and in vivo studies.

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Asymbiotic Seed Germination in Terrestrial Orchids: Problems, Progress, and Prospects

Nora E. Anghelescu, Yavar Vafaee, Kolsum Ahmadzadeh, and Jen-Tsung Chen

1 Introduction

The species within the Orchidaceae family are among the largest and most diverse groups of flowering plants (Gaskett and Gallagher 2018). Scientific evidence indicates that the most recent common ancestor of extant orchids lived about 76–84 million years ago (the Late Cretaceous) (Ramirez et al. 2007). Throughout history, orchids have fascinated mankind, dating back thousands of years. Based on a Greek myth, *Orkhis* was a prince who fell in love with a priestess of Bacchus, but the creatures guarding her, tore him apart. The flowers that grew from his bloodshed were named after him (Ramirez et al. 2007). Therefore, this explains the origin of the name of one temperate genus, genus *Orchis* L., which later gave the name of the entire Orchidaceae family. The word "*orchid*" can also be traced back to the works of Theophrastus between 370 and 285 BC (Yam and Arditti 2017a). Due to the shape of the tuberoids of some orchids, they were considered to be aphrodisiacs, and another myth suggests that the tuberoids were the favorite food of satyrs (Gaskett and Gallagher 2018). With 899 genera, 27,801 species, and about 70,000 to 100,000

N. E. Anghelescu
Faculty of Horticulture, University of Agronomic Sciences and Veterinary Medicine of Bucharest, Bucharest, Romania

Y. Vafaee (✉) · K. Ahmadzadeh
Department of Horticultural Sciences and Engineering, Faculty of Agriculture, University of Kurdistan, Sanandaj, Iran

Medicinal Plants Breeding and Development Research Institute, University of Kurdistan, Sanandaj, Iran
e-mail: y.vafaee@uok.ac.ir

J.-T. Chen
Department of Life Sciences, National University of Kaohsiung, Kaohsiung, Taiwan
e-mail: jentsung@nuk.edu.tw

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interspecific, cultivated hybrids, Orchidaceae is the second most species-rich family among the flowering plants, after Asteraceae, comprising 10% of all systematically verified angiosperms and 40% of all monocotyledon species (Gaskett and Gallagher 2018; The Plant List 2020). From an evolutionary and phylogenetic point of view, orchids are among the most evolved plant species with a broad range of inter- and intra-specific variation, reflected as a wide morphological diversity including plant architecture and flower size, shape, color and smell, and variations that can be rarely seen in other plant families (Zhang et al. 2018; Otero and Flanagan 2006). All characteristics of the species undergoing active speciation are present among orchids, which live in a delicately balanced equilibrium with their ecosystem (Dressler 1982). Orchids represent the main interest in many scientific studies due to their amazing flower beauty, small and dust-like seed, unique pollination strategies and reproduction system, as well as due to their complex symbiosis association with mycorrhizal fungi (Zhang et al. 2018; Schluter et al. 2011). It is important to note that these outstanding features are considered evolutionary forces to retain or improve orchids' diversity and survival (Gaskett and Gallagher 2018; Rasmussen 1995; Shefferson et al. 2020). Orchidaceae is one of the most adaptive plant families, which has provided their species with the possibility of long-term survival (Shefferson et al. 2020). A typical adaptation mechanism among Orchidaceae species is the formation of a multilayer epidermis of dead cells, called velamen, present in the roots of many orchids, especially tropical orchids, protecting the root cortex from excessive drying and helping the water absorption (Zotz and Winkler 2013; Gravendeel et al. 2004). Another adaptation mechanism of orchids is their extraordinary flowers, which have a close and special relationship with pollinating insects (Schluter et al. 2011; Waterman and Bidartondo 2008). The third mechanism is the symbiotic relationship with mycorrhizal fungi, which makes orchids more tolerant of non-suitable habitats, thus helping their global spreading (Gao et al. 2020; Selosse et al. 2022). Therefore, members of the Orchidaceae family comprise a substantial variety of life forms including epiphyte, lithophyte, aquatic, and terrestrial, which are compatible with diverse niches from tropical forests to high alpine regions, except Antarctica, with the greatest species diversity in the tropical and subtropical region (Zhang et al. 2018; Tsiftsis et al. 2018; Acharya et al. 2011). They are found all over the world, from deserts and semi-scrubs to rainforests and tundra ecosystems (Acharya et al. 2011; Renz 1978). Renz (Renz 1978) indicated two distinct border lines of Mediterranean orchids grown in the Iranian plateau and Himalayan range (Fig. 1).

Among different life types of orchids, terrestrial species are typically grown in soil and produce fleshy underground round or palmate-shaped tubers. Many terrestrial species within the Orchidaceae family are on the red list of rare and endangered species of wild plants and animals (CITES) (Valletta et al. 2008; Hinsley et al. 2017) and some of them are at risk of extinction because of climate change, deforesting, land manipulation, tuber overexploitation, and illegal trade (Vafaee et al. 2021; Ghorbani et al. 2014a, 2014b). The harvest of underground tubers of terrestrial orchid species in the Anatolia and Middle East, the two main hotspots of terrestrial orchid species, has intensified due to increased global demand. For instance,

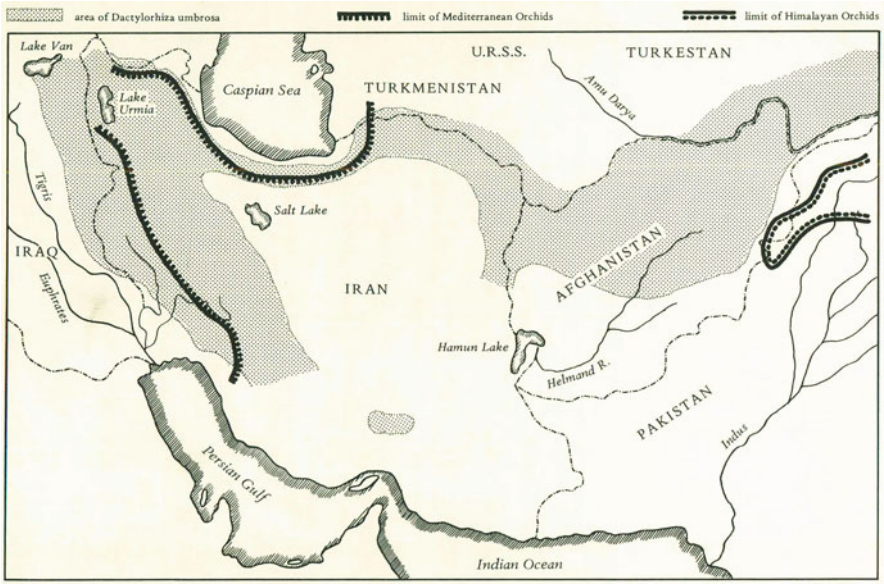

Fig. 1 The supposed distribution of distinct border lines for Europe-Mediterranean and Himalayan orchids (Renz 1978)

approximately 7–11 million orchid plants are annually harvested from the main terrestrial orchid diversity regions in Iran, and this pressure has put these medicinally valuable species in danger of extinction (Ghorbani et al. 2014a). In the presence of special mycorrhizal fungi, which provide essential nutrients for orchids, only a little ratio of orchid seeds can germinate in nature (Acharya et al. 2011; Renz 1978). Exploiting the large number of seeds produced within each capsule (about 0.2–2 million seeds (Valletta et al. 2008)), the asymbiotic seed germination procedure can be employed for large-scale propagation of terrestrial orchids through the generation of a high number of in vitro raised plantlets over a short period (Jolman et al. 2022). The present book chapter introduces terrestrial orchid species, describes their biology and conservational status, and focuses on in vitro conservation efforts performed on terrestrial orchids, with major emphasis on asymbiotic seed germination barriers. Moreover, it summarizes the performed research on asymbiotic seed germination of terrestrial orchid species highlighting the important variables including media components in particular organic supplements and plant growth regulators (PGRs).

2 Conservational Status of Orchids

Based on the state of the World's Plants and Fungi report released by Royal Botanical Gardens Kew, it has been highlighted that about 40% (two-fifths) of all plant species are at risk of extinction on a world scale which shows a double increase in the number of threatened species from 2016 to 2020 (Nic Lughadha et al. 2020). With the highest documented species number after Asteraceae, the Orchidaceae family is on the front line of extinction. In this regard, five orchid species have already been extinct, 87 are near threatening, 195 species are classified as vulnerable, 197 species are identified as critically endangered, and a total of 747 species are classified as threatened based on the IUCN Global Red List 2020 (Wraith et al. 2020). An individual tropical tree can harbor hundreds of epiphytic orchid species, so a small loss in habitat will impose profound negative impacts on orchid diversity and survival (Go et al. 2020). Several major and minor factors can, directly and indirectly, lead to the destruction of natural orchid habitats and declining their diversity and survival, as summarized by Hágsater and Dumont (Hágsater and Dumont 1996). Geographic distribution, habitat specificity, and population size all affect the efficiency of these factors on a given orchid species. The main causes are habitat destruction, modification, and fragmentation due to lodging, agriculture, artificial plantations, and overexploitation for ornamental, medicinal, and food purposes (Hágsater and Dumont 1996; Sezik 2002). Rare species are generally thought to have more specific habitat priorities than non-threatened species. A further factor responsible for orchid decline is environmental destruction, which can increase extinction risk through intensified climate change, soil erosion, and drought, among other factors (Gale et al. 2018; Swarts and Dixon 2009). Intense fires, floods, or severe environmental fluctuations are among the natural catastrophes threatening rare orchid species (Wraith et al. 2020; Phillips et al. 2020). Small and spatially isolated fragments of natural habitat destabilize populations and impede pollen and seed exchange (Kropf and Renner 2008; Cozzolino et al. 2005). Genetic diversity can be lost in fragmented populations, leading to decreasing the attraction of a diverse range of pollinators. For example, it has been shown that some terrestrial orchid species grown in Iran including *Himantoglossum affine* (Boiss.) Schltr., *Orchis simia* Lam. and *Anacamptis collina* (Banks & Sol. ex Russell) R. M. Bateman, Pridgeon & M. W. Chase are at risk of extinction due to environmental and anthropogenic impacts (Gholami et al. 2021a, 2021b; Kaki et al. 2020; Vafaee et al. 2017; Nosrati et al. 2011). Orchid conservation traditionally is based on three procedures including developing action plans, determining the population or conservation status at the species or genus level, and propagating and reintroducing cultivated individual plants of the threatened species into nature/the wild (Gale et al. 2018).

3 Terrestrial Orchid

Genealogically and phenologically, temperate, terrestrial orchids are similar to tropical orchids, the main differences being the underground fleshy tubers formed in soil and their rather smaller flowers (Djordjević and Tsiftsis 2022). The significant feature of terrestrial orchid species includes their complicated ecology, rareness, and capability to survive in almost all habitats (Rasmussen 1995; Swarts and Dixon 2017). Beyond their reproductive structures and pollination mechanisms, terrestrial orchids are unusual in many ways (Shefferson et al. 2020). It is important to study the terrestrial orchids, mostly temperate species, from a mycotrophic viewpoint if we are going to understand their biology. It is well documented that terrestrial orchids are more associated with mycorrhizal fungi than epiphytic counterparts. This is because the seedlings of these species stay underground and remain dependent on mycorrhizal fungi for a long time. In contrast, orchid seedlings growing epiphytically access light at their early-life stages and can start photosynthesis once the seedlings are established (Rasmussen 1995). Based on the World Conservation Union, terrestrial species account for one-third of all taxonomically verified orchids, while more than half of extinct, vulnerable, and critically endangered species belong to this life type of orchids (Swarts and Dixon 2009, 2017). Due to the multiplicity of threatening factors, terrestrial orchids have already experienced more injuries and are also more vulnerable to experience extinction in the future (Swarts and Dixon 2009). The terrestrial orchid genera which have attracted more attention in terms of conservational activities are *Cypripedium* L. (Bernhardt and Edens-Meier 2010), *Orchis* Tourn. ex L. (Fay et al. 2007), *Ophrys* L. (Devey et al. 2008), *Platanthera* Rich. (Knudson et al. 2015), *Dactylorhiza* Neck. ex Nevski (Hedrén 2001), *Himantoglossum* Spreng. (Dulić et al. 2019), *Goodyera* R.Br. (Wong and Sun 1999), *Cephalanthera* Rich. (Hasegawa et al. 2017), *Epipactis* Zinn (Squirrell et al. 2002), and *Serapias* L. (Bellusci et al. 2009). In Fig. 2, the flower and inflorescence morphology of some endangered terrestrial species have been shown.

4 The Life Cycle of Terrestrial Orchids

Terrestrial orchids have a long-life cycle in nature where they need 2–5 years to enter the reproductive phase and to produce mature seeds (Balilashaki et al. 2020; Delforge 2006). Seeds, protocorms, juveniles, dormant adults, vegetative adults, and flowering individuals account for the six primary stages of the terrestrial orchid life cycle (Shefferson et al. 2020; Harrap and Harrap 2009) (Fig. 3). The life cycle of terrestrial orchids starts with symbiotic seed germination, which is a complex process requiring special microclimate and micro-edaphic conditions besides the relationship with mycorrhizal fungi (Rasmussen et al. 2015; Fatahi et al. 2022a). Cell division of the embryo within the dust-like seed leads to the formation of the protocorm, a special structure containing leaf and shoot primordia (Cardoso et al.

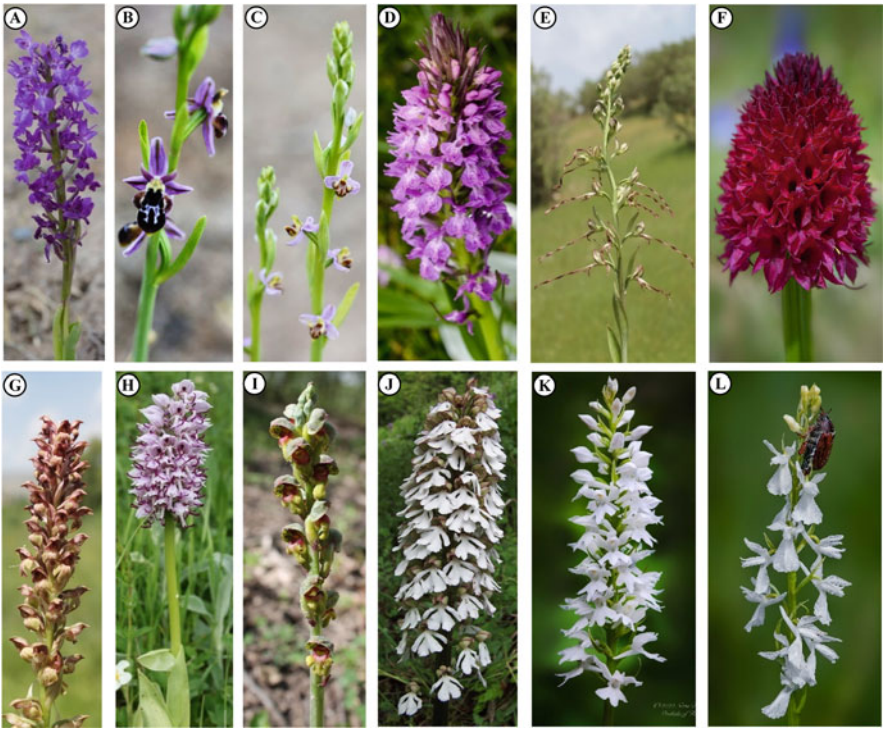

Fig. 2 The flower and inflorescence morphology of some most endangered terrestrial orchid species. (**a**) *Orchis mascula* (L.) L.; (**b**) *Ophrys reinholdii* subsp. *straussii* (H.Fleischm.) E.Nelson; (**c**) *Ophrys schulzei* Bornm. & Fleischm.; (**d**) *Dactylorhiza umbrosa* (Kar. & Kir.) Nevski; (**e**) *Himantoglossum affine* (Boiss.) Schltr.; (**f**) *Nigritella nigra* subsp. *bucegiana* Hedrén, Anghel. & R. Lorenz, subsp. nov.; (**g**) *Anacamptis coriophora* (L.) R. M. Bateman, Pridgeon & M. W. Chase; (**h**) *Orchis simia* Lam.; (**i**) *Steveniella satyrioides* (Spreng.) Schltr.; (**j**) *Orchis purpurea* Huds., (**k**) *Dactylorhiza fuchsii* (Druce) Soó subsp. *carpatica* (Batoušek & Kreutz) Kreutz var. *albiflora*; (**l**) *Anacamptis palustris* (L.) R.M.Bateman, Pridgeon & M.W.Chase subsp. *elegans* (Heuff.) R. M. Bateman, Pridgeon & M. W. Chase var. *albiflora*. Photos a–e, g–j © Yavar Vafaee, Abdolbaset Ghorbani, Iran; Photos f, k, l © Nora E. Anghelescu, Romania

2020). The protocorm is the primordial stage of terrestrial orchids' life cycle, which develops underground, and is found in the Orchidaceae and Pyroloideae families (Shefferson et al. 2020; Yeung 2017). Upon the development of protocorm, a high density of rhizoids will be generated, which will help the absorption of the essential nutrients from the surrounding medium (Piria et al. 2008). The length of the first post-germination winter determines the seedling's ability to transition from the protocorm to the young plantlet stage. In this term, some species even require more than one winter season (Rasmussen 1995). In the next stage, the protocorms develop root-tuber structures (also known as mycorrhizae), from which small plantlets will start developing, after spending the dormant phase (Harrap and Harrap 2009). The seedlings can live underground for months or even years, where they

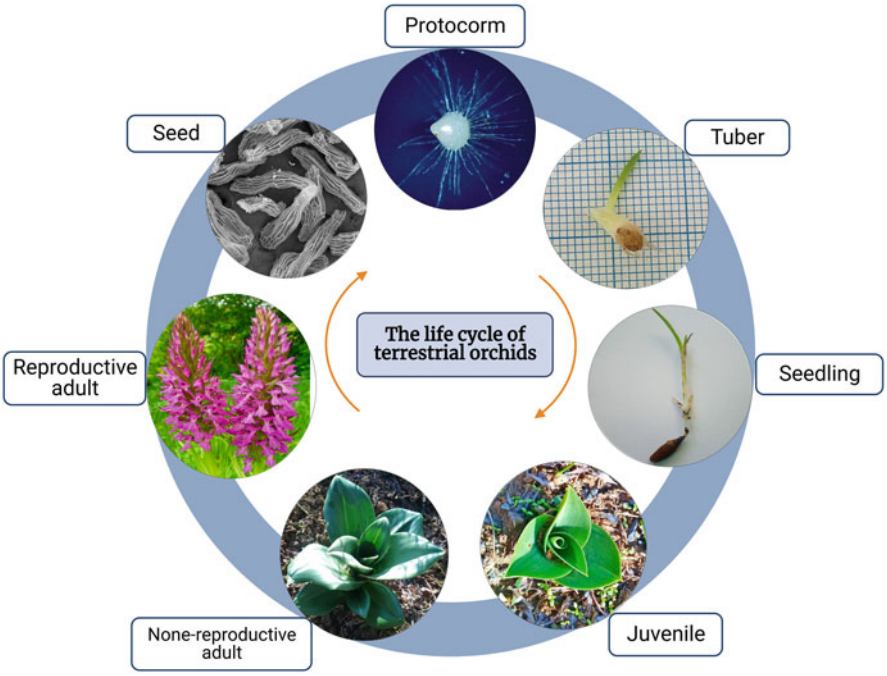

Fig. 3 The life cycle of terrestrial orchids. After Shefferson, Jacquemyn (Shefferson et al. 2020). (The diagram created with Biorender.com)

exclusively depend on mycorrhizal fungi to obtain their required nutrients, being called mycotrophs (Harrap and Harrap 2009). It is important to consider various factors such as the depth of the germination, the porosity of the soil, concentration of humus, climate and genetic variation that may affect how long the underground phase lasts (Rasmussen 1995).

Like many other higher plants, orchids exploit asexual propagation means besides sexual reproduction (Yam and Arditti 2017a). This includes vegetative reproduction through the root-tuber structure which results in the generation of genetically identical individuals. Vegetative reproduction can be seen in almost all types of terrestrial orchid root systems as classified by Tsiftsis, Štípková (Tsiftsis et al. 2018) including rhizomatous (*Cephalanthera* Rich., *Corallorhiza* Gagnebin, *Epipactis* Zinn, and *Epipogium* Sw.), intermediate (*Dactylorhiza* Neck. ex Nevski, *Gymnadenia* R.Br., and *Platanthera* Rich.) and tuberous orchids (*Anacamptis, Himantoglossum* Spreng., *Ophrys* L. and *Orchis* Tourn. ex L.). In tuberous orchids, during the autumn of the second year, root and shoot meristems started to activate, producing a young plantlet that remains dormant during the winter (Malmgren 1996). During the spring of the third year, the axillary bud of the mother tuber produces a new tuberoid that survives in the next dormant season and generates a new shoot in the growing season (Figs. 4 and 5). In Iran and Turkey, the collectors

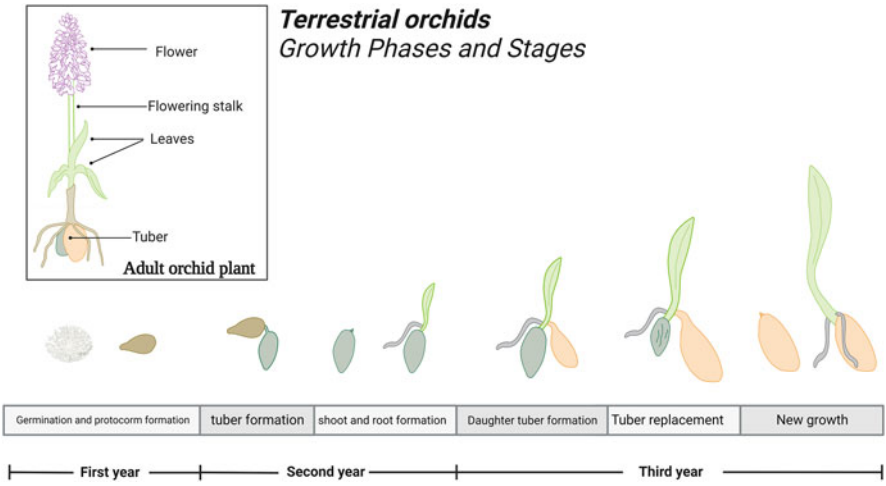

Fig. 4 The growth phases and stages of terrestrial orchid species. The brown and gray parts are the protocorm and mother tuber, respectively. The pink part is new formed daughter tuber. After Rasmussen (1995) and Harrap and Harrap (Harrap and Harrap 2009). (The picture created with Biorender.com)

Fig. 5 The development of the daughter tuber on the mother tuber as a vegetative reproduction system in (**a**). *Ophrys reinholdii* subsp. *straussii* (H. Fleischm.) E. Nelson and (**b**). *Gymnadenia conopsea* (L.) R.Br. Photos © Yavar Vafaee, Iran

pick the daughter young tuber and leave the mother tuber for the next growing season, a traditional conservational activity mitigating the overharvesting pressure on terrestrial orchid species (Ghorbani et al. 2014a, 2014b).

At the time of harvest, each plant has an old tuber, which has a stem and flower, and a fresh, fleshy tuber, which is for the next year's plant growth (Fig. 5). Old tubers are rough and wrinkled. In most cases, collectors collect the plant before the seeds are formed, which is a limitation of reproduction in wild populations (Kreziou et al. 2016).

5 Salep and Tuber-Derived Products

Besides medicine, orchid products are widely used in the food industry to make traditional ice creams and beverages with special rheological properties (Kurt 2021; Şen et al. 2018; Kurt and Kahyaoglu 2015). The tubers of terrestrial orchids are rich sources of glucomannan (GM), which consists of linear chains of glucose and mannose connecting with 1–4 beta glycosidic bonds (Kurt 2021) (Fig. 6). In Mediterranean and Middle East countries, the underground tubers usually harvest and boil in water or milk, and then dry to prepare salep powder (Şen et al. 2018; Sen et al. 2019). During salep preparation, no cleaning and purification process is performed and the resulting powder is used directly in various formulations (Ece Tamer et al. 2006; Jagdale et al. 2009). However, salep has other constituents including starch, protein, and ash, which usually consider factors reducing the quality of the salep powder. A typical salep sample can include 8–48% glucomannan, 5–44% starch, 2.7–12% protein, and 1.5–6.8% ash (Şen et al. 2018). As an anti-constipation constituent, GM generally causes bowel movements for 12–24 h (Kurt 2021; Kurt and Kahyaoglu 2015). On the other hand, GM is a natural water-soluble fiber that can regulate blood sugar, help hypoglycemia alleviation, and reduce stress (Tekinşen and Güner 2010). It can also act as a preventive agent for chronic diseases and obesity (Jagdale et al. 2009).

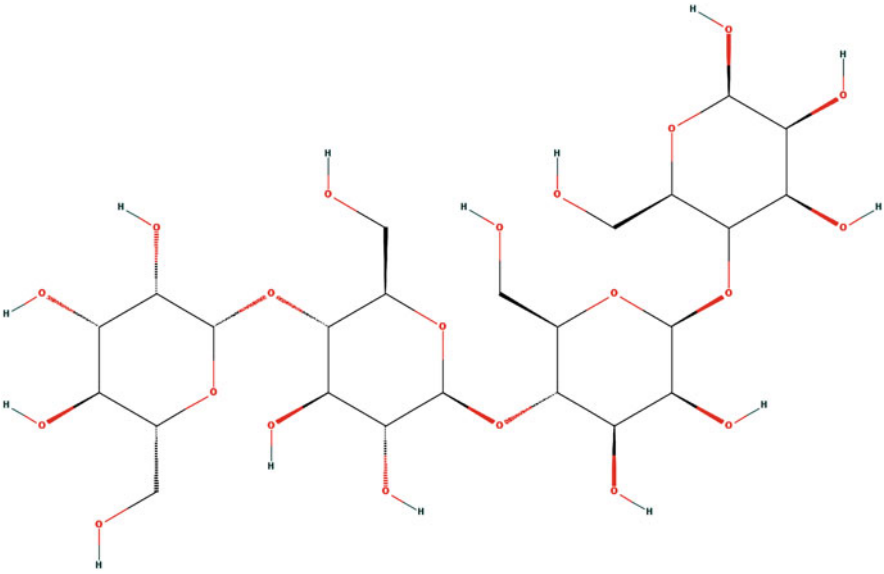

Fig. 6 Chemical structure of glucomannan, a polysaccharide found in a high ratio in terrestrial orchid species. (Source: Kim, S., et al., PubChem 2023 update. Nucleic Acids Research, 2023. 51 (D1): p. D1373–D1380)

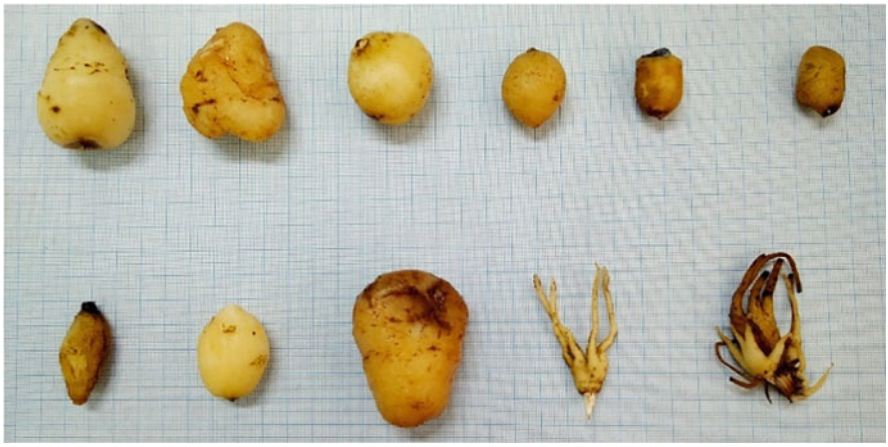

Fig. 7 Tuber morphology of tuber in selected terrestrial orchid species. Scale: 1 cm

In recent years, the high demand for salep-based beverages and ice creams and also for its food and medicinal products has attracted the attention of collectors to supply tuber material from nature (Ghorbani et al. 2014b; Sezik 2006). Salep is a white flour obtained by grinding the dry tubers from terrestrial orchid species (Kurt 2021). About 24 genera and 90 species of terrestrial orchids within the Orchidaceae family are used to produce salep powder (Sen et al. 2019). The genera *Anacamptis* Rich., *Himantoglossum* Spreng., *Orchis* Tourn. ex L., *Ophrys* L., and *Serapis* L. are orchids with round or oval tubers and the genus *Dactylorhiza* Neck. ex Nevski with palmate and finger-shaped tubers are among the most used taxa to produce salep powder (Ece Tamer et al. 2006; Ghorbani et al. 2017). Approximately 30 tons of orchids are annually harvested in Turkey (Kurt 2021) which accounts for 30–120 million individual terrestrial orchid plants. The shortage of natural populations of terrestrial orchids in Turkey has shifted the harvesting pressure to neighboring countries. In this regard, a volume of 7–13 million terrestrial orchid plants is harvested in Iran belonging to 30 species and sub-species mainly growing in the Alborz and the Zagros Mountain basins (Vafaee et al. 2021; Ghorbani et al. 2014a, 2014b). Figure 7 shows the tuber morphology of some terrestrial orchid species grown on the Iranian plateau.

Hot salep is a viscous milky drink with unique rheological features that is widely consumed in Turkey during the winter season (Karaman et al. 2012). It is prepared by boiling salep powder and milk with sugar and then sprinkling cinnamon on top (Ece Tamer et al. 2006; Dogan and Kayacier 2004). In Greece, it is also widely used in local markets as a traditional warm beverage in winter. It is interesting that before the introduction of coffee, the salep drink was common in Europe (Kreziou et al. 2016). Although there are alternatives such as carboxymethyl cellulose (CMC) due to the unique and special organoleptic and rheological features of salep and also because of CMC side effects, there is still an increasing demand for original salep powder (Kurt 2021; Sen et al. 2019; Kargar Jahromi et al. 2018).

6 Seed and Embryo in Terrestrial orchids

Despite their microscopic size, orchid seeds are produced in large numbers where an individual orchid capsule may contain about 0.2–2 million seeds (surprisingly about four million seeds per capsule in *Cycnoches ventricosum* Bateman) (Sonkoly et al. 2016; Arditti and Ghani 2000). As a result, orchid seeds are among the smallest known seeds in the plant kingdom. All orchids including terrestrial species have tiny and dust-like seeds which makes the tracing of seed dispersal and monitoring of germination and plantlet growth challenging (Rasmussen and Whigham 1993; Ren et al. 2017). These stages could comprise seed releasing from dehiscent capsules to the symbiotic establishment and seedling development (Rasmussen 1995; Rasmussen et al. 2015; Süngü Şeker et al. 2021). However, with the advent of current domestication platforms for terrestrial orchids, it is possible to trace and monitor seed dispersal and establishment at pilot levels (Rasmussen 1995). On the other hand, considering the geographical distribution of orchids and the physical properties of seeds, some reasonable assumptions can be made about seed dispersal (Rasmussen 1995). Aside from their size (from 0.05 in *Anoectochilus imitans* Schltr. to 6 mm in length in *Epidendrum secundum* Jacq., showing 120-fold size differences) and shape, orchid seeds particularly in terrestrial species represent a remarkable diversity in their testa architecture and sculpture (Vafaee et al. 2021; Gholami et al. 2021b; Arditti and Ghani 2000; Barthlott et al. 2014). Having a large number of air spaces, seeds are ideally adapted to being dispersed by wind (Hedrén et al. 2021). This allows orchids to disperse their seed kilometers far from their main niches (100 and 250 km for *Orchis militaris* L. and *Orchis simia* Lam., respectively (Rasmussen 1995)) leading to higher dispersal rates, maintenance, and extension of genetic diversity throughout geographical and ecological boundaries and in the same time reducing parental investment per seed (Hedrén et al. 2021). The seed morphometric characteristics play an important role in the systematic and taxonomic analyses of terrestrial orchid species, and species-specific patterns have been found for various epiphytic and terrestrial orchid species (Vafaee et al. 2021; Barthlott et al. 2014). The morphometric variation of seed testa in orchids could be attributed to the ways of dispersion and dormancy status (Barthlott et al. 2014). The color of orchid seeds varies greatly from whitish to dark brown which is determined by the seed coat and especially by the embryo. Figure 8 shows the seed morphology and structure of some threatened terrestrial orchid species collected from the Iranian plateau.

Compared to other flowering plants, orchids exhibit a unique seed development pattern (Fang et al. 2016). The pattern of orchid embryo development is unique among flowering plants for several features including lack of cotyledon and endosperm, the various morphology of suspensor, and the simple seed coat (Lee et al. 2007). The seeds of most flowering plants are known for having an embryo that differentiates into cotyledon(s), radicle, plumule, and hypocotyl, but in orchids, embryo development is not as advanced as other flowering plants (Yeung 2017; Lee et al. 2007; Kauth et al. 2006). In orchids, the embryo is poorly differentiated and the meristems and cotyledons are usually absent at the time of seed maturity

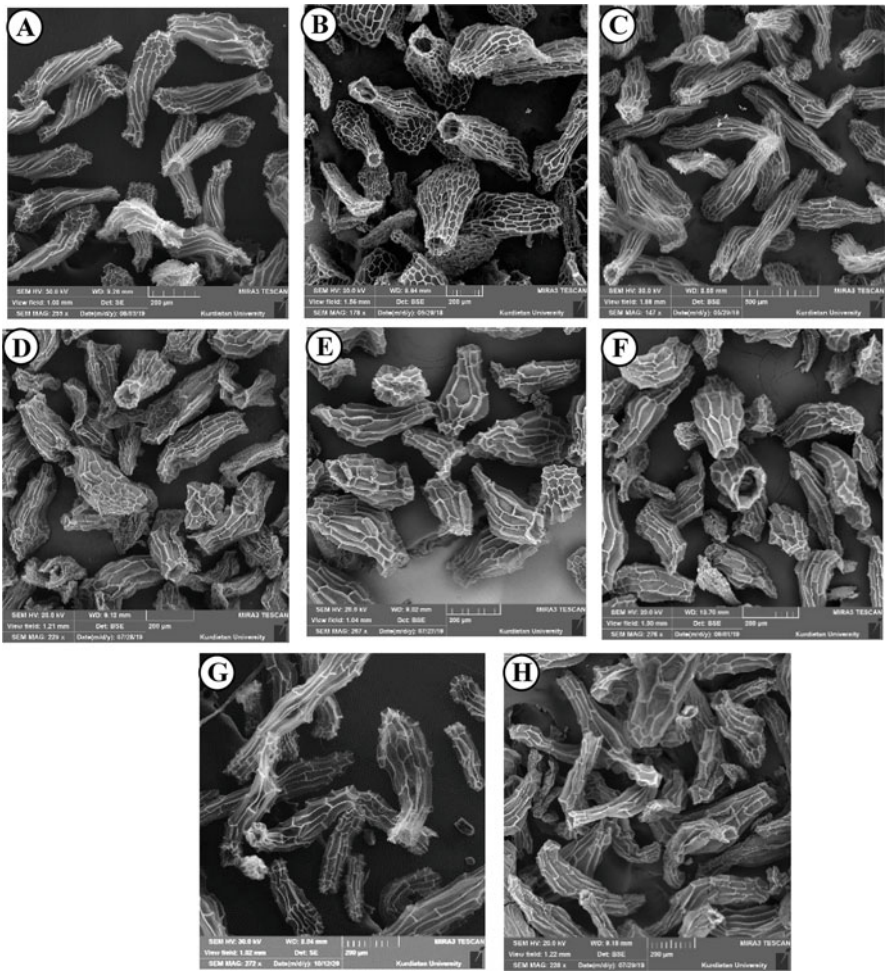

Fig. 8 The seed morphology of some threatened orchid species. (**a**) *Anacamptis coriophora* (L.) R. M. Bateman, Pridgeon & M. W. Chase; (**b**) *Dactylorhiza umbrosa* (Kar. & Kir.) Nevski; (**c**) *Ophrys reinholdii* subsp. *straussii* (H.Fleischm.) E. Nelson; (**d**) *Orchis mascula* (L.) L.; (**e**) *Himantoglossum affine* (Boiss.) Schltr.; (**f**) *Orchis simia* Lam.; (**g**) *Ophrys cilicica* Schltr. (prev. *Ophrys kurdistanica* Renz); (**h**) *Himantoglossum comperianum* (Steven) P.Delforge (part of research studies performed at the Research Center for Terrestrial Orchid, RCTO, university of Kurdistan)

(Balilashaki et al. 2020; Yeung 2017). Although in some orchid species, there may be more than one embryo (polyembryony), for example, the presence of more than 12 embryos in the seed of *Thecostele alata* (Roxb.) E.C.Parish & Rchb. f. species (Barthlott et al. 2014). An embryo gradually expands and fills the endosperm cavity, as the polar-chalazal complex degenerates at the beginning of seed development (Yeung 2017; Lee et al. 2007). An increase in embryo volume occurs during the

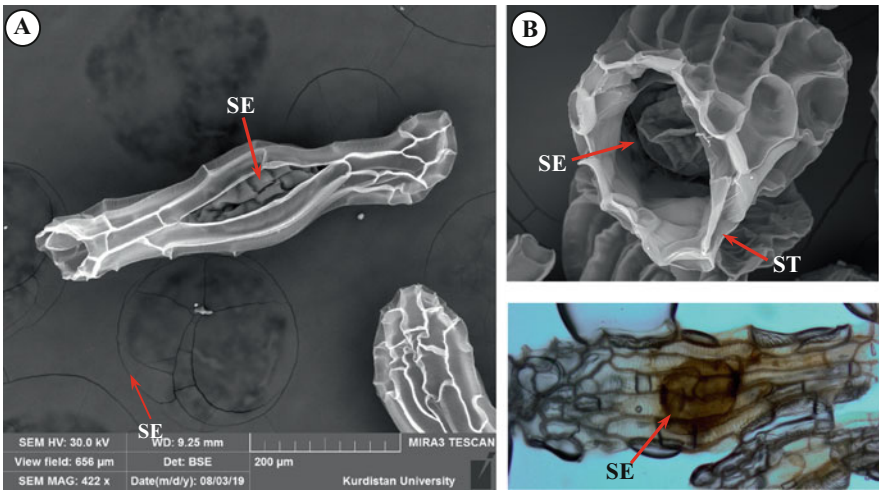

Fig. 9 The testa and embryo as revealed by SEM in (**a**). *Himantoglossum comperianum* (Steven) P.Delforge and (**b**). *O. simia* Lam. and using light microscopy in (**c**). *Ophrys reinholdii* subsp. *straussii* (H.Fleischm.) E.Nelson. *SE* seed embryo; *ST* seed testa

generation of globular embryos due to cell divisions in the outermost as well as the inner layers of the embryo proper (Lee et al. 2007). The seed coat develops from the integuments (maternal tissues) into a thin layer with varied surface characteristics. In Fig. 9, the SEM and light microscopic images of seed-containing embryos have been shown in some typical terrestrial orchid species.

7 Symbiosis with Mycorrhizal Fungi

Recent investigations have revealed that an increasingly large number of green orchids, in the genera *Cypripedium* L., *Cephalanthera* Rich., *Corallorhiza* Gagnebin, *Epipactis* Zinn, *Epipogium* Sw., *Limodorum* Boehm., *Gymnadenia* R. Br., *Neottia* Guett., *Orchis* Tourn. ex L. and *Platanthera* Rich (Shefferson et al. 2020; Abadie et al. 2006), obtains large amounts of their carbon from associations with ectomycorrhizal fungi. Current research using molecular techniques has begun to elucidate the type of fungi found in association with orchids. All of the fungi identified thus far that form orchid mycorrhiza typically belong to the division Basidiomycota Moore, R.T. Rhizoctonia-forming fungi or higher fungi, which occurred in the most ancestral orchid lineages, and today are most widespread in the family. More specifically, the mycorrhizal fungi mainly come from four families, Ceratobasidiaceae G.W. Martin (genus *Rhizoctonia* D.C., genus *Ceratobasidium* D.P. Rogers), Sebacinaceae K.Wells & Oberw. (genus *Sebacina* Tul. & C.Tul.),

Tulasnellaceae Juel (genus *Tulasnella* J.Schröt., genus *Epulorhiza* R. T. Moore) and Russulaceae Lotsy (genus *Russula* Pers.). They are usually saprotrophs, which feed on decaying wood, leaf litter or dung, ectomycorrhizal fungi attached to tree roots, and parasites on other plants (Rasmussen et al. 2015; Favre-Godal et al. 2020). The ectomycorrhizal fungi are generally symbiotic with the roots of neighboring photosynthetic trees. They obtain simple carbohydrates from the photosynthetic leaves of the trees and, in return, they provide minerals, amino acids, water, etc., to them (Selosse et al. 2022). Studies showed that the orchids managed to hitch-hike the hyphae of the ectomycorrhizal fungi and thus gain direct access to the flow of readily synthesized nutrients that come from the photosynthetic leaves. By associating with these tree-symbiotic fungi (the ectomycorrhizal fungi), the orchids ultimately became parasites on the trees, directing the abundant flow of nutrients straight into their roots (Selosse et al. 2022). This mutualistic association provides the fungus with relatively constant and direct access to carbohydrates, such as glucose and sucrose (Selosse and Cameron 2010). The carbohydrates are translocated from the tree source, usually the leaves, to root tissue and onto the plant's fungal partners. In return, the plant gains the benefits of the mycelium's higher absorptive capacity for water and mineral nutrients due to the large surface area of fungal hyphae, which are much finer than plant roots, thus improving the plant's mineral absorption capabilities. The mycelium can send extremely fine filaments far out into the soil, which acts as root extensions (Dearnaley et al. 2016). These filaments are far more effective in nutrient and water absorption than the plant roots themselves. The mycorrhizae enable them to grow much more quickly than they would otherwise. It has been estimated that the mycorrhizae increase the nutrient absorption of the plant by a factor of 100–1000 times (Li et al. 2021). This phenomenon used to be termed epi-parasitism or hyper-parasitism (to be a parasite on another parasite). Yet orchids are not alone in benefitting from such a relationship with ectomycorrhizal fungi. It is now known that 90% of plant species interconnect and have mutually beneficial relationships with mycorrhizae, but for these to exist, the soil must be undisturbed. These fungi have been fundamental to plant growth for the last 460–400 million years (Wang 2009; Kanchan et al. 2022).

7.1 The Damaging Effect of Phytoalexins

Nevertheless, at certain moments in time, the friendly, mycorrhizal fungus has the potential to grow excessively and turn the tables on the orchid, becoming parasitic on the roots. If the infection would not be stopped on time, the fungus may extend its pelotons to the entire rhizome, the base of the stem, and leaves. In protocorms, this phenomenon may be lethal, the fungus being able to infect the whole protocorm body, ultimately destroying it. Having said that, this sudden and seemingly uncontrolled invasion generally does not take the orchid by surprise because these plants have adapted to produce highly effective, "home-made," specific fungicides, known as phytoalexins. These poisonous substances allow the orchid to keep control

of the expansion of the hyphae, without destroying or killing them. In most cases, the fungi remain alive, for at least a certain period, and benefit the orchid by releasing the needed nutrients. Phytoalexins also limits the penetration of hyphae to specific areas, such as the aboveground organs (leaves, stem, flowers). They are synthesized locally, before the initial fungal infection, initially by the protocorms' rhizoids, and later, by the entire root system. The production increases significantly in response to fungal infection or wounding (Pavarino 1909; Bernard 1911). Several phytoalexins were isolated from orchids. To mention a few, orchinol, discovered in 1957 and isolated from *Orchis militaris* L., was shown to be widespread in many European terrestrial orchids, as well as loroglossol and hircinol that were isolated from *Loroglossum hircinum* (L.) Rich. [today *Himantoglossum hircinum* (L.) Spreng.], both discovered by Bernard in 1910 (Bernard 1921; Bernard and Costantin 1916). Thus, the orchid can control and regulate the timing and degree of fungal association, presumably providing a sufficient reason for the fungi to colonize and re-associate with it. The degree of colonization changes over the season, indicating that the orchid is controlling the uptake of nutrients while preventing parasitism by the fungus. While fungal food sources have become a life condition for orchids, one might ask how strong is the impact of orchid predation on fungal survival and evolution (Jones and Smith 2004). Up until now, it has not been demonstrated that orchid poisonous effect substantially affects fungal health and vigor. However, in some areas, it has been reported that the aboveground production of mushrooms (the fruiting bodies of fungi) was much lower in mycelia that supported orchids, as compared to mycelia of related fungal species, which do not associate with them. This might indicate that fungal fitness was reduced by the poisonous effect of orchids on their fungal partner. Moreover, the damaging effect was, subsequently, affecting other exosystemic interactions, damaging entire hyphal networks, and reducing plant species' resistance and development. It remains to be fully demonstrated if the reduction in fungal diversity in specific habitats was solely due to the poisonous effect of a sudden increase in the production of phytoalexins or if it was also due to the simultaneous, combined effect of other factors such as changes in the substrates pH, temperature, humidity, etc. Despite these observations, it is well known that orchid mycorrhiza, as well as all the other complex mycorrhizal networks, can heal and recover rapidly. This would diminish, at least partially, the effects orchids have on their specific fungi. At the same time, during their evolution, the fungi have not developed any significant avoidance or defense mechanisms against orchid poisonous effects. This leads to the conclusion that fungi are generally able to gradually recover and, in time, re-establish the ectomycorrhizal associations specific to certain, particular ecosystems (Merckx and Bidartondo 2008). However, in case of temporary loss/disappearance of specific symbiotic fungal partners, germination of seeds may be significantly affected, even if the loss of fungal parents lasts for 1–6 months to 1 year. This generally would be sufficient to affect entire generations of germinating seeds or protocorms in their first stages of development when they are entirely dependent on healthy, strong fungal associations.

7.2 Destruction of Ecosystems/Natural Habitats

As mentioned in the previous section, orchids are highly dependent on the activities of both the specific fungi and the trees that sustain them, from the initial, early stages of development and, in many cases, throughout their adult life. This explains why particular orchids are only found in woodlands that contain specific types of trees (Yeh et al. 2019; Jacquemyn et al. 2017). For instance, the chlorophyll-deficient *Corallorhiza trifida* Châtel. is associated with the ectomycorrhizal fungi of the genus Tomentella (Thelephoraceae family), which populate the roots of birch and willow in some areas and pine trees in others (De Angelli and Anghelescu 2020). Recent studies showed that *Corallorhiza trifida* Châtel. derives about 52% of its nitrogen and 77% of its carbon from the associated fungi and therefore it is particularly sensitive to the type of trees and fungi associated with. Another example is *Limodorum abortivum* (L.) Sw., which in some areas has a particular association with pine trees, while in others with beech or oak trees (Bellino et al. 2014; Wang et al. 2021a). It seems that most orchid species grow in woods not because of the shade and moisture they provide, but because of the presence of the specific fungi that are dependent on certain trees. This makes the orchids particularly sensitive to any environmental change or destruction. Dr. Kenji Suetsugu (Kobe University), in 2016, discovered the elusive Japanese orchid, *Lecanorchis tabugawaensis* Suetsugu & Fukunaga, explains the significance and importance of fungi-dependent plants for the ecosystems in which they live: "*Due to the sensitivity of mycoheterotrophic plants, it has long been suggested that their species richness provides a useful indicator of the overall floral diversity of forest habitats. A detailed record of the distribution of these vulnerable plants, therefore, provides crucial data for the conservation of primary forests*."

Wherever the natural habitats/ecosystems remain unaltered by human presence and activity, a perfect balance is established between the varied plant and animal species living together in the same natural habitat. As long as there is no human disruptive intervention, each ecosystem self-regulates. But any interference by humans can have unpredictable consequences, which can lead to the destruction of the existing balance in that ecosystem. Due to their complex way of life, the associations with a mycorrhizal fungus, and the sophisticated yet low-efficiency reproductive cycle, orchids need stable ecosystems (Rasmussen et al. 2015). For these sensitive plants, any change or alteration to their environment can lead to a rapid reduction in numbers and eventually to their disappearance (Hágsater and Dumont 1996). Where there is the correct blend/mixture of fungi present within the soil, orchids, as well as other plant species can flourish. When this equilibrium is disturbed, for instance, by deep cultivation, land drainage, or slash-and-burn agriculture, the composition of the soil and the mycorrhizal growth mat changes. As a consequence, the symbiotic fungi could disappear and the subsequent uptake and sharing of nutrients from the environment to the orchid can be severely inhibited. Habitat destruction and habitat change are the major reasons for this, but the collectors illegally removing plants, photographers, botanists, and visitors trying to

get a closer look, all contribute to their decline (Gale et al. 2018). All ecosystems are complex, resilient systems that connect thousands of species of plants, allowing them to intercommunicate and adapt. But they are also vulnerable, not only to natural disturbances but also to a myriad of anthropic factors. Instead of hurting and destroying them, we could reinforce and help them recover. The great thing about natural environments is that they have an enormous capacity to regenerate (Dukes 2007). Nevertheless, we should not be surprised if new research into the social networks of plants will reveal the surprising benefits that orchids provide to their partners—the fungi and, ultimately, the whole ecosystems in which they live (Anghelescu 2021).

8 Barriers of Seed Germination in Terrestrial orchids

8.1 Seed Testa

The physical features of seeds in terrestrial orchid species and their biological roles have been studied and reviewed by many researchers (Vafaee et al. 2021; Süngü Şeker et al. 2021; Aybeke 2013, 2007; Calevo et al. 2017; Chase and Pippen 1988; Gamarra et al. 2015a, 2015b, 2012; Ortúñez et al. 2006). Seeds in terrestrial orchids are species-specific as they have unique features like embryo/airspace size ratio, unique testa sculpture, and the presence of plant hormones and other regulators which impact both symbiotic and asymbiotic seed germination (Arditti and Ghani 2000; Barthlott et al. 2014; Yang and Lee 2014). The seed testa phenotypic diversity in orchids could be attributed to the dispersion strategy and seed dormancy (Ren et al. 2017; Yang and Lee 2014; Prutsch et al. 2000). In this regard, air space within the seed testa surrounding the embryo increases its air travel and floatability on the water surface. However, the lignified, pectin layer can act as a barrier to water uptake and embryo enlargement, thus preventing seed germination in terrestrial orchids (Vafaee et al. 2021; Şeker and Şenel 2017).

Therefore, the lignified testa should be removed or softened during the seed germination process to facilitate protocorm-like bodies and rhizoid formation, which are the perquisites for the successful development of the in vitro raised plantlets (Fatahi et al. 2022a, 2022b). Therefore, the hard seed testa is one of the causes of extended dormancy observed in orchids, particularly in terrestrial species occurring in seasonal climates (Arditti and Ghani 2000). This is because, unlike tropical and epiphytic species with a seed testa composed of one layer, terrestrial, mature seed testa has 2–3 layers of dead cells (Yang and Lee 2014). Seed testa structure, cuticle thickness, seed cell number, and presence or absence of a distinct cell size gradient also provide information on how easy mature seeds are to germinate symbiotically and asymbiotically. One of the strategies to soften and eliminate the strong and impenetrable testa is the treatment with sodium hypochlorite (NaOCl), which simultaneously disinfects and scarifies the seeds. Depending on the species, the concentration and treatment time differ for many terrestrial species.

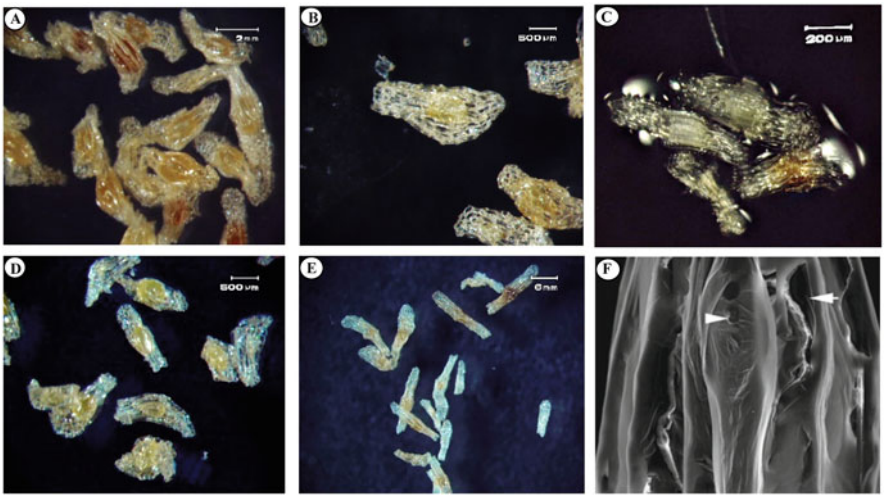

Fig 10 Test color change of seed testa in (**a**). *Anacamptis coriophora* (L.) R. M. Bateman, Pridgeon & M. W. Chase; (**b**) *Dactylorhiza umbrosa* (Kar. & Kir.) Nevski; (**c**) *Ophrys reinholdii* subsp. *straussii* (H. Fleischm.) E. Nelson; (**d**) *Himantoglossum affine* (Boiss.) Schltr.; (**e**) *Ophrys schulzei* Bornm. & Fleischm.; (**f**) Seed testa rupturing in *Paphiopedilum armeniacum* S. C. Chen & F. Y. Liu by the impact of NaOCl (Lee 2011). Photos © Yavar Vafaee, Kolsum Ahmadzadeh

In this regard, Malmgren has proposed the optimal NaOCl concentrations and disinfection times for Euro-Mediterranean orchid species (Malmgren 1996). Ponert Vosolsobě (Ponert et al. 2011), by studying different European temperate orchid species, found that higher concentrations of NaOCl and longer disinfection times have a negative effect on the germination of *Dactylorhiza fuchsii* (Druce) Soó and *Dactylorhiza majalis* (Rchb.) P.F.Hunt & Summerh., while other species like *Dactylorhiza baltica* (Klinge) Nevski, showed high germination rates. As ethanol eliminates suberin, cutin, and other wax derivatives off the orchid seed surfaces, its implementation could also improve seed germination (Jolman et al. 2022). Experimentally, the important point is the color change of disinfected seeds from brown to a milky, transparent/translucent color, which indicates successful sterilization. After the color change, the seeds should be immediately sown on a culture medium surface, as over-disinfection can lead to seed death. Fig. 10 shows how the color change occurs in the seed of several terrestrial orchid species and what exactly happens on the seed testa surface during the disinfection processes.

8.2 Toxicity of Inorganic Nitrogen Sources

During orchid seed germination, nitrogen plays a crucial role in the synthesis of macromolecules including proteins, nucleic acids, and enzymes. In this connection, the form of nitrogen used in media is one of the most important factors affecting seed

germination. Living cells are stimulated to synthesize proteins by exogenous ammonium, which activates glutamate dehydrogenase. Nitrate reductase enzymes also respond to the reduced forms of nitrogen, making the nitrates absorbed more efficiently (Rasmussen et al. 2015; Rasmussen and Whigham 1993). However, inorganic forms of nitrogen have been included in some well-known media for orchid micropropagation, the inhibition effect of organic nitrogen has been described in both tropical and temperate orchids where both orchid groups prefer organic nitrogen in the form of amino acids rather than ammonium ($NH4^+$) or nitrates ($NO3^-$) (Rasmussen 1995; Rasmussen et al. 2015; Dijk and Eck 1995; Figura et al. 2020; Nadarajan et al. 2011; Van Waes and Debergh 1986a). Terrestrial orchids are growing in natural habitats with low strength of available nutrients and therefore the reported nitrate sensitivity in asymbiotic seed germination of terrestrial orchids may be part of their adaptive strategy (Figura et al. 2020, 2021; Ponert et al. 2013). It seems that each terrestrial orchid species shows a unique response to the presence of nitrate and ammonium, therefore for the selection of an appropriate medium, we should consider the ability of target species in metabolizing nitrogen sources (Jolman et al. 2022). As an alternative conclusion, it has been stated that some terrestrial orchid species possess low nitrate reductase activity or delayed activation (Fatahi et al. 2022b; Johnson and Kane 2007; Van Waes and Debergh 1986b; Bektaş et al. 2013). The presence of both nitrate and ammonium not only can have an inhibitory impact on asymbiotic seed germination but also negatively affect the association of mycorrhizal fungi with orchids during in vitro symbiotic seed germination. According to Cuenca and Azcón (Cuenca and Azcón 1994), arbuscular mycorrhizal plants can enhance the nitrate absorption of fungi through nitrogen metabolism (a symbiotic relationship). By increasing nitrate concentrations above optimal levels, symbiosis loses its beneficial effect on plant growth, and colonized plants exhibit varying behaviors depending on the fungal species (Azcón et al. 2001). Further investigation is required to understand the details of nitrate and ammonium metabolism during symbiotic and asymbiotic seed germination at physiological and molecular scales. Our studies performed on terrestrial orchid species from the Iranian plateau show that even common media like Murashige and Skoog (MS) (Murashige and Skoog 1962) with low strengths (1/4 or 1/8 strength) can inhibit seed germination. As is represented in Fig. 11, even the protocorms of rarely germinated seed turned black and died.

8.3 Seed and Plantlet Dormancy

Terrestrial orchids have an annual cycle, whereby a period of growth is followed by the loss of leaves, stems, and adventitious roots (Schiebold 2018). In many species, the duration of the protocorm stage can extend from a few months, up to several years, until leaves are produced. Thus, the absence of a compatible mycorrhiza may last several years (Rasmussen et al. 2015; Dearnaley 2007). The period needed from seed germination to reaching the adult stages (when the first flower is produced)

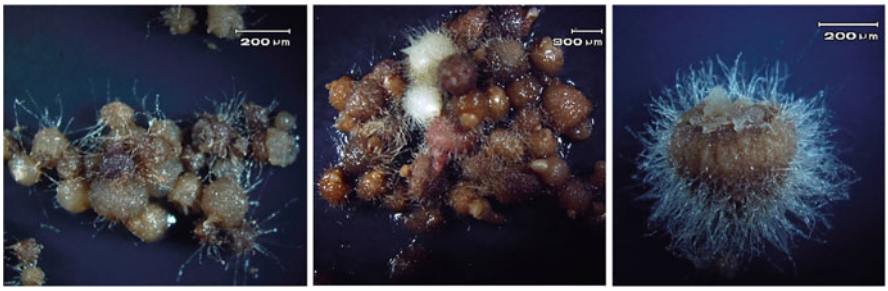

Fig. 11 The negative effect of high concentration of nitrate and ammonium present in MS medium on asymbiotic seed germination of *Ophrys reinholdii* subsp. *straussii* (H. Fleischm.) E. Nelson. (**a**) 1/4 MS; (**b**) 1/8 MS; (**c**) An individual browning protocorm. Photos © Yavar Vafaee, Kolsum Ahmadzadeh.

varies considerably and depends on the species. Consequently, the maturation time for *Cypripedium calceolus* L. is 9 to 11 years, for *Corallorhiza trifida* Châtel. is 5 to 9 years, for *Epipogium aphyllum* Sw. is 10 years, for *Ophrys apifera* Huds. is about 6 to 8 years, for *Neottia nidus-avis* (L.) Rich and *Cephalanthera damasonium* (Mill.) Druce is 9 to 11 years, for *Neottia ovata* (L.) Bluff & Fingerh. is 15 to 20 years, for *Dactylorhiza sambucina* (L.) Soó is 12 years, for *Dactylorhiza majalis* (Rchb.) P.F. Hunt & Summerh. and *Dactylorhiza incarnata* (L.) Soó is 16 years, for *Orchis mascula* (L.) L. is 8 years, for *Spiranthes spiralis* (L.) Chevall. is 3 to 10 years, for *Neotinea ustulata* (L.) R. M. Bateman, Pridgeon & M. W. Chase is 10 to 15 years, etc. (Rasmussen 1995). The orchid remains below ground until conditions become suitable for further growth. In the absence of a suitable fungus, the orchid protocorms may remain viable in the soil, postponing their germination (Allen 1992; Allen et al. 1995). They survive by utilizing their minimal reserves very slowly, waiting for a food source of simple nutrients to save them. When these are provided (by fungal association), the development continues (Selosse and Cameron 2010; Selosse et al. 2022). Nevertheless, it is commonly known that only a very small percentage of germinating seeds succeed and became adult plants. This is usually due to the absence of the mycorrhizal partner that lacks from those particular habitats. Despite the high survival potential of dormant protocorms, the prolonged absence of the fungal symbiont usually leads to protocorm starvation and ultimately to its death. Less than 5%, or even 1% in some temperate species, manage to survive and successfully reach the age of reproduction when they are able to produce fruits and viable seeds. The rest (seeds or protocorms), even perfectly viable, in the absence of the fungus, remain dormant for good. The absence of fungi, as stressed previously, may be due mainly to detrimental human intervention (anthropic factors), which usually led to major habitat and climatic changes (substrate pH, temperature changes, flooding, soil desiccation, deforestation, agriculture, tourism, estate expansion, etc.), all ultimately leading to mycorrhizal network destruction and plant species interaction disruption.

9 Asymbiotic Seed Germination

However, almost all terrestrial orchid species needs a symbiosis relationship with mycorrhizal fungi to germinate seed, develop protocorm, and establish plantlets in nature, these events can also be proceeded both symbiotically (in the presence of fungal symbiont) or asymbiotically (without fungal symbiont) (Ponert et al. 2013). The establishment of a reciprocal relationship with mycorrhizal fungi could be obligatory in some terrestrial orchid species for successful germination and protocorm formation as they supply a big part of water, mineral nutrients, and vitamins (Jolman et al. 2022). On the other hand, it has been represented that several fungal species are joining the symbiotic relationship in terrestrial orchid roots continuously or seasonally (Rasmussen 1995). One of the reasons for endangering and threatening some terrestrial orchid species is the absence of mycorrhizal fungi symbiosis due to climate change or habitat destruction (Li et al. 2021). In this regard, one of the main problems to start a symbiotic seed germination experiment is the need for a diverse range of ectomycorrhizal fungus species which usually have unfavorable features like slow growth, difficult cultivation, and high host specificity (Rasmussen et al. 2015; Fatahi et al. 2022b; Kömpe 2022). Moreover, during the symbiotic seed germination of terrestrial orchids, the nutritional and cultivation condition requirements of both orchids and mycorrhizae should be provided (Ponert et al. 2011). It is, moreover, not suitable for a wide range of physiological studies on orchids because it is almost impossible to separate any effect of the fungus from the direct effect of the factor under study. Unlike symbiotic germination, during asymbiotic seed germination, the required nutrients are obtained by orchid seeds through an artificial medium (Knudson 1922). Considering the obstacles of symbiotic culture establishment, asymbiotic germination procedures possess advantages including an easier cultivation process, fast and large scale in vitro plantlet production, and direct investigation of important variables affecting different biological aspects of orchids' life (Jolman et al. 2022; Swarts and Dixon 2009, 2017). To date, the asymbiotic seed germination of different terrestrial orchid species has been optimized as each terrestrial orchid taxon needs a specific and accurate combination of organic and inorganic medium ingredients. Depending on the genus, species, and even sub-species, there are drastically different developmental requirements, in particular, based on the climate origin like tropical and temperate that necessitate the exploitation of technically different germination procedures (Jolman et al. 2022; Diantina et al. 2020). An extensive list of the performed research works on asymbiotic seed germination of terrestrial orchid species highlighting the exploited basal media, organic components, and PGRs, the highest reported seed germination rate, and the country origin of the studied species has been shown in Table 1.

Table 1 The performed research studies on the asymbiotic seed germination of endangered terrestrial orchid species

Species	Basal medium/media	Organic supplements	PGRs	Max. germination (%)	Seed origin	Accl. ±	Reference
Anacamptis longicornu	MS, OM	–	–	95.5	Italy	+	Arcidiacono et al. (2021)
Anacamptis morio	KC, OM, MM	Pep, PJ	–	88.91	Turkey	+	Hurkan et al. (2018)
Anacamptis pyramidalis	KC, MM	Pep, CW, PJ, CH, Gl	BA, Kin, 2-iP	73.79	Serbia	–	Ostojić et al. (2022)
Anacamptis pyramidalis	KC, OM, MM	Pep, PJ	–	74.42	Turkey	+	Hurkan et al. (2018)
Chloraea crispa	MS, BM2	CH, Gl	BAP, IBA	30.50	Chile	–	Quiroz et al. (2017)
Cypripedium macranthos	MS	CW	–	68.10	South Korea	–	Huh et al. (2016)
Cypripedium macranthos	MS, BM1, HP	Pep	BA, NAA	8.90	Japan	+	Shimura and Koda (2004)
Cyrtopodium punctatum	PT, MM, KC, MS, P723	CH, Pep, Gl	–	27.30	USA	+	Dutra et al. (2008)
Dactylorhiza hatagirea	MS, MM, BM2, VW, PT139, KC, LD	CH, Gl	IBA, Kin	37.12	India	+	Warghat et al. (2014)
Dactylorhiza romana	KC, OM, MM	Pep, PJ	–	66.30	Turkey	+	Hurkan et al. (2018)
Dactylorhiza urvilleana	OM	Gy, TR	Zeatin	62.39	Turkey	–	Bektaş (2016)
Epipactis flava	VW, MS, BM1, MM, KC	CW, PE, CH	–	70.40	Thailand	+	Kunakhonnuruk et al. (2018)
Epipactis veratrifolia	FT	Pep	–	79.60	Iran	–	Dianati Daylami et al. (2017)
Eulophia flava	MS	CW	BA, NAA	26.39	Thailand	–	Vasupen et al. (2022)

Eulophia spectabilis	MS, KC, BM1	CW, CH, Gl, Ar	BAP, Kin	91.30	India	+	Nanekar et al. (2014)
Eulophia promensis	MS, P723	Pep	BAP, NAA	100	Bangladesh	+	Hossain (2015)
Gastrodia cunninghamii	MS, NG, WA, BM1	CH, Gl	–	~23	New Zealand	–	Diantina et al. (2020)
Gymnadenia conopsea	KC, MM	Pep, CW, PJ, CH, Gl	BA, Kin, 2-iP	69.88	Serbia	–	Ostojić et al. (2022)
Habenaria macroceratitis	MM	BP	–	100	USA	–	Stewart and Kane (2010)
Himantoglossum adriaticum	MM	Pep	BA	5.10	Italy	–	Del Vecchio et al. (2019)
Himantoglossum affine	MM	Pep, CW, AV, PJ, CH	–	99.46	Iran	+	Fatahi et al. (2022a)
Himantoglossum calacaratum subsp. *jankae*	KC, MM	Pep, CW, PJ, CH, Gl	BA, Kin, 2-iP	82.56	Serbia	+	Dulić et al. (2019)
Liparis koreojaponica	ND	–	–	15	Japan	+	Tsutsumi et al. (2011)
Liparis kumokiri	ND	–	–	30	Japan	+	Tsutsumi et al. (2011)
Microtis arenaria	BM1, MS, P723, Pa5, W3	CW, BP, CH, Gl	BA	99.20	Australia	+	Dowling and Jusaitis (2012)
Neotinea tridentata	KC, OM, MM	Pep, PJ	–	55.03	Turkey	+	Hurkan et al. (2018)
Ophrys apifera	MM	PSE, CW, BP, YE	Zeatin, BA, GA, Kin, TDZ	9.10	Italy	+	Pierce et al. (2013)
Ophrys benacensis	MM	CM, PB, PJ	–	39.80	Italy	+	Pierce et al. (2010)
Ophrys sphegodes	MS, OM	–	–	12.0	Italy	+	Arcidiacono et al. (2021)

(continued)

Table 1 (continued)

Species	Basal medium/media	Organic supplements	PGRs	Max. germination (%)	Seed origin	Accl. ±	Reference
Ophrys sphegodes	KC, MM	Pep, Gl, CH	–	62	Serbia	–	Dulić et al. (2018)
Ophrys spp.	MM	CW, PJ	–	96	Greece	–	Kitsaki et al. (2004)
Anacamptis coriophora	OM, KC, LM, PM	Pep, Tr	IAA, IBA, 2,4-D, NAA, BA, 2-iP, Kin, TDZ	44.20	Turkey	–	Bektaş et al. (2013)
Orchis mascula	MM, OM	YE, CM	BA	5.12	Italy	–	Valletta et al. (2008)
Orchis militaris	MM, KC, Ha	CW, BS	BA, 2-iP, IBA	82.60	Russia	–	Nabieva (2021)
Orchis simia	MM	Pep, CW, AV, PJ, CH	–	94.51	Iran	+	Fatahi et al. (2022b)
Paphiopedilum armeniacum	MS	CaH, PE, BH	NAA	96.20	China	+	Wang et al. (2021b)
Paphiopedilum spicerianum	MS, RE,	CW	BAP, NAA	21.65	China	+	Chen et al. (2015)
Paphiopedilum tigrinum	mHa, 1/2 MS	CW	BA, Kin	90.17	China	+	Yao et al. (2021)
Paphiopedilum venustum	BM, BM1, KC, MM	Pep, CW, PJ, CH, Gl	–	82.75	India	+	Kaur and Bhutani (2016)
Paphiopedilum wardii	MS	CW	NAA	65.33	China	+	Zeng et al. (2012)
Pelatantheria scolopendrifolia	1/2 MS, PM, BM1, BM2	CH, Gl	–	94.10	South Korea	–	Kim et al. (2021)
Phragmipedium warscewiczii	KC, MS	–	–	2.90	Costa Rica	+	Muñoz and Jiménez (2008)
Phragmipedium longifolium	KC, MS	–	–	41.30	Costa Rica	+	Muñoz and Jiménez (2008)

Phragmipedium pearcei	KC, MS	–	–	38.70	Costa Rica	+	Muñoz and Jiménez (2008)
Platanthera chapmanii	P723	Pep	–	15.50	USA	–	Poff et al. (2016)
Pleione bulbocodioides	1/2 MS	CW, PE, BE	2,4-D, BA, Kin, TDZ	73.32	China	–	Zhou et al. (2021)
Prasophyllum pruinosum	BM1, MS, P723, Pa5, W3	CW, BP, CH, Gl	BA	65.50	Australia	+	Dowling and Jusaitis (2012)
Pseudorchis albida	MM	CW, BP, ME, YE	BA, GA3, TDZ	50.50	Italy	–	Pierce and Cerabolini (2011)
Pterostylis banksii	MS, NG, WA, BM1	CH, Gl	–	~34	New Zealand	–	Diantina et al. (2020)
Pterostylis nutans	BM1, MS, P723, Pa5, W3	CW, BP, CH, Gl	BA	91.20	Australia	+	Dowling and Jusaitis (2012)
Satyrium nepalense	MS, KC	–	BAP, Kin, TDZ	86.70	India	+	Mahendran and Bai (2009)
Spiranthes spiralis	KC, MM	Pep, CW, PJ, CH, Gl	BA, Kin, 2-iP	36.65	Serbia	+	Dulić et al. (2019)
Thelymitra nervosa	MS, NG, WA, BM1	CH, Gl	–	~35	New Zealand	–	Diantina et al. (2020)
Thelymitra pauciflora	BM1, MS, P723, Pa5, W3	CW, BP, CH, Gl	BA	60.90	Australia	+	Dowling and Jusaitis (2012)

BM1 Basal medium/media, *Ha* harvais, *HP* hyponex-peptone, *KC* Knudson C, *LD* Lindemann, *MM* Malmgren, *MS* Murashige and Skoog, *ND* New Dogashima, *NG* Norstog, *Pa5* burgeffs N3f, *PT139* Mitra, *PT* Phyto Technology orchid medium, *RE* Robert Ernst, *PM* Phytamax, *OM* Orchimax, *W3* Western 3, *WA* water-agar Organic supplements: *Ar* arginine, *BH* banana homogenate, *BP* banana powder, *BS* birch sap, *CaH* carrot homogenate, *CM* coconut milk, *Gy* glycine, *ME* malt extract, *PE* potato extract, *PJ* pineapple juice, *PSE* pea seed extract, *Tr* tryptone, *PGRs* plant growth regulators, *2,4-D* 2,4-dichlorophenoxyacetic acid; N6-(2-Isopentenyl) adenine, *BA* benzyl adenine, *BAP* 6-benzylaminopurine, *IAA* indole-3-acetic acid, *IBA* indole-3-butyric acid, *GA3* gibberellic acid, *Kin* kinetin, *NAA* naphthalene acetic acid, *TDZ* thidiazuron

9.1 Asymbiotic Seed Germination Stages

There are different processes and stages between symbiotic and asymbiotic germination in terrestrial orchids as symbiotic germination requires an extra stage for mycorrhizal association and symbiont development. In terrestrial orchid species, during asymbiotic germination process, the embryos enlarge and produce small structures called protocorms, which have root and shoot meristematic centers. The protocorm can develop completely only in the presence of adequate storage resources for the shoot and root formation. By activation of root and shoot meristem, the plantlets start to grow under in vitro conditions. Based on the description in the literature, asymbiotic seed germination in terrestrial orchids can be divided into five stages (Bektaş 2016; Nabieva 2021):

Stage I: "no-germination" stage. Unimbibed seed with intact testa.

Stage II: "swelling" stage. Embryo swelling and enlargement followed by testa rupturing.

Stage III: the "pre-germination" stage. Complete rising of the embryo from ruptured seed testa and formation of first rhizoids.

Stage IV: "Rhizoid" stage. The formation of rhizoids on the surface of the protocorm.

Stage V: "Protocorm or germination" stage. Enlargement of protocorm and formation of protomeristem.

Stage VI: "Shoot" stage. Further enlargement and development of the first green leaf.

As a part of studies performed in the Research Center for Terrestrial Orchid, RCTO, university of Kurdistan), the asymbiotic seed germination of *Ophrys reinholdii* subsp. *straussii* (H.Fleischm.) E. Nelson (a threatened Euro-Mediterranean terrestrial orchid species) has been shown in Fig. 12 highlighting the main stages of germination.

10 Organic Supplements and Asymbiotic Seed Germination

As artificial media give different results depending on the target species, screening media and supplements would be helpful to determine the best nutrient formulation that maximizes seed germination in terrestrial orchids (Swarts and Dixon 2009, 2017; Cardoso et al. 2020). In this regard, terrestrial orchid species seeds are sensitive to the inorganic form of nutrients in particular nitrogen and therefore a variety of organic additives and compounds have been used for asymbiotic seed germination of orchids (Utami and Hariyanto 2020; de Menezes Gonçalves et al. 2016; Kaur 2021). There are several such compounds, including peptone, coconut water, pineapple juice, casein hydrolysate, yeast extract, and amino acid mixtures. It is very important to use a suitable organic compound such as pineapple juice, coconut milk, boiled potatoes, or other similar compounds. These compounds

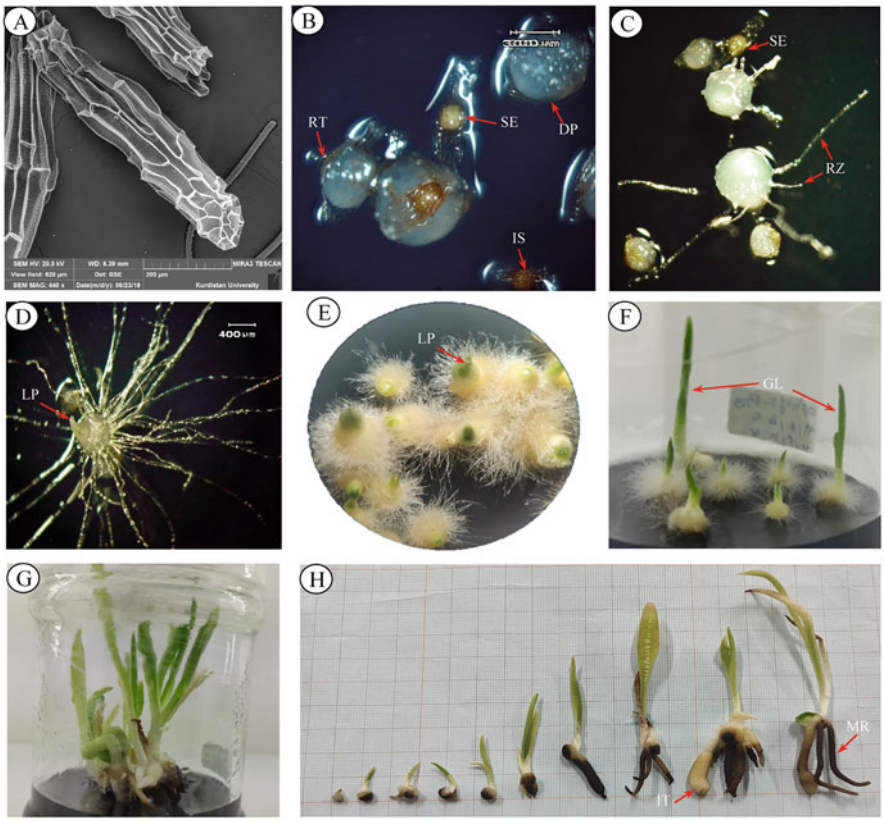

Fig. 12 Seed germination stages and plantlet growth and development in *Ophrys reinholdii* subsp. *straussii* (H.Fleischm.) E.Nelson. (**a**) SEM micrograph of seed testa micromorphology (Stage I); (**b**) Embryo enlargement (stage II) and its excise from seed testa (stage III) 18–22 days after seed sown; (**c**) Rhizoid formation (stage IV) 24–30 days after seed sown; (**d, e**) Enlargement of protocorm and formation of protomeristem (stage V); (**f**) Developing green leaves (stage VI); ready for acclimatization 3–4 months after seed sown; (**g**) A clump of in vitro raised plantlets with healthy and green leaves and small tuber; (**h**) The stages of in vitro protocorm development, rooting, and plantlet growth. *SE* swelling embryo; *RT* rupturing testa; *DP* developing protocorm; *RZ* rhizoids; *LP* leaf primordium; *IT* in vitro formed tubers; *MR* main root. Photos © Yavar Vafaee, Kolsum Ahmadzadeh

contain vitamins and plant hormones, which are often the most suitable compounds for orchid propagation. The use of organic compounds is important in the in vitro culture of various orchids as they provide vitamins and plant growth regulators. In many cases, they have a positive effect on seed germination and plantlet growth regardless of whether their components are known or unknown. Here, we discuss the nature and the application of some organic supplements used for in vitro germination and propagation of terrestrial orchids. The use of inexpensive organic complex supplements can reduce the costs helping the large-scale in vitro micropropagation

of endangered terrestrial orchid species and their reintroduction to nature (Fatahi et al. 2022a, 2022b).

10.1 Peptone

Different raw materials can be digested by acids or enzymes to produce a protein hydrolysate named peptone (Nhut et al. 2008). Peptone is a product of animal tissue and products digestion and is composed of low molecular weight constituents (23% glycine, 16.16% total nitrogen, 15.38% peptone nitrogen, 11% glutamic acid, 9.42% monoamine nitrogen, 8% arginine, 5.9% aspartic acid) (Yam and Arditti 2017b). Peptone not only is autoclavable and dialyzable but also stable under acidotic and alkaline conditions (Jan et al. 1994). There are numerous reports on the exploitation of peptone as one of the main organic media constituents not only in plant tissue culture but also in animal and insect cell culture to supply carbon and nitrogen. In orchids, peptone is used to improve symbiotic and asymbiotic seed germination. In this regard, in *Himantoglossum affine* (Boiss.) Schltr. as a Euro-Mediterranean terrestrial orchid species, the highest germination rate (98.77 ± 0.37%) was obtained with media containing pineapple juice plus peptone. Besides its positive impact on seed germination and protocorm development and growth, peptone also positively affects terrestrial orchid plantlet growth as it is a rich source of amino acids and vitamins such as thiamin, biotin, pyridoxine (Utami and Hariyanto 2020). In this regard, individual use of organic nitrogen compounds resulted in higher germination efficiencies, and plantlets grown on media supplemented with peptone had the highest plantlet length and weight compared to other organic nitrogenous compounds (Fatahi et al. 2022a). Evaluation of different levels of peptone and banana homogenate on in vitro micropropagation of terrestrial orchid *Paphiopedilum venustum* (Wall. ex Sims) Pfizer revealed that BM-1 medium (Van Waes and Debergh 1986b) containing 1 g/L peptone resulted in the highest shoot efficiency of shoot multiplication (Kaur and Bhutani 2016). The adding peptone to seed germination medium for *Paphiopedilum hirsutissimum* (Lindl. ex Hook.) Stein and *Paphiopedilum insigne* (Wall. ex Lindl.) Pfizer resulted in a 30% higher germination percentage (Zeng et al. 2016). *Orchis simia* Lam. is a threatened terrestrial orchid growing in central and southern Europe with fragmented populations due to climate change and overexploitation. Fatahi, Vafaee (Fatahi et al. 2022b) by studying different organic compounds supplying nitrogen found that commercially available amino acid mixture (Vamine) and casein hydrolysate were more efficient than peptone on seed germination and plantlet growth. They stated that altogether the using of organic nitrogenous supplements can replace the need for mycorrhizal fungi regardless of the nature of used organic nitrogenous compounds. Malmgren medium containing peptone, coconut water, and glutamine resulted in the highest germination rate in lizard orchid, *Himantoglossum calcaratum* (Beck) Schltr. subsp. *jankae* (Somlyay, Kreutz & Óvári) R.M.Bateman, Molnar & Sramkó (Dulić et al. 2019) showing the important role of amino acids in

the successful asymbiotic seed germination of terrestrial orchids. With lower percentages, the seed of *Himantoglossum adriaticum* H.Baumann was successfully germinated on the same medium supplemented with 0.5 g/L peptone (Del Vecchio et al. 2019).

10.2 Coconut Water

Coconut water is the liquid obtained from the center part of the endosperm, while coconut milk is the liquid obtained from the solid and fleshy part (George et al. 2008; Yong et al. 2009). Compared to coconut water, coconut milk which is also the source of coconut oil has not been commonly used in terrestrial orchid tissue culture. There are a variety of compounds found in coconut water, including amino acids, organic acids, plant growth regulators, vitamins, sugars, sugar alcohols, minerals, nucleic acids, and unknown growth substances, all of which can support and trigger in vitro plant growth and development (George et al. 2008; Yong et al. 2009). Plant growth regulators (PGRs) including auxins (mainly indole-3-acetic acid IAA), cytokinins (trans-zeatin, trans-zeatin O-glucoside, N^6-isopentenyladenine, and dihydrozeatin), gibberellins (GA1 and GA3), and abscisic acid are among most prevalent PGRs in coconut water (Yong et al. 2009; Shekarriz et al. 2014). Various mineral ions including Ca, Fe, Mg, P, and K can be found in coconut water (Vasupen et al. 2022). Additionally, it contains B1, B2, B3, B5, B6, B7, and B9 vitamins (George et al. 2008; Yong et al. 2009). Due to the presence of PGRs in particular cytokinins, coconut water is extensively has been exploited in the propagation of terrestrial orchid species through asymbiotic seed germination (George et al. 2008). By providing faster energy to the cells and by triggering cell division through its cytokinin content, CW implementation in culture media results in better responses (Jolman et al. 2022). Coconut water could promote seed germination and development of seed to post protocorm stage in *Eulophia flava* (Lindl.) Hook. f (Vasupen et al. 2022). However, the supplementation of Malmgren medium with coconut water resulted in lower asymbiotic germination rates in *Himantoglossum affine* (Boiss.) Schltr. (an endangered Euro-Mediterranean tuberous orchid), causing the shortest time to germination compared to other used organic supplements (Fatahi et al. 2022a). *Orchis militaris* L. is a cold-hardy terrestrial Euro-Siberian species considered recalcitrant to in vitro seed germination response. Adding 5% coconut water to Malmgren medium led to a higher number of protocorms and seedlings, producing first secondary roots and true leaves in *Orchis militaris* L. (Nabieva 2021). The perennial tuberous and rhizomatic orchid species, *Pleione bulbocodioides* (Franch.) Rolfe, also known as Cremastra Pleione, is an endangered species due to tuber overharvesting and natural low-rate propagation. It has been reported that the rate of protocorm formation is higher in *Pleione bulbocodioides* (Franch.) Rolfe uses coconut water than other organic compounds like peptone and banana extract. Among three studied concentrations of coconut water (50, 100, and 150 mg/L), the protocorm induction percentage was at the highest value

(50.35 ± 0.60%) using 100 mg/L coconut water (Zhou et al. 2021). The *Spiranthes spiralis* (L.) Chevall. seed sown on Knudson C medium supplemented with coconut water had a high germination rate which has been attributed to a high content of cytokinins supporting cell division and thus growth promotion (Dulić et al. 2019). Similar findings have been obtained with asymbiotic seed germination in threatened terrestrial orchid *Cypripedium macranthos* Sw. on the MS medium nourished with coconut water (Huh et al. 2016).

10.3 Pineapple Juice

Sucrose, glucose, and fructose are among the most abundant ingredients of pineapple, which comprise about 81–86% of its total soluble solids. On the other hand, there is 2–3% fiber in pineapple which is high value within fruit crops (Malmgren 1996). Ascorbic acid is the most prevalent organic acid in pineapple while there is also a low level of citric acid present in pineapple juice (Kitsaki et al. 2004; George et al. 2008). In this term, bromelain is a protease present in pineapple contributing 80% of total proteolytic activity that can break down other proteins. Minerals found in pineapples include calcium, chlorine, potassium, phosphorus, sodium, and copper (Utami and Hariyanto 2020; George et al. 2008). Besides nutritional roles in providing macro- and micronutrients as well as PGRs, pineapple juice can also reduce phenolic compound production in the environment (Rasmussen 1995), which has improved the propagation efficiency of European orchids through asymbiotic seed germination (Malmgren 1996). A number of temperate terrestrial species have also been found to benefit from pineapple juice in terms of root differentiation and growth (Rännbäck 2007). Malmgren stated that adding 15–125 mL pineapple juice supplies about 30–40 mg/L potassium and also enough concentrations of microelements (Malmgren 1996). Pineapple juice is a permanent organic supplement added to the asymbiotic seed germination medium in different species members of terrestrial orchid genera including *Cypripedium* L., *Dactylorhiza* Neck. ex Nevski, *Nigritella* Rich., *Gymnadenia* R.Br., *Orchis* Tourn. ex L., *Platanthera* Rich., etc. Pineapple juice could be successfully applied for asymbiotic seed germination in endangered tuberous orchid species including *Orchis simia* Lam. (Fatahi et al. 2022b), *Himantoglossum affine* (Boiss.) Schltr. (Fatahi et al. 2022a), *Cypripedium* spp. (Rännbäck 2007), *Ophrys benacensis* (Reisigl) O. Danesch, E. Danesch & Ehrend, (Pierce et al. 2010), *Himantoglossum calcaratum* (Beck) Schltr. subsp. *jankae* (Somlyay, Kreutz & Óvári) R. M. Bateman, Molnar & Sramkó and *Spiranthes spiralis* (L.) Chevall. (Dulić et al. 2019), *Anacamptis pyramidalis* (L.) Rich., and *Gymnadenia conopsea* (L.) R. Br (Ostojić et al. 2022). Moreover, a high ratio of seed germination and plant development have been obtained with Malmgren medium containing pineapple juice, however, the best results were obtained with BM1 medium supplemented with casein hydrolysate. On the other hand, Kitsaki and Zygouraki (Kitsaki et al. 2004) studied during the germination of different terrestrial orchid species belonging to the *Ophrys* L. genus including *Ophrys umbilicata* Desf.,

Ophrys sphegodes subsp. *spruneri* (Nyman) E.Nelson, *Ophrys speculum* Link, *Ophrys tenthredinifera* Willd., *Ophrys sphegodes* subsp. *mammosa* (Desf.) Soó ex E.Nelson, *Ophrys lutea* Cav., *Ophrys fusca* subsp. *iricolor* (Desf.) K.Richt., *Ophrys ferrum-equinum* Desf., *Ophrys* × *delphinensis* O. Danesch & E. Danesch, *Ophrys scolopax* subsp. *cornuta* (Steven) E. G. Camus, *Ophrys argolica* H. Fleischm. and *Ophrys apifera* Huds., the medium containing pineapple juice as the inorganic supplement resulted in the best plantlet development (Kitsaki et al. 2004).

10.4 Casein Hydrolysate

The enzymatic or acidic hydrolysis of different natural products such as milk, plant and animal tissues, and microbial cultures can result in the production of hydrolysates. There are mixed recommendations for using hydrolysates in the in vitro orchid culture (Lee and Yeung 2018). There are several commercially available hydrolysates. Casein hydrolysate is a fraction product obtained from the enzymatic digestion of mammalian milk. Casein hydrolysate is an important component in various media formulations since it provides a mixture of proteins, amino acids, and peptides as reliable natural sources (George et al. 2008). Some micronutrients and vitamins are also present (Dulić et al. 2019). Due to its high concentration of essential and non-essential amino acids, vitamins, and phosphates, casein hydrolysate is known as a germination and growth-inducing factor in the orchid tissue culture (Fatahi et al. 2022a; Kaur 2021). BM media are typical media used for asymbiotic seed germination of some terrestrial orchids, developed by Van Waes and Debergh (Van Waes and Debergh 1986b), which contains 500 mg/L casein hydrolysate. However, other commercial media used for multiplication, maintenance, and sub-culturing terrestrial orchids usually contain 1–2 g/L of casein hydrolysate depending on the species (Utami and Hariyanto 2020). In this term, among different combinations of organic additives, 500 mg/L casein hydrolysate plus 15% coconut water (CW) represented the best seed germination results in *Eulophia spectabilis* (Dennst.) Suresh is a therapeutically important endangered orchid species from India (Nanekar et al. 2014). Using MS and Mitra media supplemented with 500 mg/L casein hydrolysate and 1 mg/L N^6-benzyladenine (BA), a high asymbiotic seed germination (75 ± 2.5%) was obtained *Crepidium khasianum* (Hook.f.) Szlach (Deb 2006). Similar results were obtained in *Ophrys sphegodes* Mill. using Knudson C and Malmgren media containing peptone, L-glutamine, folic acid, and casein hydrolysate (Dulić et al. 2018). *Chloraea crispa* Lindl. (Quiroz et al. 2017), *Anacamptis pyramidalis* (L.) Rich. and *Gymnadenia conopsea* (L.) R.Br. (Ostojić et al. 2022), *Orchis simia* Lam. (Fatahi et al. 2022b), *Himantoglossum affine* (Boiss.) Schltr. (Fatahi et al. 2022a), *Himantoglossum calcaratum* (Beck) Schltr. subsp. *jankae* (Somlyay, Kreutz & Óvári) R. M. Bateman, Molnar & Sramkó (Dulić et al. 2019), and *Eulophia spectabilis* (Dennst.) Sur*esh* (Nanekar et al. 2014) are among other species whose asymbiotic seed germination has benefited from the improving impacts of casein hydrolysate.

10.5 Amino Acid Mixtures

Based on the fact that the enzymatic systems of amino acid metabolism and biosynthesis of the developing embryo change and evolve (Lee et al. 2007), they can be exploited as easy-to-metabolize alternative nitrogen sources (Rasmussen et al. 2015; Utami and Hariyanto 2020; Kaur 2021). However, they may not be available and considered primary nitrogen sources, they can be metabolized and used to synthesize new essential structural and enzymatic proteins (Rasmussen et al. 2015). The metabolism of ready mixtures of amino acids (which are commercially available) can be performed by orchids' embryos and PLBs more efficiently compared to other inorganic nitrogen sources (Valadares et al. 2014). This is because under in vitro conditions, they can redirect or skip some nitrogen assimilation pathways (Rasmussen 1995; Rasmussen et al. 2015). The response of asymbiotic seed germination to amino acids supplementation is different depending on the terrestrial orchid species (Fatahi et al. 2022b). Researchers believe that nitrogen in the amino acid form may facilitate seed germination or growth of protocorms than available inorganic nitrogen sources (Malmgren 1996; Kauth et al. 2008; Stewart and Kane 2007). This fact has been also shown in asymbiotic seed germination of *Himantoglossum affine* (Boiss.) Schltr. where a ready amino acid mixture with the commercial name Vamine was more effective in the induction of seed germination than peptone and casein hydrolysate (Fatahi et al. 2022a). The slow growth of orchids was attributed to the sluggish nitrogen metabolism using inorganic nitrogen forms like NH_4^+ for seed germination, in the first step ammonium is converted to amino acids (Wu et al. 2013). Since all amino acids are not required during seed germination, a combination of selected important amino acids can be used more effectively to achieve high seed germination ratios. It has been shown in *Orchis simia* Lam. that bigger protocorms (4.5-fold bigger) were obtained on media supplemented with pineapple juice (PJ) in combination with Aminoven (a commercially available amino acid mixture) compared to protocorms grown on other media. Enhanced seed germination and subsequent plant growth in *Habenaria macroceratitis* Willd. on modified Malmgren modified medium have been reported (Stewart and Kane 2010). The advantage of using amino acid mixtures instead of undefined organic supplements containing nitrogen such as peptone, casein hydrolysate, and in particular coconut water and pineapple juice is that commercial amino acid mixtures contain given concentrations of known amino acids. On the other hand, unlike inorganic nitrogen sources, the recommended levels of amino acids even at higher concentrations are not suitable for terrestrial orchid seed germination.

11 Conclusions

Since all amino acids are not required during seed germination, a combination of selected important amino acids can be used more effectively to achieve high seed germination ratios. It has been shown in *Orchis simia* Lam. that bigger protocorms

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Progress and Prospect of Orchid Breeding: An Overview

Khosro Balilashaki, Zahra Dehghanian, Vahideh Gougerdchi, Elaheh Kavusi, Fatemeh Feizi, Xiaoyun Tang, Maryam Vahedi, and Mohammad Musharof Hossain

1 Introduction

Orchid is the general name of Orchidaceae, which belongs to perennial herbaceous plants with unique and attractive flower shapes and colors, and is the second-largest family of flowering plants with high ornamental, medicinal, and other economic value. Orchidaceae is the most evolved, highly specialized, diverse, and widespread plant family belonging to Monocotyledons, with about 801 genera and 28,237 species (Shriram and Kumar 2022). Orchids are virtually found on all continents except icy Antarctica and hot deserts but their greatest variation is to be found in the tropical and subtropical regions, mostly Asia, South America, and Central America.

K. Balilashaki (✉) · F. Feizi
Department of Horticultural Science, Faculty of Agricultural Science, University of Guilan, Rasht, Iran
e-mail: khosrobali@alumni.ut.ac.ir

Z. Dehghanian
Department of Biotechnology, Faculty of Agriculture, Azarbaijan Shahid Madani University, Tabriz, Iran

V. Gougerdchi · E. Kavusi
Department of Plant Breeding and Biotechnology, Faculty of Agriculture, University of Tabriz, Tabriz, Iran

X. Tang
College of Art Colleges of Landscape Architecture, Fujian Agriculture and Forestry University, Fuzhou, China

M. Vahedi
Department of Horticultural Science, Faculty of Agricultural Sciences and Engineering, College of Agriculture and Natural Resources, University of Tehran, Tehran, Iran

M. M. Hossain
Department of Botany, University of Chittagong, Chittagong, Bangladesh

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So far, over 1,06,000 hybrids have already been registered and developed (Hossain et al. 2013) and more than 1000 new hybrids are added per year.

The shape of orchids is simple and elegant, the fragrance of flowers attacks people, is one of the precious flowers, deeply loved by people. As a flower with high ornamental value and miniature potted plants, orchids are widely used in trade and commercial markets, and the current market demand is increasing (Chao et al. 2018). The world business value of orchids exceeded billion dollars, and the countries of Thailand, Singapore, and Malaysia dominated the world orchid market. The global orchid trade value was estimated at US$ 504 million in 2013 (Cheamuangphan et al. 2013), and the figure is undoubtedly increased many folds recently. In addition, *Gastrodia elata*, *Dendrobium nobile*, *Cypripedium henryi,* and other species of orchids are excellent Chinese herbal medicine, with great medicinal value (Wang et al. 2020).

With the prosperity and development of the orchid market in the world, many countries are engaged in orchid breeding, and the demand for technological renewal and industrial upgrading of orchid breeding is increasing. At present, the breeding methods of orchids are still mainly based on the combination of wild resource domestication and traditional breeding. However, there are many practical problems with traditional breeding techniques, such as prolonged bleeding times and a huge workload. With the renewal and development of technology, several new breeding methods have emerged. In recent years, compared with the time-consuming traditional breeding, the method of using the CRISPR/Cas9-KO system to carry out orchid molecular breeding has produced ideal alleles in less than 20 months, which greatly accelerates the efficiency of breeding (Semiarti et al. 2020). The genetic modification of orchids by *Agrobacterium*-mediated transformation and gene gun technique has been successfully and continuously applied, which has made great progress in the improvement of important traits of orchids, such as flower color, fragrance, cut flower shelf-life, and so on (da Silva et al. 2016). This article mainly reviews the history and methods of orchid breeding, which might be a reference for future studies on orchid breeding.

2 The History of Breeding

As an ornamental plant, orchids have a history of more than 2000 years. Undoubtedly, the Chinese described orchids for medicinal use (Bulpitt 2005). Between 551 and 479 B.C., the elite people of Japan grew orchids for their beauty and fragrance (Hossain et al. 2013). In ancient times, orchids are often found in poetry, with the rise of private gardens, orchid cultivation is more and more extensive, and after thousands of years of choice and utilization, the formation of a variety of orchid varieties resulted in a wealth of orchid culture.

Orchids have a long history of cultivation and are extremely rich in germplasm resources. The ancient laborers began the original breeding work by selecting the most satisfactory or strange types. For thousands of years, a wealth of experience has

been accumulated, and many numbers of fine garden plant species have been created. Because of the characteristics of history and culture, breeding is not alone as a technique but is usually included in cultivation and reproduction methods.

Chinese ornamental and cultivated orchids are much earlier than those cultivated in the West. As early as 2400 years ago in the Spring and Autumn period, there was already a description of orchids. The oldest book on orchids in Japan is "Igansai-ranpin" written by Jo-an Matsuoka in 1728 A.D. in which species of *Aerides*, *Bletilla*, *Cymbidium*, *Dendrobium,* and *Neofinetia* were described. The Samurai grew *Neofinetia falcata*, the merchants grew *Cymbidium*, and possibly the peasants grew *Bletilla* (Bulpitt 2005). In ancient times, people first collected wild orchids, as for artificial cultivation of orchids, until the court began. After Wei and Jin dynasties, orchids expanded from court cultivation to private gardens of literati class and were used to decorate gardens and beautify the environment. It was not until the Tang Dynasty that the cultivation of orchids developed into general gardens and florists (Deng 1990).

The cultivation of orchids became very widespread during 960–1126 AD, and records of the descriptive features, ecology, and distribution became abundant (Schiff 2018). The Jin Zhang Lan Pu written by Zhao Shigeng of the Southern Song Dynasty in 1233 can be said to be the earliest monograph on orchids in China, and it is also the first monograph on orchids in the world (Chen et al. 2011). The book is divided into three volumes and five parts, describing the morphological characteristics of more than 30 species of orchids. About 10 years later, Wang Guixue wrote a book (Wang's Treatise on Orchids) in 1247, which described the types and grades of orchid genealogy and the use of soil or soil mixture as potting medium (Luo et al. 2012). Early articles on orchids and their cultivation were relatively short.

The orchid cultivation in Ming Dynasty entered the prosperous period, the orchid variety in the south of the Yangtze River increased continuously, the cultivation experience became more and more abundant, and the orchid gradually became the common appreciation of the general people. Qing Dynasty was the most prosperous period of orchid cultivation in China. With the continuous emergence of genealogy and new horticultural varieties in the past dynasties, a number of Yilan with rich experience have emerged. Based on summing up the previous experience, they have put forward new ideas and wrote valuable orchid Monographs. The period of the Republic of China is also an important period of development in the history of Chinese orchids. During this period, after 2 or 300 years of exploration, the valve type theory of orchids has been completed, and a large number of orchid varieties have been discovered. In the twentieth century, Chinese orchids have entered a more prosperous period, the number of all kinds of orchid books published; the wide range of newly developed national orchid varieties, the huge contingent of orchid enthusiasts, and the active orchid trading have exceeded the previous dynasties (Reinikka 1972).

Ancient horticulturists gradually mastered fine cultivation and management techniques, constantly used sexual reproduction combined with selection to breed new varieties, and used asexual propagation methods to preserve special variation types

and other horticultural ideas and methods for traditional breeding. It also laid a foundation for the formation and development of modern orchid variety groups.

In Europe, the cultivation of orchids only started in the late eighteenth century. In 1778, Dr. John Fotherrdill brought *Cymbidium ensifolium* and *Phaius tankervilleae* back to England for the first time (Reinikka 1972). In 1780, Vere Kensington introduced *Cymbidium pendulum* into Europe. Since then, Orchid plants have been found in Asia and Oceania and sent to the United Kingdom, and then spread to Europe and the United States through the United Kingdom.

The Orchid was initially ignored in England, but it has been noticed by the British since 1889 by the hybrid of *Cymbidium eburneum* and C. *lowianum*. From 1904 to 1905, there were many new orchids introduced from Vietnam to Europe, such as *C. insigne*, *C. erythrostylum,* and *C. eburneum*. Since then, there has been more and more crossbreeding, and new hybrids have been emerging. There were only four hybrids in 1904 and 88 in 1908, which increased to as many as 170. One of the parents of *C. insigne* is praiseworthy, it shows obvious genetic factors, leaving future generations with good characteristics, such as growth habits, flower shape and color, and easy to cross with other orchids of the genus.

In crossbreeding, most of the parents in Europe are large flower types. They are easier to cross and obtain hybrid offspring, so they are valuable and excellent parents. These hybrids have many flowers, large flowers, long flowering, and beautiful colors, so they are very popular as cut flowers. As a result, the number of new hybrid varieties has increased greatly every year, and tens of thousands of new hybrid varieties have been increased at present. New and excellent hybrid varieties continue to replace the inferior old varieties. The application of new cultivation techniques, aseptic test-tube plantlets, tissue culture, and other rapid propagation progress, so that enterprises and companies engaged in orchid cultivation all over the world.

Since the nineteenth century, European orchid scholars have done a lot of work. In 1833, J. Lindley (father of orchid cultivation) sorted the genus Orchid and gave the first classification of orchids. He also left an unfinished book, "Folia Orchidaceae" considered a classic of Botany.. Blume established the related genus *Cyperorchis* and *Irdorchis*. English naturalist Charles Darwin wrote the book "Fertilisation of Orchids" in 1862, this book was the first essential contribution to the knowledge and comprehension of the strategies used for the species to ensure propagation. Lewis Castle published another book "Orchids: Their Structure, History and Cultivation" in 1877 that offers a concise history of the orchid coupled with simple directions for breeding. For the first time, Reinchenbach made a comprehensive summary of the genus Orchid, describing 19 species of orchids. In 1903, Rolfe, a British scientist, first classified Orchidaceae in China, including nine species of orchids. In 1919, R. Schlechter, a German orchid scholar, summarized 33 species of Orchidaceae in East Asia. In 1924, he made a taxonomic study on *Cymbidium* and *Cyperorchis* all over the world and established the following taxa (groups). The renowned British plant explorer and phytogeographer J.D. Hooker (1888–90) described 1250 species of orchids from the Indian subcontinent in his famous book "Flora of British India."

In Southeast Asia, the history of planting orchids is also earlier. In addition to the cultivation of orchids produced in the region, there are also *C. ensifolium* and *C. sinense* from China. In recent decades, with the improvement of orchid planting technology and the continuous emergence of hybrid orchids, many orchid growing enterprises and companies have been formed, such as Thailand and Singapore, where commercial orchids have entered the international orchid market and have a fixed position in the world orchid industry.

3 Botany and Structure of the Flower

Orchid flowers display a bewildering array of shapes, sizes, and colors, yet all have a distinctive "orchidness" that sets them apart from other plant groups (Apriyanti et al. 2013). The flower of Orchids consists of four main parts including an outer whorl of three sepals, an inner loop of three petals, a single large column in the center, and an enlarged bottom petal called a labellum. The overall flower shape is bilaterally symmetrical a necessity for reliable insect pollination.

The pollen of orchids is also very special. The pollen structure of orchids is called pollen block or pollinia (a coherent mass of pollen grains). It is not the pollen that will spread out, and it will not cause the discomfort of some pollen allergies. Pollen block is the general name of flower powder mass, pollen mass stalk, sticky disk handle, and sticky disk connected, and it is the organ of male and female (Johnson and Edwards 2000). This structure is an efficient structure for orchids to transmit powder to insects. When the insect comes to the orchid flower to collect honey, his body touches the sticky plate, which sticks the orchid pollen block to the insect, takes it to the next orchid, and completes the pollination.

4 Pollination

Pollinators are resources on which plants rely and sometimes compete, hence systems of pollination can be thought of as ecological niches. The pollinator assembly of a plant determines its pollination niche, even though plants interacting with the same pool of pollinators may have diverse pollination niches due to different pollinator use (Joffard et al. 2019). Orchids have a wide range of pollination tactics and flower characteristics. One-third of these orchid species are charming, in that they do not provide a reward for pollinators but instead use signs that pollinators traditionally associate with food or sex promises to attract them (Reyes et al. 2021). Pollinators have played a significant role in the Orchidaceae family's diversification and are critical for the conservation of most orchid species that rely mainly on insects for sexual reproduction (Schatz et al. 2017). While some orchid–pollinator relationships are described as highly specialized, such as in sexually deceptive or euglossine bee-pollinated orchids, others, such as food deceptive orchids, appear to be far more

opportunistic. Pollination niche breadth and overlap may differ between pollination tactics and maybe biogeographical zones. Pollinators of several hundreds of orchids have been documented in detail in several places, particularly in the Europe - Mediterranean region, where orchid–pollinator interactions have been widely recorded (Claessens and Kleynen 2016).

The morphological structure of the orchid flower is an obstacle to easy fertilization and consequently, the pollen lumps can't be carried by the wind, either. Although the birds play a role in the pollination of some species, insects are the most common pollinators in nature. Identifying orchid flowering periods and pollination biology will aid in the creation of capsules, ensuring a dependable and sufficient seed source for future trials. This data is critical for determining the best harvesting period for high-yielding seed germination. Pollen and the age of the flower are both important factors in pollination success (Indan et al. 2021). Reward-based generalized food deception, pollination, autonomous self-pollination, and Batesian food-source mimicry are the four known ways of pollination in *Cymbidium* orchids (Indan et al. 2021).

4.1 Capsule Development

In orchids, pollination mechanisms are highly specialized, and many species have species-specific pollination systems. Specialization, unfortunately, makes species increasingly reliant on and vulnerable to the absence of mutualism partners. The investigation of plant–pollinator interactions is important with considering attempting to maintain self-sustaining populations in the wild over the long term. Pollination during the early flowering phase is recommended because pollen is most receptive between the first and seventh days after blooming, increasing the likelihood of capsule development. Using immature flowers that are less than a week old ensures that the stigmatic surface is open to pollen. Flowers close after 2 weeks, and pollen becomes brown and unresponsive. Hand-pollination technique increased the success of capsule productions as well because the time between pollination and subsequent developmental events in embryos differs enormously in both genus and species, and the time it takes for an orchid capsule to achieve complete maturity differs by species (Utami and Hariyanto 2019). Winter was shown to be the best season for pollinating *Phalaenopsis* hybrids, resulting in an 80–88% capsule formation rate. The germination effectiveness of seeds taken from capsules of varying maturity levels was further reduced by the pollination season. Seeds recovered from winter pollinated capsules consistently outperformed seeds gathered from other seasons in terms of germination (Balilashaki et al. 2015). In *Phalaenopsis* hybrids, it was founded that seeds derived from 5-month mature capsules needed the least amount of time to germinate than seeds derived from 3 months or 7-month mature capsules (Balilashaki et al. 2015).

4.2 Seed Germination

The ultimate competent system of orchid breeding is seed propagation (Shekarriz et al. 2014). Isolation of compatible mycorrhizal fungi is required for symbiotic seed germination. Asymbiotic seed germination, on the other hand, does not necessitate the isolation of mycorrhizal fungi. Moreover, asymbiotic seed germination is a comparatively simple and effective procedure. Nonetheless, there are still some situations where symbiotic germination seedlings are preferable. If there is a probability that symbiotic seedlings will develop more quickly than asymbiotic seedlings, symbiotic seed germination may become the favored method for producing orchids (Deng 1990). Orchid seeds are incredibly tiny which may be 0.1 mm (in Oberonia) to the highest 6 mm (in Epidendrum) in size is the world's smallest seed. There is a nearly 400-year gap between the first sighting of orchid seeds and Knudson's successful asymbiotic germination in 1921. Since then, orchid hybridization has been used for propagation and breeding all over the world. Initial growers' hybrids, on the other hand, could not have predicted their success (Yam and Arditti 2009). Modern hybrid seedlings are usually created by crossing two superior parental cultivars to improve and refine morphological and reproductive characteristics as well as disease resistance (Tang and Chen 2007). Typical orchid hybrids could serve as role models for reintroduction projects, which could help endangered and threatened species. The most important stage in this process is to figure out effective asymbiotic germination techniques to create seedlings for further research. There is little data on seed germination in *Phalaenopsis* species, and none on asymbiotic or symbiotic seed germination parameters. The type of basal medium and capsule age was found to affect ability (seed maturity) (Indan et al. 2021).

5 Hybridization

Crossing orchids to other species is one option for preventing genetic extinction. Hybridization has the effect of combining the best qualities of both parents in the hybrid offspring. In assessing the effectiveness of a hybridization procedure, selecting a parent with high compatibility to be crossed is critical, one major block to the successful crossing is that the crossed parents should have close genetic closeness and in assessing the effectiveness of a crossbreeding effort (Hartati et al. 2019). Even though crossbreeding is a straightforward and effective method for cultivating orchid hybrids, there are various things to consider when doing so, including the hybrid combination's fertility, qualitative analysis of goal features, and the selection of superior hybrid offspring (Reinikka 1972). F1 progenies formed from two parents with opposing goal features typically show significant phenotypic differences. However, it has been noted that in the case of *Cymbidium*, hybrid seeds, particularly those of distant hybrids, are difficult to cultivate because of their distant genetic link, with the degree of difficulty rising in the order intraspecific intrageneric/

intergeneric. Failure of distant hybridization is caused by parents' incompatibility and postfertilization embryo abortion (Li et al. 2021).

Since the beginning of orchid collection and cultivation, natural hybrids resulting from crossbreeding between species have been observed in the wild. *Phalaenopsis intermedia* is the oldest hybrid that produces by a cross between *P. aphrodite* and *P. rosea* (De and Bhattacharjee 2011).

5.1 Artificial Hybridization

Orchid developers around the world have experimented with many species and hybrids, with variable degrees of success. Orchid hybrids are the progeny of a cross between two genetically different individuals. In this group of plants, intraspecific, intrageneric, and intergeneric hybrids have been developed. In orchids, intergeneric crossings are very common, and many hybrids involving two, three, four, and five genera have been registered and listed (De and Bhattacharjee 2011). Although free breeding is prevalent in orchids, it is not possible to make hybrids between any two genera. The majority of orchid breeding success has been attributed to the art of paint breeding, orchid breeders' intuition and tenacity, and, on a few times, pure luck. Raising progeny from seed to flowering stage takes several years. Orchid seeds, unlike those of other crops, require specific care to germinate and the maturation of seeds takes a long time. Furthermore, the number of seeds produced in a capsule is so large that obtaining a representative sample of the progeny is impossible. As a result, information on the ability to combine characteristics and their inheritance in orchids is limited (De and Bhattacharjee 2011).

6 Breeding Methods

6.1 Crossbreeding

Crossbreeding is one of the most common and effective methods in orchid breeding. Orchid interspecific and even some intergeneric crossing is easy to succeed, but because orchid seeds do not have endosperm and organized embryo, they need to rely on symbiotic fungi to germinate in nature. Early hybrid breeding is very difficult to obtain hybrid progenies. In 1854, RHS founded the international login system for orchid hybrids. The first orchid hybrid to be logged in is *Calanthe dominyi* (*C. furcate* × *C. masuca*). Knudson found that sugar can replace fungi to promote seed germination, and established the technique of in vitro propagation of any plant in pure (that is, aseptic) culture (Knudson 1922). The registration of new orchid hybrids showed explosive growth. Several factors must be considered when performing crossbreeding, these factors are fertility of the hybrid combination, qualitative analysis of target traits, and the selection of superior hybrid offspring (Reinikka

1972). The F1 progenies derived from two parents with contrasting target traits (such as a parent with large flowers in size but short flowering time and the other with long flowering time but small flowers in size) usually exhibit large phenotypic differences (Zhang et al. 2011). Recently embryo rescue technique has shown the light of hope to regenerate distant hybrids effectively. By this technique, immature embryos are cultured in vitro and controlled embryo abortion (Luo et al. 2012).

6.2 Ploidy Breeding

Orchids are prone to 2n gametes during meiotic, which leads to polyploidy of hybrid progenies. Polysomaty and polyploidy are common occurrences among orchids, represent a powerful force for evolution, and have been found in several important genera: *Paphiopedilum*, *Coelogyne*, *Cymbidium*, *Dendrobium*, *Calanthe*, *Oncidium*, *Paphiopedilum*, *Vanilla*, *Vanda,* etc. (Hossain et al. 2013). At present, the offspring sterility produced by interspecies crossing with different chromosome sizes or ploidy levels is one of the problems encountered by *Phalaenopsis* breeders. Therefore, the occurrence of endopolyploidy in *Phalaenopsis* was studied, and a simple and effective technique was developed to determine the nuclear DNA content and double the number of chromosomes. In addition, flow cytometry has been used for endopolyploidy in different tissues of *Phalaenopsis* species. It was found that different patterns of endopolyploidy occurred in different tissues of *Phalaenopsis* species at various stages of development. According to these results, a simple and effective protocol was developed for the production of polyploid plants by sectioning protocorms or protocorm-like bodies (PLBs) without using anti-microtubule agents (Chen et al. 2011). Through this technique, a series of tetraploid species of *Phalaenopsis* were developed. For example, *Phal*. Doris and *Phal*. Zada, the super parents of *Phalaenopsis* breeding, are tetraploid hybrids. Among the registered *Phalaenopsis* hybrids, 90.2% have *Phal*. Doris lineage and 43.5% have *Phal*. Zada lineage.

6.3 Selection Breeding

Breeding according to selection uses the natural diversity of genotypes as the original material for selection. After selection breeding, three important genetic parameters must be considered: heritability, genetic correlations between traits, and interactions of genotypes ×environment. The three parameters should be used to deal with the relationship between heritability, variation, and selection given that plant phenotypes are determined by the environment as well as by the genetic material (Murthy et al. 2018).

6.4 Molecular Marker-Assisted Breeding (MMAB)

In addition, MMAB was also carried out. The application of MMAB technologies for practical breeding and selection has several advantages as it is fast, accurate, and free from the influence of environmental conditions (Jiang 2015). MMAB is to reduce linkage liability, aggregate favorable genes, speed up the breeding process, and improve breeding efficiency by using molecular marker analysis closely linked to the target gene. Among the different molecular markers, scientists and breeders have given prevalence to RFLP, AFLP, SNP, SSR. The genetic relationship among 81 selected *Dendrobium* species and hybrids was studied. AFLP markers could be used to determine the variation between materials. This study provided useful information on the genetic diversity of some *Dendrobium* orchids and will likewise be useful for monitoring the germplasm, developing new hybrids, and protecting new plant varieties. Molecular marker-assisted breeding has been widely used in crop breeding, but there are few reports in orchid breeding, and there is still a lack of molecular markers closely linked to the target traits. The breeding and application of functional genes have become a hot research direction in orchid plants in recent years. Professor Yu Hao of the National University of Singapore has successfully established a genetic transformation system for *Dendrobium*, which is expected to carry out molecular breeding and quality-oriented improvement (Sawettalake et al. 2017; Chai and Yu 2007). Recently, the point mutants of C3H and C4H genes of *Dendrobium candidum,* and the mutants of MADS44, MADS36, and MADS8 of *Phalaenopsis* were successfully obtained by using the CRISPR/Cas9 gene-editing system (Tong et al. 2020).

6.5 Transgenic Breeding

To improve the important characteristics of orchids, such as new flower color, fragrance and shape, flowering control, abiotic stress tolerance, disease, and pest resistance, transgenic technology has been applied to orchids. It is often difficult to introduce new traits into orchids through mutation or conventional breeding, but genetic transformation can be relatively easy to achieve (Nirmala et al. 2006). The success of orchid genetic engineering, like other plants, depends on the totipotency of plant cells, that is, the inherent ability of plant tissues to produce cells that can regenerate fully dynamic plants (Hossain et al. 2013).

Although the flowering time, flower fragrance, and color of orchids can be controlled by genetic transformation, it remains to be determined whether the flowering period is prolonged or not. Moreover, the disastrous impact of viral diseases on the yield and quality of orchids remains a major concern for orchid breeders and producers.

6.6 Conventional Breeding

Flower color, shape, and smell are the main unique identifiers for orchids since they are the main determinatives of customer choice. Conventional breeding techniques, though, have resulted in the loss of perfume in many new floricultural cultivars. Cut flower and decorative orchid breeders have concentrated on generating plants with enhanced vase life, transportation qualities, and overall aesthetic attributes (like color and shape). *Phalaenopsis* orchids have 2–3 years of growth courses. Using conventional hybridization to transfer beneficial characteristics into commercial cultivars is a lengthy and time-consuming procedure that will require years to complete. Furthermore, intraspecific and/or interspecific incompatibility hampers variety enhancement work. All five *Phalaenopsis* subgenus has identical chromosome numbers ($2n = 2x = 38$), which may be classified into small, medium, and large chromosomal groupings based on chromosome sizes and nuclear DNA content (Chen et al. 2011). Species with short chromosomes, including *P. amabilis*, *P. aphrodite,* and *P. equestris*, are the source of the majority of commercial cultivars. *P. amboinensis* and *P. violacea* are among the species with big chromosomes and powerful scents. Because of interspecific incompatibility, productive crosses among species with tiny and big chromosomes are challenging. Seed germination, as an important component of traditional breeding, is directly relevant to the performance and efficiency of crossbreeding. To develop an effective germination mechanism, in-depth research into the developmental features and germination processes of distant hybrid seeds is very important. Because once hybrid seeds are gained, an appropriate cultivation strategy is required to maintain the population constant or expand it. Because orchid grains are hard to replicate in the natural environment, in vitro propagation system is the most significant breeding procedure for orchids. Seed maturation, culture situations, and culture medium are all important elements of in vitro propagation success. Many orchid species have been studied in vitro, such as those of the genera *Cymbidium*, *Phalaenopsis*, *Dendrobium*, *Oncidium*, *Dactylorhiza*, and *Calanthe (*Bezerra et al. 2020*)*. At the moment, the major goals of in vitro propagation are the production of genotype variation and reducing the breeding course, and substantial progress has been achieved toward these goals. Adaptation of bioreactor system in micropropagation has opened a new era in plant propagation.

6.7 Breeding Via Mutation

Mutation breeding is suited for breeding ornamental plants because species can be easily reproduced, simplifying the generation of spontaneous and induced mutants (Yamaguchi 2018). Mutation provides several benefits, such as a high mutation rate, the disruption of trait relationships, efficient enhancement of individual characteristics, and the reduction of the breeding course (Li et al. 2021). Over time, this kind of

breeding has already been utilized to create orchids with distinct phenotypic characteristics, increased medicinal component concentration, and improved adaptation and tolerance (De et al. 2014). Polyploidization is a usual technique of mutation breeding. Most orchid species, such as *Cymbidium (*Wang et al. 2011*)*, *Dendrobium* (Zhang et al. 2011), *Oncidium* (Cui-Cui et al. 2010), and *Phalaenopsis* (Chang et al. 2019), have been successful through polyploid breeding. Orchids' high heterozygosity can boost the apparent mutation rate and result in a slew of superior mutation kinds in a short time. Unexpected mutations can occur, resulting in harmful mutations, but in most cases, only single alterations are acquired (Reinikka 1972). Furthermore, the success of mutation breeding is determined by parameters including explant type, genotype, induced mutation technique, and *optimal* dose for each mutagenic treatment.

6.8 Hybrid Breeding

Due to the intrinsic beauty of flowers and the capacity to transfer these characteristics to hybrids, several species have gained worldwide attention in breeding programs. Several species are important, such as *Cymbidium devonianum*, *C. lowianum*, *C. tracyanum*, *C. elegans*, and others (Tiwari et al. 2022).

6.9 Molecular Breeding

A research project aimed at creating a solid approach for orchid molecular breeding utilizing the CRISPR/Cas9 knockout technology. *Phalaenopsis amabilis* protocorms cultured on New Phalaenopsis medium supplemented with peptone were utilized as the plant materials. Ti plasmids had been filled with T-DNA construct of pRGEB32 vector carrying PDS3 sequence, and protocorm was immersed in the *Agrobacterium tumefaciens*. Transformants were detected and verified. From PDS3T2 lines, 0.96% of PDS transformants were produced. Several transformants have paler leaves than non-transformants. The CRISPR/Cas9 system appears to have effectively altered the target gene in orchids, indicating that it might be used for practical gene editing in orchids (Semiarti et al. 2020). The study determined that the *Agrobacterium tumefaciens*-mediated transformation method might be used to deliver CRISPR/Cas9 to a *Dendrobium macrophyllum* orchid protocorm. The T-DNA Ubi::: Cas9:: VAR2/prGEB32, and afterward the protocorm were cultivated for 4 weeks in the Vacin and Went culture medium +6 mg/L hygromycin antibiotics for transformants, the *A. tumefaciens* strain EHA 105 was infected.

Researchers found that transformation efficiency was maximum (0.66%) during the 15-min infection phase, but it dropped to 0.43% and 0.23% after 30 and 45 min. Cas9 (402 bp), HPT (545 bp), VAR2 (723 bp), the *D. macrophyllum* genome, and

TrnL-F (1200 bp) were amplified. When examining the sequence, a substitution mutation was observed at the target site (Setiawati et al. 2020). MMAB offers the advantages of fast, accurate, independent of environmentally friendly settings using molecular biotechnologies for practical breeding and breeding (Jiang 2015). As a result of their frequency and potential, the following are the most relevant molecular markers: RFLP AFLP, SSR, and SNP. The first three were broadly applied with great success for orchid reproduction (Li et al. 2015), established a set of markers (Gen-SSR) for the genetic connections, and the cartooning investigations of other orchid species at *Cymbidium ensifolium*. When combined with functional annotations of unigenes, these marker types assist to recognize candidate genes with unique environmental roles. The sequencing of *Paphiopedilum concolor* root transcriptome in a simple sequence of repeats provides critical insight into the mechanisms of the growth and development of the roots (Li et al. 2015). The genes linked with flower color, floral shape, and resistance in *Phalaenopsis*, which were utilized by Chung et al. (2017) to find out, was a major reference point in genetic engineering generally for the *Phalaenopsis* and Orchidaceae (Chung et al. 2017). The efficacy of flower color forecast for several *Phalaenopsis* species was tested by applying gene-specific single-nucleotide amplified polymorphism markers to facilitate the reproduction of a novel *Phalaenopsis* variety. The Phalaenopsis aphrodite genome was confirmed and integrate with an SNP-based genetic link and optical map. This has developed a unique asset to not only increase the reproductive performance of horticultural orchids but also attributed to major studies of epiphyte genomic adaptation for future reference (Chao et al. 2018). The first SNP integrated high-density map with large coverage in the genome of *Dendrobium* was published by Lu et al. (2018). Many QTL sites laid the basis to map more features of medicinal relevance for the future. When it comes to gene-mining and genome studies, *Bletilla striata*'s EST-SSR transcriptome has provided a solid foundation for phylogenetic and operational gene-mining studies (Xu et al. 2019). Researchers have laid the groundwork for fine-tuning the expression quantitative trait locus (eQTL) mapping of *Dendrobium* (*D. nobile*, *D. wardianum*) by RNA sequencing, eQTL analysis, and development of high-density genetic maps (Li and Chan 2018). Wang et al. (2019) examined in vitro the fluctuation of SNP and insertion-deletion frequencies in *Oncidium* "Milliongold" somaclones that had been regenerated by protocorm-like bodies (PLBs) (Wang et al. 2019). Most species lacking reference genome sequences might benefit from SLAF-seq, according to the study's findings. Molecular marker technology has been extensively applied to the study of orchid phylogeny and genetic relationships, but only a few studies have combined molecular marker technologies with phenotypic characteristics. Another technology (Genome-Wide Association Research) has also been used in studies on cabbage, tomato, and tea (Deng 1990; Xing et al. 2019; Fei et al. 2020), but in orchids only to a modest extent. As a result, greater study on these characteristics is needed to offer more precise genetic data for orchid breeding. *Camellia* is a genus of flowering plants in the Theaceae family, and many of its species are economically valuable. A large number of single sequence repeats (SSRs) in the *Camellia* genus have been produced in the last decade, yet there are not enough SSRs available to the public in this genus. During the investigation, a

total of 4,63 kb of data was collected, including 28,854 putative SSRs. They synthesized and initially screened 172 primary pairs of 10 *C. japonica* accessions and found that 111 polymorphic accessions matched those depending on taxonomy and regional categorization. Additionally, 51 polymorphic SSR markers have been randomly selected for future genetic interactions of 89 accessions in the *Camellia* region. Each *C. japonica* genotype was significantly split and grouped, as demonstrated by the genetic structure study's clustering algorithms. For the molecular genetic reproduction of camellias, the results give high-quality SSR resources (Li et al. 2021). Genomic diversity was found to be widespread among the species (PPB: 90.1%; HE: 0.3414; H: 0.5013). Despite this, genetic diversity within groups was limited. With PPB: 76.2%; HE: 0.2966; $H = 0.4319$, Shiko-2 was the most variable, whereas XS was the least variable (PPB: 67.3%; HE: 0.2344; H: 0.3478). Nei's gene diversity statistics, Shannon's information measure, and AMOVA (analysis of molecular variance) with 21.3%, 21.4%, and 22.5%, respectively, indicated a very high degree of genetic differentiation among populations. The genetic and geographic distances were shown to be significantly related ($r = 0.8154$, P 0.05) (Deng 1990). An important part of developing microsatellite loci to improve commercial moth orchid breeding is molecular identification (*Phalaenopsis* species). There are Microsatellite Primer Sets for the *Phalaenopsis aphrodite* subspecies, which include genomically-SSR and EST-SSR primers. To better understand *Phalaenopsis* transferability, *P. aphrodite* subsp. *formosana* will be utilized. Magnetite beads and NGS (next-generation sequencing) collected 10 or 28 polymorphic EST-SSRs and gSSR (genomic-SSR) markers that indicate 21 *Phalaenopsis* species, including several subgenus *Phalaenopsis* with strong transferability. They found that these microsatellite markers differed from those found in the *Phalaenopsis* subgenus. The genetic connections among species of the *Phalaenopsis* subgenus may therefore be isolated and integrated. They can help to identify parentages of *Phalaenopsis* and to investigate the hybridization of *Phalaenopsis* (Bolaños-Villegas et al. 2021).

6.10 Gene Transfer Breeding

Mutation breeding and crossbreeding are generally challenging methods for introducing new traits, like new colors or disease resistance, into orchids, but transgenic technology makes it possible (Nirmala et al. 2006). It is most usual to utilize *Agrobacterium*-mediated and microprojectile techniques to breed orchids (Fig. 1). *Dendrobium* (Kuehnle and Sugii 1992) were the first orchids to undergo successful transformations by particle bombardment. Efficient transformation methods have been devised for certain major commercial orchids, such as *Phalaenopsis* (Tong et al. 2020), *Vanda* (Shrestha et al. 2007), *Cymbidium* (Chin et al. 2007), *Dendrobium* (Chen et al. 2018), *Cattleya* (Zhang et al. 2010), *Erycina pusilla* (Li and Chan 2018), etc.

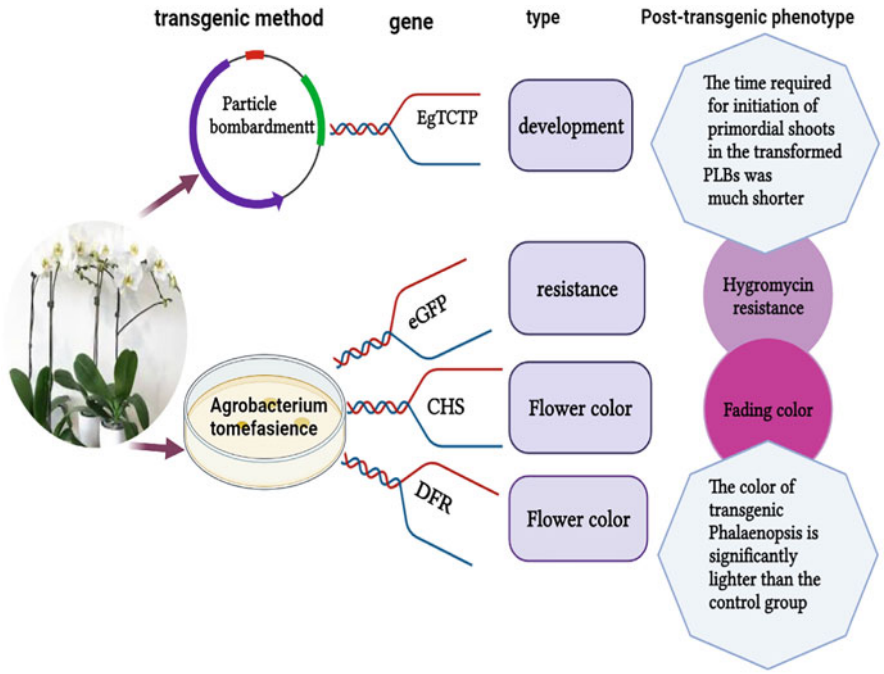

Fig. 1 Gene transfer methods to breed *Phalaenopsis* orchids are based on *Agrobacterium*-mediated and particle bombardment techniques and their transformation pathways to introduce superior traits in orchids species

By using particle bombardment, Yang et al. (1999) successfully transferred a plasmid containing *GUS* and *NPTII* markers to orchids to create kanamycin-resistant transgenic plants (Yang et al. 1999). Scent-related genes were identified by using RAPD molecular markers. Transgenic *Cymbidium* plants were created when *NPTII*, the plasmid containing the *GUS* marker gene, was introduced from *Agrobacterium* to *Cymbidium* (Chin et al. 2007). As Chai and Yu (2007) review, transgenics have become a key means for creating new genotypes of orchids and have resulted in important progress in flora, plant architecture, and biotic and abiotic resistance. *Agrobacterium tumefaciens* has created the *Phalaenopsis* protocorm as a vector expressive receptor material and pCAMBIA1301 (containing the GUS report gene and the hygromycin resistant gene hpt) (Chai and Yu 2007). Researchers used the pollen tube route as well as the ovary injection methods for the transmission of the *cbf1* resistant gene into *Phalaenopsis*. On Fd and OnFNR have shown substantial impacts on soft rot and both genes can play an important role in the resistance to *Oncidium* soft rot (Tong et al. 2020).

The transformation of the *Oncidium* PLBs via *Agrobacterium*-mediated transformation for temporary expression was carried to PR1 (an important downstream gene of acquired plant resistance). Plants that were transformed became stronger (Gao et al. 2020). In *A. thaliana*, the introduction of *Dendrobium* Chao Praya Smile

DOAP1 led to early flowering as well as early termination of inflorescence meristem into flower meristems [4; 61].

Use of protocorms produced from seeds of *Phalaenopsis aphrodite* and *Phalaenopsis* cultivars as an alternate transformation method. eGFP was driven by ubiquitin promoter in the T-DNA vector construct utilized for transformation. Hygromycin was used to select the altered protocorms, which were then effectively regenerated. BC1 progeny demonstrated resistance to hygromycin when backcrossed to the transgenic line, proving the transgene is heritable. It has been shown that all backcross F1 explants that survived were positive transformants utilizing PCR and western blot analysis (Hsing et al. 2016). With the use of particle bombardment, *Agrobacterium*-based transformation systems and direct gene transformation procedures for genetically modified *Oncidium* orchids have been developed (Li et al. 2015; You et al. 2003). When it comes to transforming *Oncidium* with ferredoxin resistant to soft rot disease (You et al. 2003), for example, the following methods are described: using the *Agrobacterium* system, using particle bombardment to suppress the flower color gene (Yee et al. 2008), and using the same *Agrobacterium* system to alter the ethylene receptor gene (Raffeiner et al. 2009). The processing of *Oncidium* has grown even more complicated by adding the phosphomannose isomerase gene to the *Agrobacterium*-mediated transformation system. Because of their hygromycin sensitivity and long-term regeneration, *Oncidium* species have had a restricted number of genetic transformation studies. GFP (Green Fluorescent Protein), phosphotransferase hygromycin (*hptII*), and CymMV-CP genes were introduced into the protocorm-like bodies of *Oncidium* orchids *Oncidium* Gower Ramsey and *Oncidium* Sweet Sugar (PLBs) using a direct gene transformation approach. Many transgenic *Oncidium* orchids were investigated in a genetic study to confirm the inheritance of transgenes.

It was possible to effectively transfer the AcF3H gene from Ascocenda flavanone 3-hydroxylase (AcF3H) to *Dendrobium* 5 N white orchid plants utilizing *Agrobacterium*-mediated gene transformation. A plant expression vector with the AcF3H gene was built in the gateway cloning method. *A. tumefaciens* AGL1, which carried the plant expression vector pGWB5-AcF3H, was co-constructed as a selective marker. The agroinfiltration method was employed to temporarily express acF3H in white *Dendrobium* 5 N and Anna petals *Dendrobium* and the findings revealed that, according to the study, no cyanidine concentration was detected for white petals *Dendrobium* 5 N after acF3H infiltration. On the other hand, the content of *Dendrobium* Anna petals was 6% higher than cyanidin showing that AcF3H was transitory (Khumkarjorn et al. 2017).

Tetraploid or diploid *Phalaenopsis* orchids have been explored with the transfers of *Agrobacterium*-mediated genes with a construct of T-DNA vector which contains the eGFP powered by the ubiquitin promoter. A hybrid between the pollinia of the transgenic plants and four separate *Phalaenopsis* orchid varieties revealed hygromycin and *hptII* positivity in PCR and GFP protein production demonstrated by Western blotting (Hsing et al. 2016). The AcF3H (Ascocenda Flavanone 3-hydroxylases) gene has been successfully transformed into white orchid plants

of *Dendrobium* 5 N utilizing *Agrobacterium* transformation genes. An expression medium for the AcF3H gene was produced for the first time utilizing gateway cloning. For the hpt gene, the protocol-like corpus (PLBs), the *A. tumefaciens* line AGL1, and the PGWB5-AcF3H vector of plant expression were co-cultivated. The highest transformation efficacy was therefore obtained by cultivating PLBs with *Agrobacterium* cells in 15 min (10.13%). To verify the transgenic plants, the seedlings were rebuilt 3 months after the transformation and PCR analysis was performed, the hpt gene and the 35S promoter region were targeted using particular primers. Transgenic crops had about 400 and 500 bp PCR products that matched the gene of hpt and the 35S promoter, respectively, but no non-transgenic crops, indicating that the AcF3H gene was present in a white orchid genome. AcF3H was temporarily expressed using agroinfiltration procedures in the white and the *Dendrobium* Anna petals of *Dendrobium* 5 N and discovered in the white petals of *Dendrobium* 5 N after AcF3H that wasn't cyanidin content in the sample. The cyanidin concentration of *Dendrobium* Anna petals, on the other hand, rose by around 6%, indicating temporary expression of the AcF3H gene. When PLBs were co-cultivated with *A. tumefaciens* AGL1, which maintains pGWB5-AcF3h for 15 min, the highest transformation efficiency (10.13%) was attained. The wild type and mutant libraries were completely clean reading 98,988,774 and 100,188,534 bp and De Novo, constructed at 98,446 uniqueness, accordingly for an average length of 989 bp. When transcription profiles were compared between the two libraries, 18.489 were discovered to be differently expressed.

Most of the Kyoto encyclopedia for the enrichment of genes and genomes was used in membrane-building and ploidy-related activities, consisting of increased flowering and changed cell sizes seen in the mutant. 29 MADS-box genes were identified as possibilities for the floral patterning of *C. goeringii*, as well as several floral and hormone-affecting regulators and genes. A short RNE sequence revealed that 132 miRNA families produced in *C. goeringii* flowers were conserved, and the multiple-tepal formation has been caused by 11 microRNAs related to 455 target genes. The combined study of mRNA and microRNA showed two transcription/microRNA pathways that contribute to multi-tepal characterization (Fig. 2): a popular floral related miR156/SPL and a miR167/ARF regulations technique for developing reproductive organs; and a multi-tepal cell-proliferation regulations cascade that likely regulates the miR319/TCP4–miR396/GRF regulation (Cheamuangphan et al. 2013).

Cymbidium faberi has a distinctive floral smell which boosts its commercial worth, one of the most renowned oriental orchids. However, until this study the molecular process of floral fragrance production was unclear. Methyl jasmonate (MeJA) is one of *C. faberi's* major organic volatile compounds (VOCs). 79,363 unigenes were selected for further examination using comparative transcriptome analysis. 9409 genes (GDEs) of which 558 were assigned to 258 pathways led to a transcriptome study of blooming and withered *C. faberi* flowers (Xu et al. 2019). The top 10 strategies for achieving a conversion of alpha-linolenic acid to MeJA included the metabolism of α-linolenic acid, pyruvate metabolism, and fatty acid degradation. In one of its DEG Jasmonic Carboxylic Acid Methyl Transferases

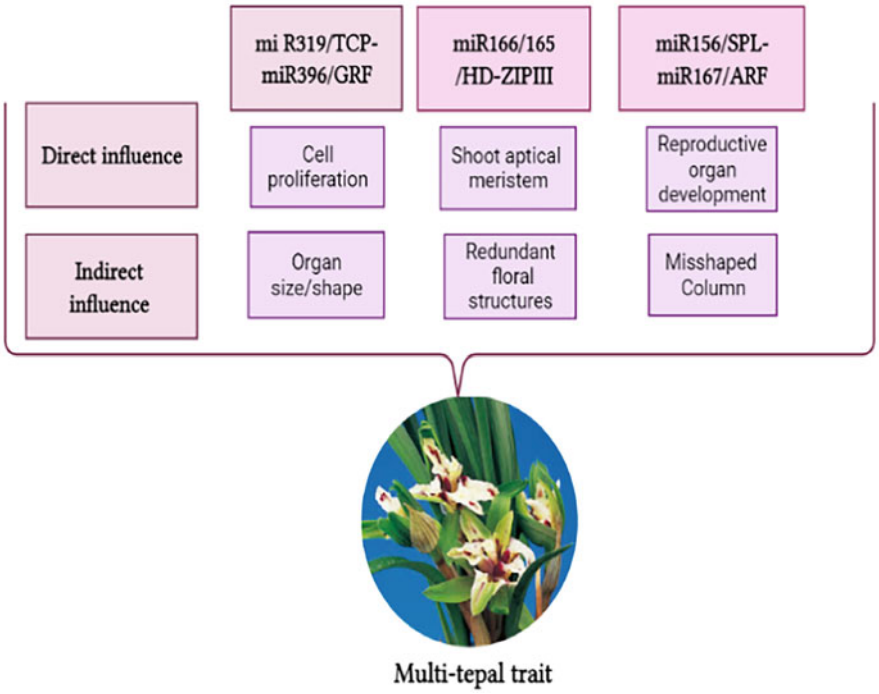

Fig. 2 A system of miRNA/transcription factors influences the multi-tepal characteristics of *C. goeringii*

(CfJMT, unigene 79,363), flora blooming *C. faberi* is expressed extensively but seldom detected in the roots or leaves. While CfJMT synthesis in tomatoes did not raise MeJA levels, the expression of internal MeJA genes, particularly for the treatment of injuries, has changed, indicating that CfJMT may be connected with abiological stress in the tomato. The molecular pathways for floral fragrance generation in *C. faberi* have been explored as part of a study that will aid in the genetic modification of modern varieties of commercially valuable oriental orchids (Xu et al. 2019).

The EHA105 *A. tumefaciens* strain, which possesses a binary plasmid, has been shown to successfully process *Erycina pusilla* plants. The promoter for the plasmid should be CaMV 35S series (CaMV 35S) (Lee et al. 2015). The hygromycin-containing medium can be used to select explants with 6-benzylaminopurine and naphthaleneacetic acid modifications. According to research, protocorm-like (PLBs) at 3 months of age is the best stage for transformation. Self-pollination allowed T1 progenies to be obtained in the 18-month MV 35S series (Li and Chan 2018; Lee et al. 2015). To stimulate protocorm development and multiplication, the self-pollinated seed capsules of *Erycina pusilla* are broken under aseptic conditions and a sterile half-strength MS medium was used to germinate seedlings in plastic plates (Lapjit and Tseng 2015). Upon germination, the protocorms and greens

should have a diameter of 1 cm. It's time to get back to the basics. CRISPR/Cas9 might be used to change MADS-box genes and alter floral morphology in *Erycina*. *Agrobacterium*-mediated RNA interference has been investigated in the past, but with little success (Lin et al. 2016). *E. pusilla* has been crossed with several important Oncidiinae orchids to produce new commercial orchid species. The clone PSYP1 as *E. pusilla* "Hsingda Golden" derived from in vitro flowering system has been granted the Plant Variety Rights in Taiwan for protection (Bolaños-Villegas et al. 2021).

7 Prospects for Orchid Breeding

There seems to be unevenness in science-based study and practice in the application of forwarding genetics since laborers select effective point mutations (Hall and Richards 2013) but do not recognize how to use them, so research labs are restricted by the lack of viable mutants to investigate. Reverse genetics, as opposed to forwarding genetics, investigates phenotypic changes in genetically inherited modifications using huge amounts of information. Omic information from orchids, which includes genomic, proteomic, transcriptome, and metabolome sequence analysis, has been getting more and more a direct consequence of developments in limited sequence alignment techniques. The above findings will serve as guidelines for genetically modified breeding and genome engineering breeding programs, laying the groundwork for orchid breeding programs. Moreover, among the most apparent disadvantages of reverse genetics is that it can reveal a large number of genes linked to favorable characteristics, making it more difficult to constrict the objective gene array. To resolve this ambiguity, it is necessary to combine genomic and other omic data, as well as breeding and morphologic records. Integrating, acquiring knowledge, and investigating will aid in the discovery of gene functions linked to essential qualities (Langridge and Fleury 2011). Besides conquering conflict and infertility, mutagenesis breeding could be used to gain large differences in flower color, anatomy, and shape. As a result, merging hybridization and mutation breeding will be a viable tactic for recognizing hybridization's maximum capabilities throughout orchid rearing. Attribution of specific genes is a prevention effort for breeding programs, but there is presently no reliable transition service for orchids. Transformation is currently accomplished primarily through *Agrobacterium*-mediated processes, particle bombardment, and gene silencing. Besides this, even though plants from essential ornate species of the genus like Phalaenopsis and Dendrobium have already undertaken genome editing, general performance is lower. As a result, it is still important to broaden studies on CRISPR/Cas9 to support access to key orchid phenotypes. Furthermore, while there is reportedly very little molecular genetic information for orchids, more transcriptomic evidence has become accessible, which will aid in the exploration of essential qualities including flower color, floral morphological characteristics, and flower aromas. As a consequence, molecular breeding is expected to become the primary method for orchid breeding. To

summarize, for certain, if researchers investigate important characteristic genetic traits utilizing forward or reverse genetics, or if we use conventional breeding, natural selection, or single-molecule breeding to produce great progeny to achieve desired attributes, every method has benefits and drawbacks, and if used individually, it seems to be unusual to advance reproduction. Thus, a variety of techniques and research directions must be incorporated to enable the production of orchids with different flower sizes and morphology, new colors, and complex flower fragrances.

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