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THREATENED ORCHIDS OF MAHARASHTRA: A PRELIMINARY ASSESSMENT BASED ON IUCN REGIONAL GUIDELINES AND CONSERVATION PRIORITISATION

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Abstract

The Maharashtra state lies between the latitudes 22°1' to 16°4' N and longitudes 72°6' to 80°9' E. spreading in an area of 307,731 km^2; it accounts for about 9.84 per cent of the total landmass of the country. Extensive field surveys of orchids were conducted during 2011-2014 in various parts of the state. A preliminary regional assessment was carried out using regional guidelines in accordance with the IUCN Red List criteria 3.1. A total of 101 orchid species were assessed of which 6 species are considered to be Possibly Extinct (PE), 7 species are categorised as Critically Endangered (CR), 7 species are Endangered (EN), 24 species as Vulnerable (VU), 25 species are considered Near Threatened (NT), 23 species are at Least Concern (LC) and 9 species are considered as Data Deficient (DD). In the present study thirty eight species of the orchids are reported as Threatened (CR, EN, VU). The main current threats are habitat degradation, mining and stone quarrying, over-grazing and trampling, windmills, invasive species, tourism, landslide, fire, over collection and drought. Three protected areas (Koyna WLS, Chandoli NP and Radhanagari WLS) are recommended for the *in situ* conservation.

Introduction

THE BIODIVERSITY across the planet is facing a rapid decline due to various threat factors which include habitat loss or degradation, over-exploitation, biological invasions, industrialisation, pollution and accelerated climate change. As a result of these anthropogenic activities, the rate of plant extinction has reached to 137 species per day (Moram *et al*., 2011). During the second half of the 20th century, species extinction rates reached an almost unprecedented level in Earth's history (Frankham, 2003). This rate is considered to be 1000–10,000 times faster than the one it could naturally occur (Hilton-Taylor, 2000) and a trend which may result in the disappearance of between 60,000 and 1,00,000 plant species during the next 50 years (Akeroyd, 2002; Bramwell, 2002). The Convention on Biological Diversity (CBD) in which India is a signatory aims to conserve biodiversity, sustainable use of its components and share the benefits arising from the utilization of genetic resources in a fair and equitable way. The Global Strategy of Plant Conservation (GSPC) was adopted by CBD at its sixth conference of the parties. The long-term objective of the GSPC is to halt the continuing loss of plant diversity. The revised Global Strategy for Plant Conservation (GSPC) (2011–2020) calls for an assessment of the conservation status of all known plant species (target 2, UNEP, 2010).

The IUCN Red List of Threatened Species is recognised as the most comprehensive and objective global approach for evaluating the conservation status of plant and animal species. It is a widely recognized tool for identifying threatened species and offers a powerful method to identify priority sites for protection by providing information on the conservation status of species in the wild (Rodrigues *et al.,* 2006). The red list data constitutes a source of information that is essential to guide conservation efforts focussed on species. It is probably the best tool for estimating the current levels of biodiversity and trying to judge whether biodiversity levels increase or decrease in the future. IUCN Red list categories and criteria 1994 and 2001 were planned for the assessment of extinction threat of the species at the global level. Over the last decade, there has been growing interest in countries using the IUCN Red List Categories and Criteria at local and regional levels because it is the regional scale where the anthropological actions and biodiversity strike (Pimm *et al*., 2001). The regional or national threat lists play a significant role in enlightening global preservation efforts, particularly when the information that they contain is integrated into the global IUCN Red List (Cuaron, 1993; Rodriguez *et al*., 2000). In response to this interest, IUCN developed guidelines on how to apply the IUCN Red List Criteria appropriately for sub-global level assessments. Since the first version of the Guidelines for Application of the IUCN Red List Criteria at Regional Levels was

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published in 2003 (version 3.0), these guidelines have been reviewed. In 2012, the guidelines for application of the IUCN Red List Criteria at Regional and National Levels (version 4.0) was released. The extinction risk of a species can be assessed at global, regional or national level. One species can have a different category in the Global Red List and a Regional Red List. For example, taxa that is common worldwide and classified as Least Concern (LC) in the Global Red List might be Endangered (EN) in a particular region. Red listing is not an end in itself but provides a comparative framework for conservation planning (Given, 2003).The application of the IUCN Red List Criteria at the regional level is a scientific and objective process for assessing how likely a species is to go extinct from a particular region.

Orchids are regarded as the flagship species in plant conservation, although sadly many species are being driven to extinction by either direct or indirect human activities. The state of Maharashtra harbours 101 orchid species. Many species are threatened with extinction either directly through loss of habitat or due to reasons such as degradation, fragmentation, over-collection etc. The aim of this article is to provide the preliminary red list assessment of orchids of Maharashtra at regional level. We hope that such regional assessment will be definitely beneficial for conservation planning at regional level as well as at national level.

Materials and Methods

Study Area

The state Maharashtra lies in the Western and Central part of the country between the latitudes 22°1' to 16°4' N and longitudes 72°6' to 80°9' E. It is bordered by Gujarat and the Union territory of Dadra and Nagar Haveli to the NorthWest, Madhya Pradesh to the North and NorthEast, Chhattisgarh to the East, Karnataka to the South, Telangana to the SouthEast and Goa to the SouthWest. It occupies an area of 307,731 km^2, which accounts for about 9.84 per cent of the total area of the country. The altitude ranges from sea level to 1646 msl. It comprises 35 districts and physiographically, this state may be divided into three natural divisions - the coastal strip (the Konkan), the Sahyadri or the Western Ghats and the Plateau. Over 80 % region of the state is occupied by Deccan Plateau. Tapi, Godavari, Bhima and Krishna are the main rivers of the state. This state has a tropical monsoon climate. Over 90% of the rainfall is due to South-Western monsoon (June to September). There is heavy rainfall in the coastal region (about 2000 mm), scanty rains in the rain shadow areas in the central parts (about 500 mm) and medium rains in the eastern parts (about 1000 mm) of the state. As per Champion and Seth (1968), the State has 16 forest types which belong to six forest type groups *i.e.*, Tropical Semi-Evergreen, Tropical Moist Deciduous, Littoral and Swamp, Tropical Dry Deciduous, Tropical Thorn and Subtropical Broad leaved Hill Forests.

Species Coverage

A total 101 species belonging to 33 genera were assessed. Of these 51 species are terrestrial, 49 epiphytic and one myco-heterotrophic. In Maharashtra, the total endemic orchid species are 36 spread over in 12 genera. Of these 25 species are endemic to Western Ghats *i.e.*, *Bulbophyllum fimbriatum* (Lindl.) Rchb.f., *Conchidium exile* (Hook.f.) Ormerod, *C. filiforme* (Wight) Rauschert, *C. microchilos* (Dalzell) Rauschert, *Dendrobium aqueum* Lindl., *D. barbatulum* Lindl., *D. lawianum* Lindl., *D. microbulbon* A. Rich., *D. nanum* Hook.f., *D. nodosum* Dalzell., *D. ovatum* (L.) Kraenzl., *Gastrochilus flabelliformis* (Blatt. & McCann) C.J. Saldanha, *Habenaria elwesii* Hook.f., *H. foliosa* A. Rich., *H. heyneana* Lindl., *H. multicaudata* Sedgw., *H. ovalifolia* Wight, *H. perrottetiana* A. Rich., *H. rariflora* A. Rich., *H. suaveolens* Dalzell, *Pinalia mysorensis* (Lindl.) Kuntze, *P. polystachya* (A.Rich.) Kuntze, *Smithsonia maculata* (Dalzell) C.J.Saldanha, *S. straminea* C.J. Saldanha and *S. viridiflora* (Dalzell) C.J. Saldanha, 4 species are endemic to Peninsular India *i.e.*, *Eulophia pratensis* Lindl., *Habenaria brachyphylla* (Lindl.) Aitch., *H. gibsonii* Hook.f. and *H. grandifloriformis* Blatt. & McCann and 7 species are Indian endemic *i.e.*, *Aerides crispa* Lindl., *A. maculosa* Lindl., *Conchidium reticosum* (Wight) Ormerod, *Eulophia ochreata* Lindl., *Habenaria hollandiana* Santapau, *H. longicorniculata* J. Graham and *Porpax jerdoniana* (Wight) Rolfe.

Data Collection

The present work is the result of extensive and intensive field explorations undertaken during the period 2011 to 2014 at different regions of Maharashtra. Prior to the field survey, a tentative list of species occurring in Maharashtra was prepared based on standard literature. The information collected was used to draft the preliminary distribution of these species, as well as to plan the time table for field studies. The geographical co-ordinates of each location were recorded during the field survey using Global Positioning System (GPS model Garmin etrex). A total of 517 GPS readings were recorded in the field and simultaneously 1641 occurrence records were

collected from different herbaria (CAL, BSI, BLAT, SUK). The period of these herbarium data collections range from the year 1888 to 2014. Records lacking geographic coordinates on specimen labels were geo-referenced using topographic maps and online mapping tools such as Google Earth or GEOLocate.

During field surveys, when a population of orchids was located, the size, the extent, the habit, the habitat, the altitude and the life forms were recorded. Mature individuals were also counted in each locality for assessing the status of the species, only those individuals which bear flowers or fruits were counted as mature (IUCN, 2010). Direct observations were made to determine the potential and actual threats to the orchid population in Maharashtra. Various threats that were observed include habitat destruction, modification and fragmentation of natural habitats, encroachments, tourism activities, windmills, mining and stone quarrying, illegal collection for medicinal purpose, grazing, fire, invasive species, and natural disasters.

IUCN Categories and Criteria

There are nine clearly defined categories [Extinct (EX), Extinct in the Wild (EW), Critically Endangered (CR), Endangered (EN), Vulnerable (VU), Near Threatened (NT), Least Concern (LC), Data Deficient (DD) and Not Evaluated (NE)] of IUCN to categorise any known taxa in the world. Extinct means that there is no reasonable doubt that the last individual has died. Extinct in the Wild means that the taxon is extinct in its natural habitat. Species under the CR, EN and VU categories are all considered as "threatened" and are a conservation priority. The category Near Threatened is applied to taxa that do not qualify for CR, EN and VU, but is close to qualifying for or is likely to qualify for a threatened category in the near future. The category Least Concern is applied to taxa that do not qualify for CR, EN, VU and NT. Widespread and abundant taxa are included in this category. The category Data Deficient highlights taxa for which sufficient information is lacking to make a sound assessment status. The category Not Evaluated applies to taxa that have not yet been evaluated against the Red List Criteria.

The IUCN has framed five quantitative Red Listing Criteria (A: Population size reduction, B: Geographic range, C: Small population size and decline, D: Very small or restricted population, E: Quantitative analysis) to determine whether a taxon is threatened or not. Any one or all of these criteria can be used to assign the threat category (IUCN, 2001, 2012). These criteria are based around the biological indicators of populations that are threatened with extinction, such as rapid population decline or very small population size. Most of the criteria also include sub-criteria that must be used to justify more specifically the listing of a taxon under a particular category.

For regional assessment, the IUCN Red List Categories and Criteria will be same as global but with three exceptions or adjustments. One, taxa extinct within a particular region but extant in other parts of the country is classified as Regionally Extinct (RE). The category Regionally Extinct (RE) is used when no reasonable doubt that the last individual has died. Listing of a species as 'Regionally Extinct' requires exhaustive surveys in all known or likely habitats. The tag of 'Possibly Extinct' has therefore been developed to identify those Critically Endangered species that are likely disappeared from the region, but for which confirmation required (IUCN, 2010). Possibly Extinct is a tag and not a new Red List category. In the present assessment for such species the tag 'Possibly Extinct (PE)' was used. Two, the category of Extinct in the Wild (EW) should be assigned only to taxa that are extinct in the wild across their entire natural range, including the region, but that are extant in cultivation, in captivity, or as a naturalized population (or populations) outside the past range. If a taxon is (globally) EW but extant as a naturalized population within the region, the regional population should not be evaluated according to the IUCN Criteria, but should still be considered of conservation importance and preserved as a relict of a taxon which is Extinct in the Wild. It may also be considered an important source of individuals for re-introduction efforts within its natural range. There is no such taxon present in the state. Three, taxa not eligible for assessment at the regional level (mainly introduced taxa and vagrants) should be assigned the category Not Applicable (NA). The addition of the categories Regionally Extinct (RE) and Not Applicable means that there are 11 possible categories for regional assessments (Fig. 1a). A brief description of the IUCN categories B & D (except A,C and E) which were used in the assessment of present study for orchids of Maharashtra is provided in the Table 1a. Criteria A, C and E were not used for the assessment because there were no data on defined rate of population decline coupled with the small population size.

Regional Conservation Assessment

The regional assessment was carried out in a three-step process. The first step begins to determine which taxa (Orchidaceae) and which regional populations

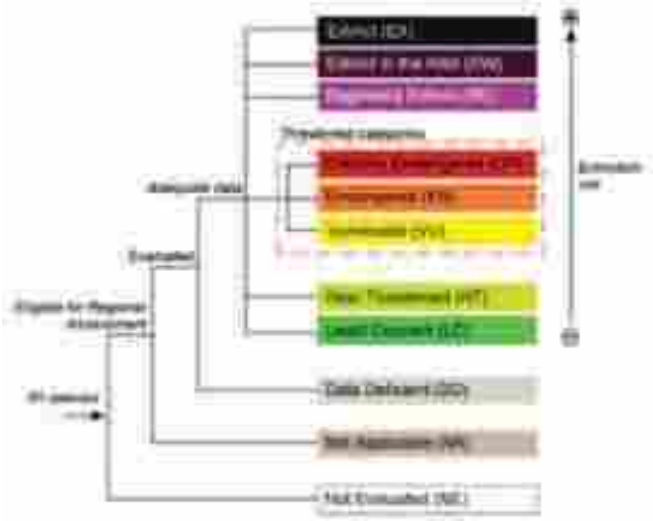

Fig. 1a. IUCN Red List Categories at regional scale (IUCN, 2012).

(Maharashtra state) to assess (step one). Next, the regional population for each taxon is evaluated according to the IUCN Red List Categories and Criteria Ver. 3.1 (IUCN 2001, 2012), and a preliminary category is assigned (step two). The effect of populations of the same taxon in neighboring regions on the regional population is then considered and the preliminary category is down-listed if appropriate (step three). If the taxon is endemic to the region or if the regional population of a species to be assessed is isolated from conspecific populations outside the region, the criterion is used without modification. Adjustments can be made to all the categories except for Extinct (EX), Extinct in the Wild (EW), Regionally Extinct (RE), Data Deficient (DD), Not Evaluated (NE), and Not Applicable (NA), which cannot logically be up- or down-listed. Taxa that have been down-listed in the regional Red List is clearly indicated by a degree sign after the category (*e.g.*, EN°, VU°).

Calculation of Area of Occupancy (AOO) and Extent of Occurrence (EOO)

Range size, according to IUCN, is measured as extent of occurrence (EOO, the smallest polygon in which no internal angle exceeds 180^0 and contains all sites of occurrence) and as area of occupancy (AOO, the area occupied by taxon, excluding cases of vagrancy, at a scale appropriate to the taxon). These two measures

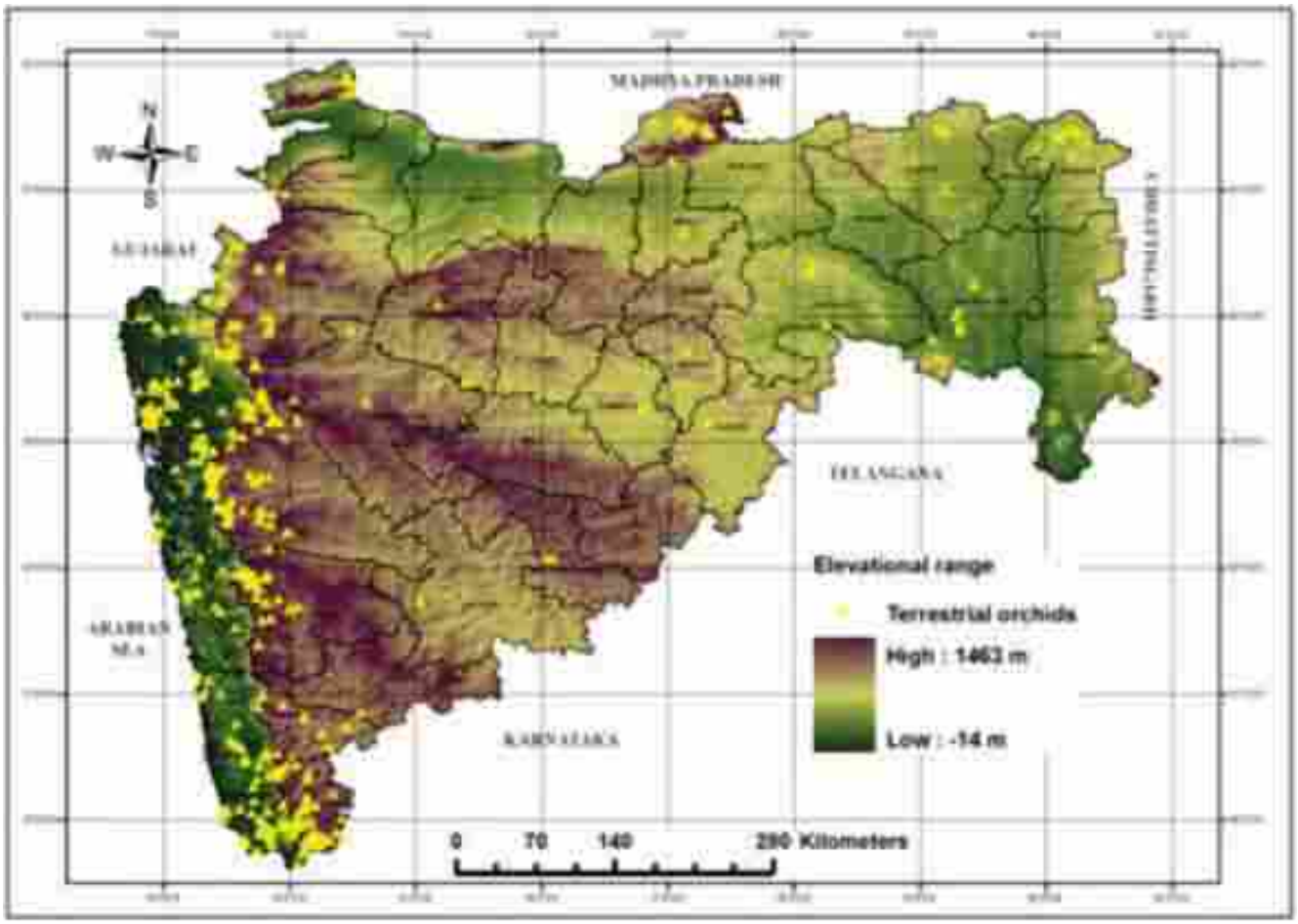

Fig. 1b. Distribution of terrestrial orchids in Maharashtra.

Table 1a. IUCN threat categories and Criteria (B and D) applied to the regional assessment of orchids in Maharashtra.

B. Geographic range in the form of either B1 (extent of occurrence) and/or B2 (area of occupancy)			
	Critically Endangered	Endangered	Vulnerable
B1. Extent of occurrence (EOO)	< 100 km²	< 5,000 km²	< 20,000 km²
B2. Area of occupancy (AOO)	< 10 km²	< 500 km²	< 2,000 km²
AND at least 2 of the following conditions:			
a) Severely fragmented, OR Number of locations	1	< 5	< 10
b) Continuing decline in any of: (i) extent of occurrence; (ii) area of occupancy; (iii) area, extent and/or quality of habitat; (iv) number of locations or subpopulations; (v) number of mature individuals.			
c) Extreme fluctuations in any of: (i) extent of occurrence; (ii) area of occupancy; (iii) number of locations or subpopulations; (iv) number of mature individuals.			

	Critically Endangered	Endangered	Vulnerable
D. Number of mature individuals	<50	<250	D1. <1,000
D2. Only applies to the VU category Restricted area of occupancy or number of locations with a plausible future threat that could drive the taxon to CR or EX in a very short time.			D2. typically: AOO<20km² or number of locations < 5

are the foundation of the 'B' criterion of the IUCN Red List system (IUCN 2001). EOO provides information on overall geographical spread while AOO provides information on the area of suitable habitat. Both EOO and AOO were calculated using the Geospatial Conservation Assessment Tool (GeoCAT; Geospatial Conservation Assessment Tool), developed by Royal Botanic Gardens, Kew. All the occurrence data of a particular species was prepared in the spreadsheet and this data import was directly done to the GeoCAT tools. Based on the location points, the extent of occurrence (EOO) and area of occupancy (AOO) values are instantly calculated and the values were compared with the thresholds set in the IUCN Criteria.

Results and Discussion

Distribution Pattern

The distribution of orchids in Maharashtra is patchy and concentrated in the high rainfall areas such as Khandala-Lonavala, Mahabaleshwar-Koyna-Chandoli, Amboli and Radhanagari. As a whole, orchids are concentrated mainly in the Western Ghats of Maharashtra (>80 species) and lowest in the Deccan peninsula. Among different life forms, the epiphytic orchids showed the same pattern, whereas the few species of terrestrial orchids were found distributed in the Deccan plateau (Figs.1b,2). Rainfall is one of the major climatic factors that affects the distribution of vegetation at a regional scale. The Western Ghats Mountain Range is very tall and blocks the moisture from the SouthWest monsoon and hence the Deccan Plateau region receives very little rainfall. Due to high mountains of Western Ghats, the rainfall decreases Northwards and Eastwards. The semi-arid region of Deccan plateau only supports few terrestrial species *i.e., Eulophia graminea* Lindl, *Eulophia pratensis* Lindl., *Habenaria commelinifolia* (Roxb.) Wall. ex Lindl., *Habenaria digitata* Lindl., *Habenaria gibsonii* Hook.f. and *Habenaria roxburghii* Nicolson and that too, mainly in rainy season. However, in marshy localities and near dam side, *Zeuxine strateumatica* (L.) Schltr. is also seen growing in the winter season. *Peristylus constrictus* (Lindl.) Lindl. is distributed in Satpura range of Toranmal and Melghat areas in Maharashtra. Epiphytic orchids such as *Luisia trichorhiza* (Hook.) Blume and *Vanda tessellata* (Roxb.) Hook. ex Don are also reported from this range. Majority of the endemic species are confined to selected hill tops or small hill areas of semi-evergreen forests, plateaus and moist deciduous forests, thus making those pockets very important with regard to conservation. Very few species are distributed in the central Maharashtra and Vidharba regions. This region falls under the rain shadow region. Endemic species such as *Aerides maculosa* Lindl., *Eulophia pratensis* Lindl., *Habenaria gibsonii* Hook.f. and *Habenaria grandifloriformis* Blatt. & McCann have very wide range of distribution. But their maximum abundance is in Western Ghats part of Maharashtra. To see the species richness along the altitudinal gradient, the state is divided into 100 m altitudinal zones for the sake of convenience. The overall distribution of orchids is shown in Fig. 3. There

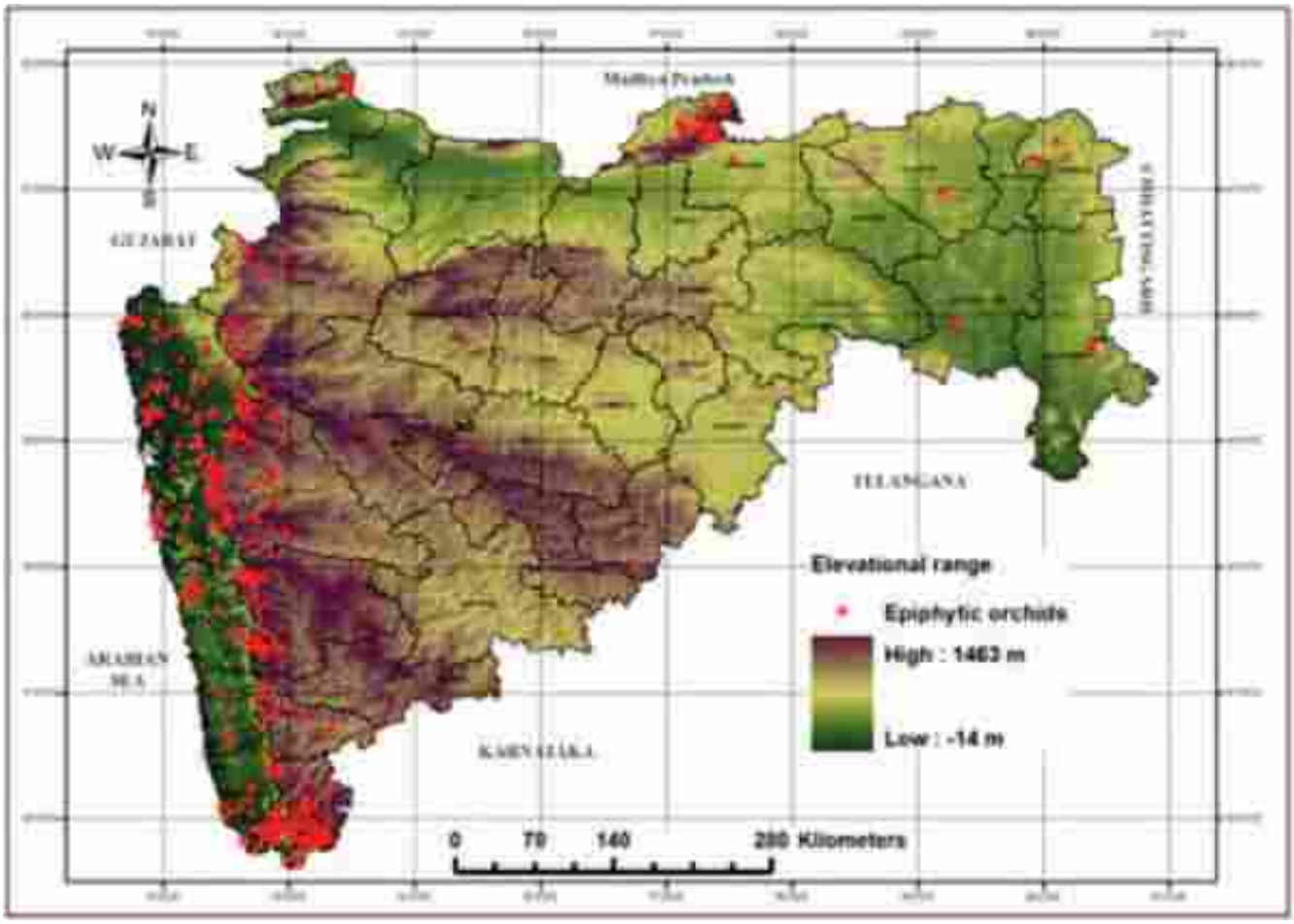

Fig. 2. Distribution of epiphytic orchids in Maharashtra.

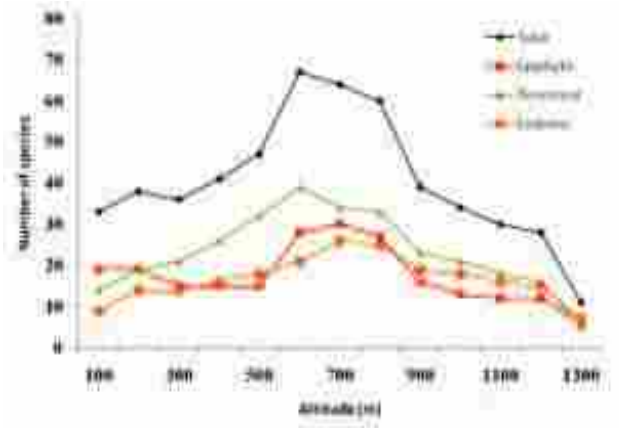

Fig. 3. Altitudinal distribution of orchids in Maharashtra.

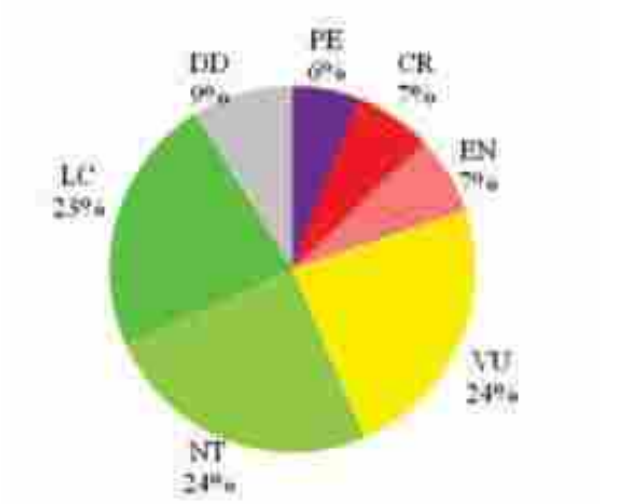

Fig. 4. Overall regional assessment status of orchids of Maharashtra.

is a significant increasing trend in the total species richness up to 600 m and after that it shows gradual decrease. Both the epiphytic and terrestrial orchids have their maximum richness in the 600 m altitude. Because of habitat heterogeneity, this altitude has maximum habitat support for orchids. However, endemic orchid species richness is more in the elevations of 700 to 800 m, because many endemic

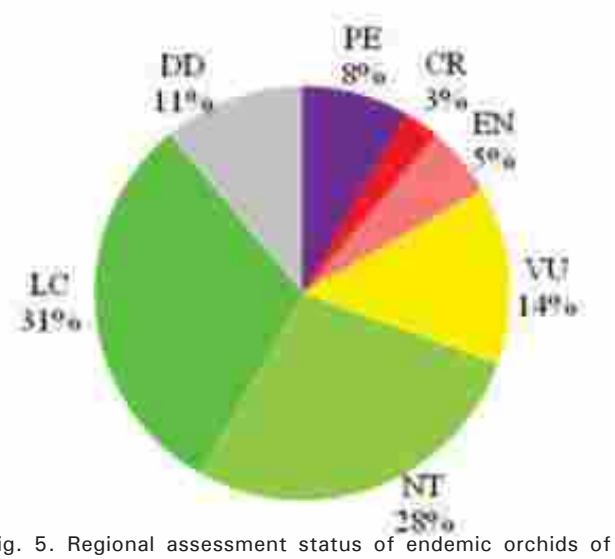

Fig. 5. Regional assessment status of endemic orchids of Western Ghats in Maharashtra.

species are restricted to these altitudes; and they are distributed in high rainfall pockets.

IUCN Threat Status

The available data and assessment for all species are shown in Table 1b. The preliminary assessment shows that 38 species are in threatened category: 7 species are Critically Endangered, 7 Endangered and 24 Vulnerable. Within the Not Threatened categories, 23 species (23%) are classified as Least Concern, 25 species (24%) as Near Threatened, and 9 species (9%) as Data Deficient. A total of 6 species (6%) have been assessed as Possibly Extinct in Maharashtra (Fig. 4).

The localities of the seven Possibly Extinct species were thoroughly explored during the survey but could not be located in the field. The possibility could be that the population size may be alarmingly small or the species may be present outside the study area. Based on these possibilities, placement of the species under the category of Regionally Extinct (RE) is doubtful until a thorough survey is made in the adjacent areas also. Therefore, as recommended in "Guidelines for Using the IUCN Categories and Criteria (IUCN, 2010), these species are tagged as "possibly extinct" and further efforts should be made in order to confirm their actual conservation status.

Out of total 36 endemic species, 3(8%) species are Possibly Extinct, 1 (3%) species Critically Endangered, 2 (5%) species Endangered, 5 (14%) species Vulnerable, 10 (28%) species Near Threatened, 11 (31%) species Least Concern and 4 (11%) species Data Deficient (Fig. 5). The majority of the assessments used category 'B' that relates to the geographical range.

Orchids Vs Protected Areas

Maharashtra state has a total of 42 well established protected areas (PAs) including one conservation reserve, covering an area of approximately 18,730 km^2 which constitutes 6.08 per cent of the state's geographical area. The present field surveys and past records show that out of 42 PAs, only 16 protected areas harbour 50% of orchid species out of the total

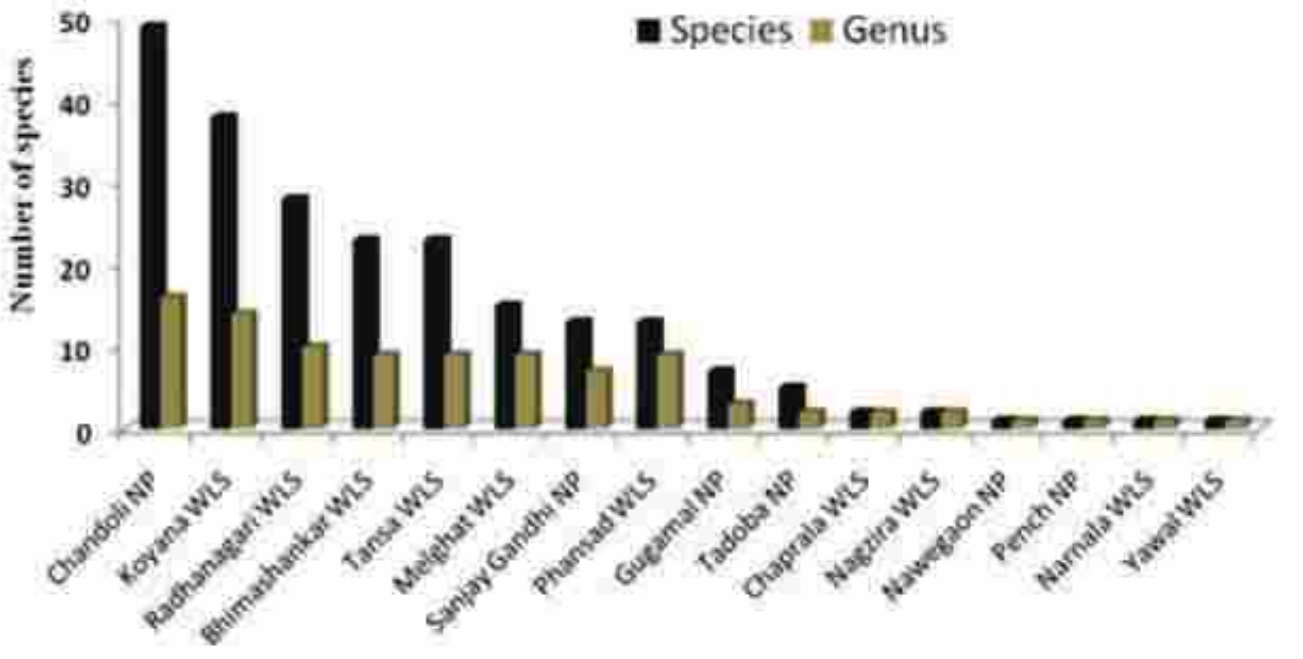

Fig. 6. Orchid species richness in the different PAs in Maharashtra.

Table 1b. Regional assessment of orchids of Maharashtra.

SL. No.	Species	Habit	EOO km^2	AOO km^2	Locations	Population (Total count of mature individuals)	Threat Category	Criteria	Threats
1	*Aerides ringens* (Lindl.) C.E.C.Fischer	E					PE		
2	*Habenaria viridiflora* (Rottler ex Sw.) Lindl.	T					PE		
3	*Pinalia mysorensis* (Lindl.) Kuntze	E					PE		
4	*P. polystachya* (A.Rich.) Kuntze	E					PE		
5	*Smithsonia maculata* (Dalzell) C.J.Saldanha	E					PE		
6	*Trias stocksii* Benth. ex Hook.f	E					PE		
7	*Cheirostylis flabellata* (A.Rich.) Wight	T		4	1		CR	B2ab(iii)	A
8	*Cleisostoma tenuifolium* (L.) Garay	E		4	1	Unknown	CR	B2ab(iii)	A
9	*Oberonia ensiformis* (Sm.) Lindl.	E		4	1		CR	B2ab(iii)	A
10	*O. mucronata* (D.Don) Ormerod & Seidenf.	E		4	1		CR	B2ab(iii)	A
11	*Pachystoma pubescens* Blume	T		4	1		CR	B2ab(iii)	A
12	*Smithsonia straminea* C.J.Saldanha	E		4	1	*ca*.210	CR	B2ab(iii); D	A
13	*Luisia tenuifolia* Blume	E		8	2		CR	B2ab(iii)	A
14	*Cheirostylis parvifolia* Lindl.	T	2,459.54	20	4	<150	EN	B1ab(i,ii,iii) + 2ab(i,ii,iii)	A
15	*Dendrobium nodosum* Dalzell	E	3,686.54	16	4	<200	EN	B1ab(i,ii,iii,iv) + 2ab(i,ii,iii,iv)	A
16	*Eulophia graminea* Lindl.	T		8	2	<10	EN	B2ab(ii,iii)	A, H, J
17	*E. epidendraea* (J.Koenig ex Retz.) C.E.C.Fisch.	T		8	2	Unknown	EN	B2ab(iii)	A, J
18	*E. pratensis* Lindl.	T	65,782.18	20	5	< 80	EN	B2ab(i,ii,iii)	C, E, H, I, J
19	*Peristylus aristatus* Lindl.	T	1,119.18	28	4	30	EN	B1ab(iii) + 2ab(iii)	C, F
20	*Zeuxine gracilis* (Breda) Blume	T	108.18	12	3	<15	EN	B1ab(iii) + 2ab(iii)	B
21	*Bulbophyllum sterile* (Lam.) Suresh	E		8	2	Unknown	VU^o	B2ab(iii)	A
22	*Cymbidium bicolor* Lindl.	E		8	2	<100	VU^o	D2	A
23	*C. aloifolium* (L.) Sw.	E	177.35	12	3	<500	VU^o	B1ab(iii) + 2ab(iii)	A, H
24	*Dendrobium crepidatum* Lindl. & Paxton	E	9,363.89	32	5	<500	VU^o	B2ab(i,ii,iii)	A
25	*D. macrostachyum* Lindl.	E	4,198.34	24	4	<600	VU^o	B1ab(i,ii,iii,iv) + 2ab(i,ii,iii,iv)	A
26	*D. nanum* Hook.f.	E	8,326.85	20	5	<400	VU^o	B2ab(ii,iii,v)	A
27	*D. peguanum* Lindl.	E	4,244.96	16	4	380	VU^o	B1ab(i,ii,iii,iv) + 2ab(i,ii,iii,iv)	A, H
28	*Epipogium roseum* (D. Don) Lindl.	MH		8	2	8	VU^o	B2ab(iii)	G
29	*Eulophia herbacea* Lindl.	T	1,492.95	12	3	Unknown	VU^o	B1ab(i,ii,iii) + 2ab(i,ii,iii)	A, C, E, J
30	*E. ochreata* Lindl.	T	214,330.22	36	5	< 450	VU^o	B2ab(i,ii,iii,iv); C2a(i)	A, C
31	*Geodorum densiflorum* (Lam.) Schltr.	T	94,140.10	20	5	80	VU^o	B2ab(i,ii,iii,iv)	B, E
32	*Habenaria crinifera* Lindl.	T	3,729.04	36	8	<400	VU^o	B1ab(iii) + B2ab(iii)	A
33	*H. multicaudata* Sedgw.	T	26,028.85	16	4	21	VU^o	B2ab(iii); D	E, H
34	*H. stenopetala* Lindl.	T	6,910.37	20	3	<10	VU^o	B2ab(i,ii,iii)	B, G
35	*H. suaveolens* Dalzell	T	15,979.10	40	8	<8000	VU	B1ab(i,ii,iii,v)	B, F
36	*Luisia trichorhiza* (Hook.) Blume	E	1,732.42	16	4	<100	VU^o	B1ab(iii) + 2ab(iii)	A, H

Table 1b. Regional assessment of orchids of Maharashtra (contd.).

SL. No.	Species	Habit	EOO km^2	AOO km^2	Locations	Population (Total count of mature individuals)	Threat Category	Criteria	Threats
37	*Luisia tristis* Hook.f.	E	87,842.57	24	5	150	VU°	B2ab(ii,iii)	A, H
38	*Oberonia brunoniana* Wight	E	1,916.74	20	4	21	VU°	B1ab(iii) + 2ab(iii)	A
39	*O. falconeri* Hook.f.	E	56,063.45	16	3	<500	VU°	B2ab(i,ii,iii)	A
40	*Peristylus lawii* Wight.	T	109,380.57	20	4	350	VU°	B2ab(i,ii,iii)	B, E
41	*Pholidota imbricata* Lindl.	E		8	2	<250	VU°	B2ab(ii,iii); D	A
42	*Porpax jerdoniana* (Wight) Rolfe	E	8,773.13	20	5	<500	VU°	B2ab(ii,iii)	A
43	*Thunia alba var. bracteata* (Roxb.) N.Pearce & P.J.Cribb	E	3,590.66	20	5	<250	VU°	B1ab(iii) + 2ab(iii)	A
44	*Zeuxine longilabris* (Lindl.) Trimen	T	2,743.86	28	4	<350	VU°	B1ab(iii) + 2ab(iii)	B
45	*Bulbophyllum fimbriatum* (Lindl.) Rchb.f.	E	10,711.25	36	9	<3,000	NT°	B1ab(iii)	A, H
46	*Conchidium exile* (Hook.f.) Ormerod	E	8,592.16	32	7	*ca.*600	NT°		A
47	*C. reticosum* (Wight) Ormerod	E	17,189.50	72	15	<4000	NT		D, F
48	*Dendrobium aqueum* Lindl.	E	14,139.33	56	10	<3500	NT°	B2a(iii)	A, D
49	*D. herbaceum* Lindl.	E	22,518.39	52	11	<2500	NT		A
50	*D. lawianum* Lindl.	E	19,098.46	44	7	<3,000	NT°	B1ab(ii,iii)	A
51	*Eulophia spectabilis* (Dennst.) Suresh	T	257,270.14	72	12	2,700	NT		A, E, I
52	*Habenaria commelinifolia* (Roxb.) Wall. ex Lindl.	T	264,881.63	60	11	400	NT		G, J
53	*H. brachyphylla* (Lindl.) Aitch.	T	169,332.95	48	12	800	NT		B, F
54	*H. diphylla* (Nimmo) Dalzell	T	15,349.36	28	7	<500	NT°		B, C
55	*H. foliosa* A. Rich.	T	25,390.14	56	12	<500	NT		C, E, I
56	*H. furcifera* Lindl.	T	125,967.96	36	12	<380	NT		A, B
57	*H. ovalifolia* Wight.	T	36,470.29	56	12	<300	NT		A, F
58	*H. plantaginea* Lindl.	T	212,479.76	64	12	<700	NT		F, I
59	*H. rariflora* A. Rich.	T	34,545.25	72	15	<3000	NT		B, D, F, G
60	*H. roxburghii* Nicolson	T	64,178.19	24	6	< 300	NT		A, J
61	*Liparis odorata* (Willd.) Lindl.	T	17,895.21	60	11	<3500	NT		A
62	*Nervilia crociformis* (Zoll. ex Moritzi) Seidenf.	T	31,243.66	92	15	<11000	NT		A
63	*N. infundibulifolia* Blatt. & McCann	T	21,353.31	40	10	<5000	NT		A, G
64	*N. plicata* (Andrews) Schltr.	T	25,954.25	40	10	<4500	NT		A
65	*Peristylus densus* (Lindl.) Santapau & Kapadia	T	19,608.50	72	13	<4000	NT		B, C
66	*Porpax reticulata* Lindl.	E	12,310.93	56	11	<6000	NT		A, H
67	*Smithsonia viridiflora* (Dalzell) C.J.Saldanha	E	12,140.81	40	7	<400	NT°		A
68	*Zeuxine strateumatica* (L.) Schltr.	T	158,863.40	44	11	<5000	NT		A, J
69	*Acampe praemorsa* (Roxb.) Blatt. & McCann	E	37,391.54	120	25	<8,000	LC		A
70	*Aerides crispa* Lindl.	E	46,210.66	120	24	<4,000	LC		A, D

Table 1b. Regional assessment of orchids of Maharashtra (contd.).

SL. No.	Species	Habit	EOO km^2	AOO km^2	Locations	Population (Total count of mature individuals)	Threat Category	Criteria	Threats
71	*Aerides maculosa* Lindl.	E	174,700.97	156	32	<15,000	LC		A, D
72	*Conchidium filiforme* (Wight) Rauschert	E	30,997.35	104	21	<10,000	LC		A
73	*C. microchilos* (Dalzell) Rauschert	E	30,467.35	92	18	<7,000	LC		A
74	*Cottonia peduncularis* (Lindl.) Rchb.f.	E	33,484.89	72	18	<1500	LC		A, H
75	*Dendrobium barbatulum* Lindl.	E	74,101.72	140	29	<15,000	LC		A
76	*D. microbulbon* A. Rich.	E	26,262.48	80	18	<11,000	LC		A
77	*D. ovatum* (L.) Kraenzl.	E	35,274.79	104	21	<7500	LC		A, H
78	*Habenaria digitata* Lindl.	T	188,278.22	96	20	2,500	LC		B, C, E
79	*H. gibsonii* Hook.f.	T	210,108.10	128	27	<2000	LC		B, C
80	*H. grandifloriformis* Blatt. & McCann	T	170,109.82	124	24	<20000	LC		B, C, F
81	*H. heyneana* Lindl.	T	26,890.01	88	18	<3500	LC		B, C, F
82	*H. longicorniculata* J. Graham	T	136,219.18	92	17	<5000	LC		B, F, G
83	*H. marginata* Colebr.	T	297,032.34	140	28	<4000	LC		B, C
84	*Malaxis versicolor* (Lindl.) Abeyw.	T	34,006.46	88	17	<5000	LC		A
85	*Nervilia concolor* (Blume) Schltr.	T	213,330.46	96	19	<6000	LC		A
86	*Oberonia recurva* Lindl.	E	29,074.29	80	15	<10000	LC		A
87	*Pecteilis gigantea* (Sm.) Rafin.	T	207,315.23	88	15	<1000	NT		B, C, E, F,G
88	*Peristylus plantagineus* (Lindl.) Lindl.	T	284,961.53	92	18	<2500	LC		B
89	*P. stocksii* (Hook.f.) Kraenzl.	T	155,879.45	84	15	<4000	LC		A
90	*Rhynchostylis retusa* (L.) Blume	E	240,677.18	84	16	<3500	LC		A
91	*Vanda tessellata* (Roxb.) Hook. ex Don	E	63,234.29	52	16	<2500	LC		A
92	*V. testacea* (Lindl.) Rchb. f.	E	325,352.18	68	15	<1500	LC		A
93	*Diplocentrum recurvum* Lindl.	E					DD		
94	*Eulophia dabia* (D.Don) Hochr.	T					DD		
95	*Gastrochilus flabelliformis* (Blatt. & McCann) C.J. Saldanha	E					DD		
96	*Habenaria elwesii* Hook.f.	T					DD		
97	*H. hollandiana* Santapau	T					DD		
98	*H. perrottetiana* A. Rich.	T					DD		
99	*Oberonia bicornis* Lindl.	E					DD		
100	*Peristylus constrictus* (Lindl.) Lindl.	T					DD		
101	*Spiranthes sinensis* (Pers.) Ames	T					DD		

Abbreviations: E, Epiphytic; MH, Mycoheterotrophic; T, Terrestrial; PE, Possibly extinct; CR, Critically Endangered; EN, Endangered; VU, Vulnerable; NT, Near Threatened; LC, Least Concern; DD, Data Deficient; A, Habitat degradation; B, Over-grazing and trampling; C, Mining and stone quarrying; D, Windmills; E, Invasive species; F, Tourism; G, Landslide; H, Fire; I, Over collection; J, Drought.

orchid species recorded in Maharashtra (Fig. 6). The best protected areas in terms of orchid species richness are Chandoli National Park with 49 species, Koyna Wildlife Sanctuary with 38 species, and Radhanagari Wildlife Sanctuary with 28 species. These protected areas can play a major role in protection of orchid diversity because within these areas, there is a restriction of collection of these species. Many PAs in Maharashtra are subject to both natural and human-induced disturbances at various scales. In recent decades, many of these have been heavily threatened by the spread of invasive alien plant species, notable among them being *Lantana* and *Eupatorium*. Mining industries are coming extremely closer to these PAs and some are even inside the PAs. Radhanagari WLS is one of the best PA for *in situ* orchid conservation but Indian Aluminium's (INDAL) Durgamanwad mine touches Radhanagari's northern boundary and affecting the habitat of rare and endemic orchids.

Major Threats to Orchids in Maharashtra

Major threats to orchids of Maharashtra include habitat degradation, mining and stone quarrying, over-grazing and trampling, windmills, invasive species, tourism, landslide, fire, over collection and drought. The graphical representation of each threat (Fig. 7) shows that 40% species are affected by habitat degradation. Destruction and fragmentation of natural habitats are the two most important factors in the current species extinction event. Although habitat destruction and degradation often appear to be the most immediate and significant effect, losses of unique evolutionary lineages and erosion of natural demographic and genetic processes associated with small population sizes as well as isolation are sure to be of consequence while considering the future of these populations (Coates, 2000).

Extension of townships, new construction on hills, creating accessibility to remote areas and 'modernisation' leading to change in life style are some noticeable threats throughout the Maharashtra. For example, private hill cities such as Aamby Valley, Lavasa, hill city *etc*. has caused damage to the natural habitat. Likewise, in Pashan lake near Pune, *Eulophia pratensis* (an endemic to Peninsular India) which was found abundant could not be located even after repeated search during this study period. This is due to expansive development of housing construction as a result of extension of Pune city. With increasing population, encroachment on forest land is a common practice. This has resulted in massive degradation of forest and illegal exploitation of resources. For example, the Yawal Wildlife Sanctuary in Jalgoan, situated on the western part of the Satpura Mountain and bordering Madhya Pradesh is under heavy pressure from encroachers of Madhya Pradesh. This sanctuary is also important because it is a part of "Satpura Tiger landscape".

The decline in number of orchid species is reported from Panchgani, Kas Plateau, and Khandala. Kas plateau, known as the valley of flowers, is facing surge in tourists. Excited visitors pluck the orchids for their homes, leaving little chance for these rare orchids to survive. The fragrant *Pecteilis gigantea* popularly known as the queen of Khandala was found very commonly fifty years ago and sold in the Khandala hill station's markets. This led to a fall in the species and now it is confined to a few spots only. Likewise, *Habenaria suaveolens* Dalzell (popular synonym is *Habenaria panchganiensis*) known as Panchgani orchid, was once abundant in Panchgani plateau has now become a rare sight due to the tourism activities such as horse rides, camel rides that almost converted the flora rich plateau to a barren land. *Eulophia graminea* Lindl. is a rare orchid in Maharashtra, which is so far reported from Sangli and Osmanabad districts. Bachulkar and Yadav (1993) had reported this orchid from sugarcane fields near Islampur (Sangli district), where they had seen only two individuals. Conversion of land for agricultural purpose especially for cultivation of cash crops also causes depletion of orchid population as in the case mentioned above. In Konkan region of Maharashtra, many of the good forests patches have been cleared for cash crops such as Areca nut, Cashew nut, and mango orchard. The plateaus of Konkan are experiencing heavy pressures and disturbances due to their rapid conversion for settlements, paddy fields, orchards, quarries, grazing lands, windmill farms and industrialisation.

Mining is a rapidly growing threat to the orchid diversity across Maharashtra. Many areas of Northern

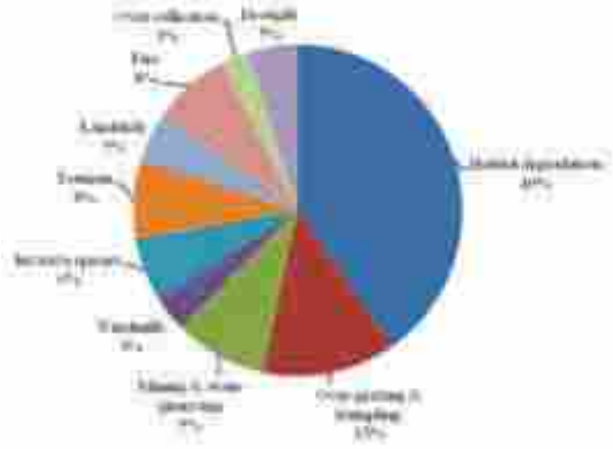

Fig. 7. Graphical representation of various threats levels.

Western Ghats of Maharashtra are heavily affected by Bauxite mining. Most of the mines are situated in the high altitude plateaus and dense evergreen forest areas above 800-1000 m.s.l. and consequentlt, the important habitat of orchids such as dense evergreen forests has been highly affected. Udgeri, Girgaon, Ringewadi, Dhangarwadi, and Manoli have their bauxite mines in the upper catchment in Warna river basin in Kolhapur district. These mines are very close to two protected areas like, Chandoli National Park and Radhanagari Wildlife Sanctuary. Such mining activities are proving to be detrimental to the last remaining wildlife habitats.

A large number of wind power projects have been commissioned on forest land in Maharashtra. Some of the key sites with optimal wind velocities are the plateaus on the Western Ghats. The rocky plateaus on the Western Ghats are terrestrial habitat islands facing extreme micro-environmental condition. These plateaus and hill sides are cut to make roads to transport heavy equipment for installing the windmills. This leads to erosion and landslides. Roads that are cut through forests and hills to enable movement of heavy-duty trailers lead to linear fragmentation of habitats. The ecological sensitive zones, plateaus and forest areas that support variety of terrestrial and epiphytic orchids in Bhimashankar Wildlife Sanctuary and Koyna Wildlife sanctuary in the northern part of the Western Ghats are facing habitat destruction due to roads, blasting and erosion as well as landslides after the monsoon with the rubble ending up in rivers and farmland below. The Western Ghats Ecology Expert Panel (WGEEP) says that according to forest department estimates, about 28,000 trees have been cut for the project (Bhushan *et al.,* 2013).

In Maharashtra, one can easily notice the local medicinal plant vendors in and around hill stations as well as near temples in the hilly regions. A variety of bulbous and tuberous plants collected from wild are sold in the name of its medicinal uses. For example, tubers of *Eulophia spectabilis* (Dennst.) Suresh, *Geodorum densiflorum* (Lam.) Schltr., *Malaxis versicolor* (Lindl.) Abeyw. are sold in the vicinity of temple in Bhimashankar WLS. *Eulophia spectabilis* Lindl. is a terrestrial orchid which is being extracted from wild leading to drastic depletion of wild populations. It is commonly known as *Amarkanda* and is widely used to cure various health problems and ailments. The corm of the plant is used in the preparation of 'salep', which is taken as an aphrodisiac (Jalal *et al.*, 2014). Since, the corm is collected by the local people, it has direct impact on the depletion of its population in wild.

Nearly 40 % of natural forest vegetation in Western Ghats has disappeared in the past 8-10 decades (Menon and Bawa, 1997). Spread of certain alien invasive weeds such as *Chromolaena odorata* (L.) R.M.King & H.Rob., *Mikania cordata* (Burm.f.) B.L.Rob., *Lantana camara* L. and *Parthenium hysterophorus* L. has led to encroachment of the habitat of ground orchids. As a consequence, it was observed that the population of orchids in many localities is on the decline. In many locations in Maharashtra, orchids are also facing threats due to landslides and floods in the rainy season.

Conservation Measures

The threat status of IUCN Red List provides an assessment of the extinction risk under current circumstances and it is not necessarily sufficient to determine priorities for conservation action. There are numerous other factors concerning conservation action such as costs, logistics, chances of success and other biological characteristics (Mace and Lande, 1991). However, assessment of taxa using Red List Criteria represents a critical first step in setting priorities for conservation action. In Maharashtra, areas such as Kaas plateau, Koyna Wildlife Sanctuary, Chandoli National Park and Radhanagari Wildlife Sanctuary have been included in the UNESCO list of natural world heritage sites which will help in conserving the natural habitats. But there is no such area which has been exclusively identified for orchid conservation. The following measures are suggested for long term orchid conservation in Maharashtra:

- 38 species, which are assessed as threatened in Maharashtra, need immediate action for conservation.
- Three protected areas (Koyna WLS, Chandoli NP and Radhanagari WLS) are recommended for the *in situ* conservation. Training on orchid identification and population monitoring should be provided to the staff of these PAs.
- Eco-Sensitive Zones (ESZ) *i.e.,* Mahabaleshwar-Panchgani and Matheran should be preferably looked upon as orchid conservation sites.
- Orchid rich localities outside the PAs *i.e.,* Amboli and Lonavala-Amby valley should be developed as orchid conservation areas (OCAs).
- Forest department, non-governmental organizations (NGOs), volunteers and local stake holders must undergo at least basic training in orchid identification and conservation.

- For *ex situ* conservation, there is a need to establish an orchid conservatory which can be used for training, rescue and vegetative propagations.

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DIVERSITY, DISTRIBUTION AND CONSERVATION OF ORCHIDS IN NARGU WILDLIFE SANCTUARY, NORTHWEST HIMALAYA

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Abstract

Like other parts of the Indian Himalayan Region, Himachal Pradesh also supports unique orchid flora. In the present investigation, extensive field surveys were conducted to study the orchid diversity of Nargu Wildlife Sanctuary during 2010-2015. During exploration of the floristic diversity, total 15 species of orchids representing 12 genera were recorded between 970-4052 m, amsl. These were analyzed for nativity and endemism. Eleven species were natives and four non-natives; 2 species were near endemic and one species (*Habenaria edgeworthii*) was endemic to Indian Himalayan Region. Among genera, *Habenaria*, *Herminium,* and *Malaxis* (2 species each) were dominant. Different plant parts *i.e.*, leaves (6 species), aerial parts and tubers (4 species each), bulbs (2 species), roots and rhizomes (1 species each) were used by the inhabitants for various therapeutic uses. Three species namely *Dactylorhiza hatagirea*, *Herminium monorchis* and *Malaxis muscifera* have been identified as Critically Endangered, 04 species (*Habenaria edgeworthii*, *Malaxis acuminata*, *Nervilia plicata*, and *Neottia listeroides*) as Endangered, 03 species (*Habenaria pectinata*, *Herminium lanceum* and *Spiranthes sinensis*) as vulnerable and 05 species (*Calanthe tricarinata*, *Epipactis helleborine*, *Goodyera fusca*, *Satyrium nepalense* and *Vanda cristata*) as near threatened. Habitat degradation, over exploitation, and complex nutrient requirement are causing rapid decrease in the population of these species in the area. Therefore, study on habitat ecology of these species requires priority attention. In addition, educational and awareness programmes on status and conservation and involvement of inhabitants in conservation would help in their conservation and management.

Introduction

THE INDIAN Himalayan Region (IHR) supports about 8,000 flowering plants and family Orchidaceae is one of the species rich families of angiosperms (Samant, 2002; Singh and Hajra, 1996). Orchids are worldwide famous for their charming and long lasting flowers and the family Orchidaceae comprises 22,500 species and 779 genera, second largest to Asteraceae; they form a diverse group of plants and represent a peak in the evolution of monocots. In India, 9% of flora (1300 species and 140 genera) is composed of orchids and is present predominantly in temperate Himalaya (Yonzone *et al.*, 2010). These are terrestrial, epiphytic and saprophytic in nature and are cultivated for beautiful flowers and widely known for their economic importance but less for their medicinal value. The diversity of orchids decreases from North East to North West Himalaya (Chowdhery and Wadhwa, 1984; Deva and Naithani, 1986; Marpa and Samant, 2012; Samant, 2002, 2009; Samant *et al.*, 1995). The north Indian hill state, Himachal Pradesh, is also very well known for its typical topography, large altitudinal range, and diverse habitats, and is representative of natural, unique and socio-economically important biodiversity. It supports 32 Wildlife Sanctuaries; 02 National Parks and 01 Biosphere Reserve. Most of the protected areas are unexplored and under explored especially for orchid diversity. On the other hand, medicinal properties and traditional uses of orchids are very poorly studied in this state till now. Further, scanty populations of these plants due to their complex nutrition requirement and anthropogenic activities make them highly vulnerable.

In general, in Himachal Pradesh, a very few studies have been carried out on Orchids (Arora, 1986; Chowdhery and Agrawala, 2013; Deva and Naithani, 1986; Duthie, 1906; Marpa and Samant, 2012; Samant, 2009; Samant *et al.*, 1995; Verma *et al.* 2013; Vij *et al.*, 1983, 2013). In general, mention of orchids has been also made in the floristic studies by many workers (Chowdhery and Wadhwa, 1984; Collett, 1902; Dhaliwal and Sharma, 1999; Kaur and Sharma, 2004; Lal *et al.*, 2004; Rana *et al.*, 2008; Singh and Rawat, 2000; Singh and Sharma, 2006; Sharma, 2008, 2013; Singh, 2007; Thakur, 2012), but a very few studies are available for the protected areas of the state. Therefore, the present attempt has been made to: i) assess orchid diversity of Nargu Wildlife Sanctuary and gather information on indigenous medicinal uses; ii) analyze orchid species for nativity, endemism, and threat categories; and iii) suggest strategy and management plan for the conservation of orchid diversity.

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Materials and Methods

Study Area

The Nargu Wildlife Sanctuary (NWLS) (31˚46' to 32˚05' N Latitudes and 76˚50' to 77˚04' E Longitudes) is located in the Mandi district of Himachal Pradesh (Fig 1). This Sanctuary was notified in 1972. It covers an area of over 278 km^2 with an altitudinal range, 970-4052 m amsl; the temperature ranges between -10°C to 35°C and mean annual rainfall is 1400 mm. It

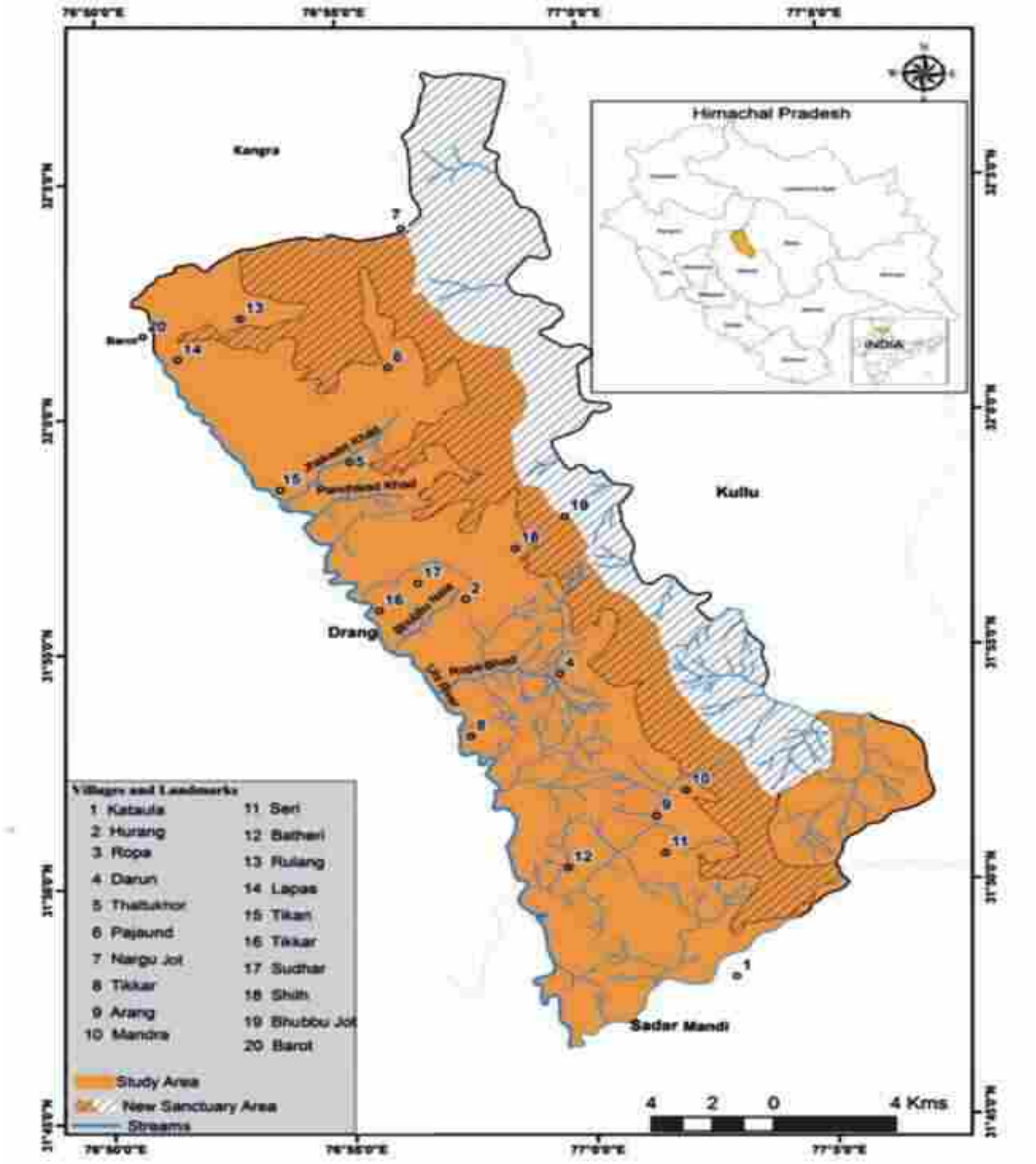

Fig. 1. Map of the study area showing Nargu Wildlife Sanctuary

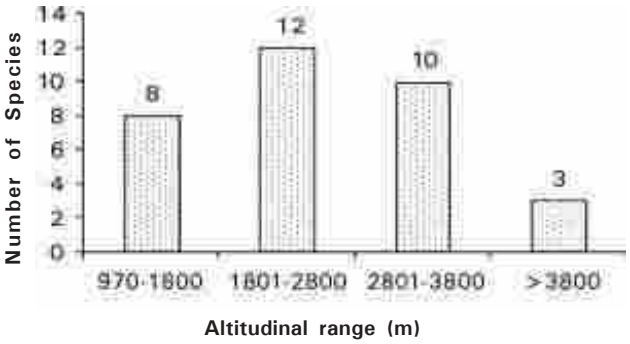

Fig. 2. Altitudinal distribution of the orchids in Nargu Wildlife Sanctuary

represents sub-tropical, temperate, sub-alpine and alpine vegetation. The Sanctuary is rich in biodiversity including a large number of mammals and birds. The NWLS has a huge permanent settlement. The inhabitants are dependent on the Sanctuary for their sustenance. The Sanctuary area is now been rationalized as per notification (No. FFE-B-F (6)-16/ 1999-Nargu; Dated November 29, 2013) of Government of Himachal Pradesh, Department of Forests, but the present study has been conducted in the old area of the Sanctuary.

Surveys, *Sampling*, *Identification and Data Analysis*

The extensive and intensive field surveys were conducted to study the orchid diversity of the NWLS during 2010-2015. The rapid sampling of the species was done and the samples of each species were collected for proper identification. For each species, information on habit, habitat, altitudinal range, population size, indigenous uses, *etc*. was collected. The species were identified with the help of flora and literature (Deva and Nathani, 1986; Dhaliwal and Sharma, 1999; Duthie, 1906; Pangtey *et al*., 1991; Samant, 1993; Singh and Rawat, 2000). Species were analyzed for nativity, endemism and threat categories. Nativity of the species was identified following Anonymous (1883-1970), Samant (2002), and Samant *et al*. (1998). Endemism of the species was identified based on their distributional range and following Dhar and Samant (1993) and Samant *et al*. (1998). Species confined to the IHR were considered as endemic, and those with a distribution extending to neighboring countries (Himalayan region of Afghanistan, Pakistan, Tibet, Nepal, Bhutan and adjacent states of the IHR) were considered as near endemic. For assessing the threat categories of the orchid species, habitat preference, population size, distribution range and use values were collectively used following Rana and Samant (2010). Information on the indigenous uses of the species is based on the available literature and interviews with the inhabitants of Sanctuary.

Results

Diversity and Distribution of Orchids

In total, 15 species of the orchids representing 12 genera were recorded between 970-4052 m, amsl. These orchid species were found in diverse habitats *viz*., shady moist forests, alpine meadows, moist rocks, boulders, *etc*. Of these, 8 species of orchids were recorded from the sub-tropical zone (970-1800 m), 12 species in the temperate zone (1801-2800 m), 10 species in the sub-alpine zone (2801–3800 m),

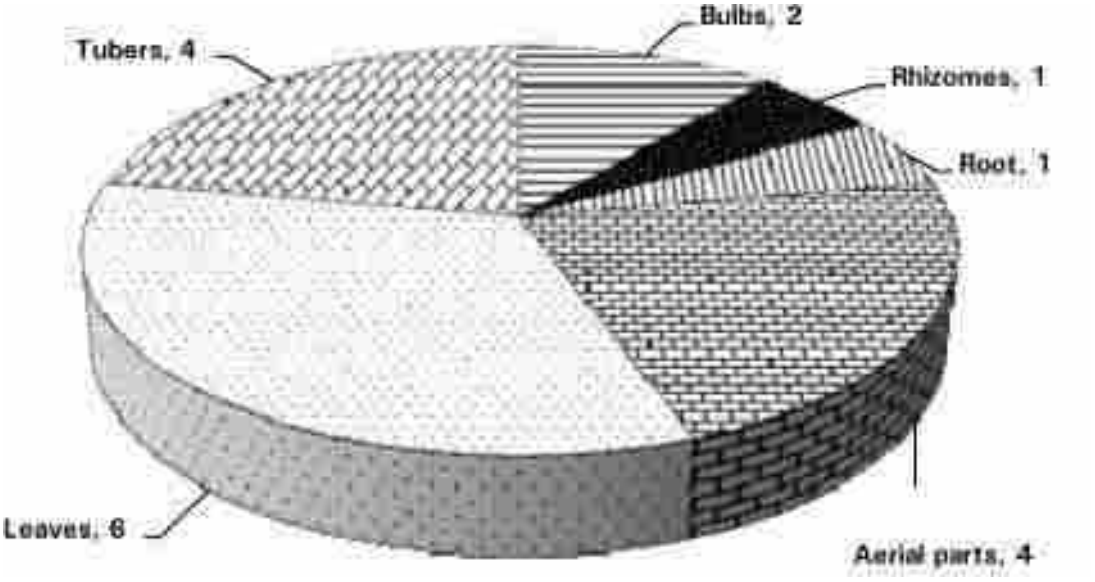

Fig. 3. Parts of medicinally important orchids used in Nargu Wildlife Sanctuary

Table 1. Diversity, distribution, indigenous uses, and conservation of orchids in Nargu Wildlife Sanctuary.

Taxa	Habitats	Altitudinal range (m)	Nativity	Status	Part/s used	Indigenous uses
Calanthe tricarinata Lindl.	SM, DR	2000-3300	Reg Himal	NT	Leaf, Bulb	Used to cure sores and eczema, and as aphrodisiac
Dactylorhiza hatagirea D. Don	SM, MAS	2800-3870	Reg Himal Europ Afr Bor Or	CR	Tuber	Used as antibiotic, blood purifier, tonic, and expectorant and for curing wound, bone fracture, cough, cold, cuts, sexual disability, rheumatism
Epipactis helleborine (L.) Crantz	SM	2500-3650	Reg Himal	NT	Leaf, Rhizome	Used as aphrodisiac and used to cure fever, blood purification
Goodyera fusca Hook.f.	DAS, BO	3000-3900	Reg Himal	NT	Aerial Part	-
*Habenaria edgeworthii*** Hook.f. ex Collett	SM	1500-3000	Reg Himal	EN	Tuber	Used as blood purifier and rejuvenator
H. pectinata D.Don*	SM	1400-3500	Reg Himal	VU	Leaf, Roots	Used for curing joint pains
Herminium lanceum (Thunb. ex Sw.) Vuijk	SM, MAS	1200-3000	Reg Himal	VU	Aerial Part	Used for curing urinary problems
H. monorchis (L.) R.Br.	SM, RI, MAS	2000-4000	Europ As Bor	CR	Aerial Part	Used as tonic
Malaxis acuminata D. Don	SM	1600-2500	Reg Himal	EN	Stem/Leaf	Used as blood purifier, aphrodisiac, spermopiotic and for curing burning sensation, arthritis
M. muscifera (Lindl.) Kze.	SM, MAS	1800-3200	Europ	CR	Bulb	Used as aphrodisiac, styptic, and febrifuge; and for curing dysentery, tonic, burns, debility, sterility
Neottia listeroides Lindl.	SM	1800-3600	Reg Himal	EN	Aerial Part	-
Nervilia plicata L.	RI	1050-1200	Reg Himal	EN	Stem/Leaf	Used as antidiabetic
Satyrium nepalense D.Don*	SM, MAS	1500-3200	Ind Or	NT	Tuber	Used as energizing tonic, aphrodisiac and for curing dysentery, malaria
Spiranthes sinensis (Pers.) Ames.	SM, DE	1100-2800	China As Temp	VU	Tuber	Used for curing tuberculosis, haemoptysis, debility, snake bite, sore throat, cough, leucorrhea, diabetes
Vanda cristata Lindl.	SM, E	1300-2100	Reg Himal As Trop	NT	Leaf	Used as tonic and expectorant

Abbreviations used: CR, Critically Endangered; E, Endangered; VU, Vulnerable; NT, Near Threatened; Afr, Africa; As, Asia; Bor, Boreal; Europ, Europe; Himal, Himalaya; Ind, India; Or, Oriental; Reg, Region; Temp, Temperate; Trop, Tropical *, Near endemic; **, Endemic; Bo, Bouldery; DAS, Dry alpine slope; DE, Degraded; DR, Dry forest; MAS, Moist alpine slope; RI, Riverine; Sh, Shrubberies and SM, Shady moist forest

3 species in the alpine zone (>3800 m) (Fig. 2). Eleven species (*Calanthe tricarinata*, *Dactylorhiza hatagirea*, *Epipactis helleborine*, *Goodyera fusca*, *Habenaria edgeworthii*, *Habenaria pectinata*, *Herminium lanceum*, *Malaxis acuminata*, *Neottia listeriodes*, *Nervillia plicata* and *Vanda cristata*) were natives and 4 species non-natives; 2 species (*Habenaria pectinata* and *Satyrium nepalense*) were near endemic and one species *i.e.*, *Habenaria edgeworthii* was endemic to the Indian Himalaya (Table1). Among genera, *Habenaria*, *Malaxis* and *Herminium* (2 species each) were dominant.

Indigenous Uses

Different plant parts *i.e.*, leaves (6 species), aerial parts and tubers (4 species each), bulbs (2 species), and rhizome and root (1 species each) were used by the inhabitants for various therapeutic uses (Fig. 3). For instance, tubers of *Habenaria edgeworthii* (known as *Riddhi* in Ayurveda) are considered to be blood purifier and energy booster; *Habenaria pectinata* were used for joint pains by the local folks. Aerial parts of *Goodyera fusca* were considered as very good appetizers. *Malaxis acuminata* (known as *Jeevak* in Ayurveda) was a key *Ashtavarga* plant and used for curing arthritis, blood purification and as aphrodisiac. *Malaxis muscifera* was considered to be a very good health tonic and a potential aphrodisiac. Likewise, other species were used for curing various ailments such as sores, eczema, paralysis, wounds, bone fracture, cough, cold, cuts, sexual disability, rheumatism, fever, blood purification, cold, dysentery, sterility, leucorrhoea, diabetes and malaria, *etc*. and also used as aphrodisiac, antispasmodic, sedative, febrifuge, appetizer and tonic (Table1 and Fig. 4). Due to extensive over use and unscientific extraction, the density of these species is decreasing at an alarming rate. The populations of *Dactylorhiza hatagirea*, *Malaxis acuminata* and *Malaxis muscifera* are decreasing very fast due to habitat degradation and their commercial exploitation.

Threat Categorization

The analysis for threat categories revealed that 03 species namely *Dactylorhiza hatagirea*, *Herminium monorchis* and *Malaxis muscifera* were Critically Endangered; 04 species namely *Habenaria edgeworthii*, *Malaxis acuminata*, *Nervilia plicata*, and *Neottia listeroides* were Endangered and 03 species namely *Habenaria pectinata*, *Herminium lanceum* and *Spiranthes sinensis* were Vulnerable; 05 species namely *Calanthe tricarinata*, *Epipactis helleborine*, *Goodyera fusca*, *Satyrium nepalense* and *Vanda cristata* were Near Threatened.

Discussion

The state of Himachal Pradesh supports relatively very less number of orchids as compared to West, Central and Eastern Himalaya (Deva and Naithani, 1986; Samant, 2002, 2009). Of the 15 species presently recorded, 11 species were native to the Himalaya and remaining 4 were non-native. A total of 2 species were observed as near-endemic for the IHR. Except *Vanda cristata*, all the other species were terrestrial and mostly preferred shady moist habitats, clearly indicating thereby that the environmental conditions are not suitable for the epiphytic orchids, in the region. As the sub-tropical and temperate regions represent the best habitats for the growth and development of the orchids, critical investigation of the habitats is essentially required, besides regular monitoring of these habitats so as to understand the dynamics of these species. The orchids are inherently slow growers and due to their complex nutritional requirements, they germinate poorly in nature which further adds to their poor populations and making them more vulnerable.

In general, IUCN Red Lists and Red Data Books, and CAMP (Conservation Assessment and Management Plan) workshops have helped in the prioritization of the species and have been playing crucial role in guiding the conservation priorities since long (Goraya *et al.*,

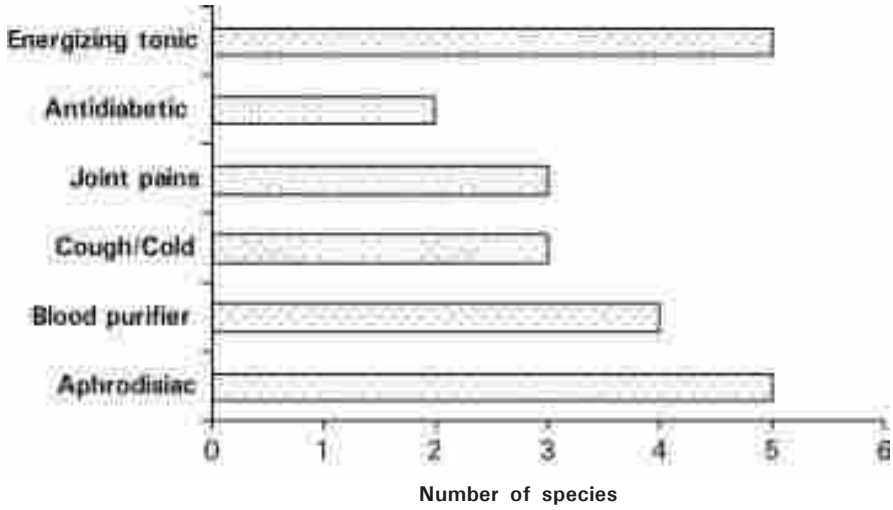

Fig. 4. Number of medicinal uses of orchids in Nargu Wildlife Sanctuary.

2013; Nayar and Sastry, 1987, 1988, 1990; Ved *et al*., 2003). However, at local level very few studies have been carried out following the IUCN criteria. The local level threat categorization of the species has been considered as the best approach for developing appropriate strategy and management plan (Rana and Samant, 2010). Following the similar approach in the present study, 03 species were identified as Criticaly Endangered, 04 species as Endangered and 03 species as Vulnerable. The major causes for this were the over exploitation and habitat degradation. Therefore, habitat monitoring, development of conventional and *in vitro* propagation protocols, mass multiplication of the species, establishment and maintenance in the *in situ* and *ex situ* conditions, educational and awareness for the inhabitants on conservation; and their participation for conservation management are suggested.

Acknowledgement

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WHAT DRIVES ORCHIDS TOWARD MYCO-HETEROTROPHY?

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Abstract

Myco-heterotrophy describes the capability of orchids to obtain carbon from fungi. Depending upon varied durations of association with fungi, orchids have been categorized as those that require fungi during germination only as compared to those that are associated with fungi throughout the life span. It has always been the subject of interest to all the scientists to evaluate the mode of nutrition between this mutualistic association for which various modern approaches have been used. There are factors which led to evolution of autotrophy to initial or partial mycoheterotrophy and subsequently, to fully mycoheterotrophy. The present paper presents the overview of mycoheterotrophy, habitat, morphology, reproduction strategies, techniques, threats and conservation of mycoheterotrophy, and major opportunities for the future research.

Introduction

MYCO-HETEROTROPHY IS a potential of a plant to procure carbon from fungi. Leake (1994) coined the term myco-heterotrophy; to understand this potential, a basic understanding about the mycorrhizal symbiosis is a pre-requisite. The term mycorrhiza is derived from the Greek words, *mykos* = *'fungus'* and *rhiza* = 'root', indicating the mutualistic association of soil fungi with plant roots that benefits each other, mycorrhizal fungi improve the nutrient uptake of the host plants and in return they receive photosynthetically fixed carbon that is essential for growth and reproduction of the fungi. Arbuscular mycorrhizae (AM) involve almost all the members of Glomeromycota and Ectomycorrhizae involve some members of Ascomycota and Basidiomycota. In some cases, saprophytic fungi (SAP) are considered to be the third source of myco-heterotrophy in the plants (Smith and Read, 2008). The mycorrhizae involved in the myco-heterotrophic interactions with Orchidaceae have been termed as orchid mycorrhiza. Two discrete types of orchid mycorrhiza are recognized as tolypophagous and ptyophagous type. The former type is most common, in which hyphae infect the rhizome or root, form pelotons (coil) in cortical cells and are digested. However, the latter one is infrequent in which hyphae that have entered a root, experience lysis at the tips and cell contents are released (Burgeff, 1932).

Orchidaceae is one of the largest families of the flowering plants, with 736 genera distributed throughout the world except polar areas and deserts. Morphologically and functionally, it is considered to be the one of the highly specialized families amongst the monocotyledons (Hajra and De, 2011). The family is divided into five sub-families: Apostasioideae, Vanilloideae, Cypripedioideae, Orchidoideae and Epidendroideae, of which Epidendroideae is the largest subfamily comprising of *ca.* 18,000 spp. and 650 genera (Chase *et al.*, 2015). As far as is known, all orchids depend on a myco-heterotrophic interaction with a symbiotic fungus for a part of their life cycle, especially for germination (Leake, 1994).

There has been a conjecture that orchids rely on symbiotic association with mycorrhiza to obtain complex organic compounds for a part of their life *i.e.*, at the time of germination only, throughout the life cycle due to unavailability of the light and scarcity of nutrients and some plant species show plasticity in trophic strategy in relation to environmental conditions. It has been speculated that the family Orchidaceae includes shifts from autotrophy to initial mycoheterotrophy to partial myco-heterotrophy and from partial mycoheterotrophy to full mycoheterotrophy. Present paper deals with the detailed description about, the strategies adopted by orchids to avail nutrients, habitats and morphology of mycoheterotrophy, tools that will help to determine the nutrient fluxes between fungi and mycoheterotrophic plants, and threats and conservation strategies.

Overview of Myco-heterotrophy

Orchids have evolved in different ways to obtain organic compounds through autotrophy, mycoheterotrophy, and partial mycoheterotrophy. Almost all the plants are autotrophs, convert carbon dioxide into the carbohydrates in the presence of sunlight. Achlorophyllous plants have lost the ability to photosynthesize and rely on symbiotic association with fungi to obtain carbon. To understand

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mycoheterotrophy, the designation of plant species according to their trophic capabilities is needed.

A fully myco-heterotrophic plant exclusively depends on fungal carbon during its life cycle. Thus, orchids that lack visible traces of chlorophyll and do not possess direct relation with autotrophic plants are considered fully mycoheterotrophic plants (Merckx, 2013).

A partial myco-heterotrophic plant combines autotrophy and mycoheterotrophy to procure carbon during at least one stage of its life cycle suggesting thereby that partial MH retain the functional photosynthetic apparatus. Orchids that are green and can survive in the extreme low light conditions are the prime candidates for partial mycoheterotrophy. Gebauer and Meyer (2003) and Preiss *et al*. (2010) reported that the dependency on fungal carbon can greatly differ between partial mycoheterotrophic species and between populations of the same species growing in different illumination conditions (Merckx, 2013; Rasmussen, 1995).

An initially myco-heterotrophic plant is completely dependent on associated fungi for its carbon supply during the early stages of development. Comprehensively, all full myco-heterotrophs are initial myco-heterophs as well, but the term is used for the species that depend on autotrophy or partial myco-heterotrophy, at maturity. Thus, all orchids except fully myco-heterotrophs are initial myco-heterotrophs (Merckx, 2013).

Habitats and Morphology of Myco-heterotrophic Orchids

Habitats

Fully myco-heterotrophic species grow in shaded habitats in closed canopy forests. These habitats are generally characterized by a lack of understory plants, where sufficient sunlight fails to reach till ground level to aid plants to perform photosynthesis. Partial myco-heterotrophs often occur in forest habitats where there is no sufficient sunlight but can also be found in open vegetations, such as bogs and meadows (Girlanda *et al.*, 2011; Matthews *et al.*, 2009). Preiss *et al.,* (2010) demonstrated that light availability is one of the major determinants of the degree of myco-heterotrophy in two partially myco-heterotrophic species of *Cephalanthera*. These observations support a strong correlation between irradiance level and dependency on fungal carbon. Hence, an evolutionary shift from autotrophy to full myco-heterotrophy seems to be accompanied by a switch towards more shaded sites.

Subterranean Morphology

Leake, (1994) observed that in fully myco-heterotrophic plants, root hair are mostly absent, roots are stout and mostly clumped, rhizomes are with a specialized fungal colonization pattern and increase in the width of the root cortex to accommodate mycorrhizal infection and to store carbohydrates and other nutrients, obtained from fungal association.

Shoots

Many myco-heterotrophic orchids have slender and thread like stems, resulting in a hyaline appearance. Vascular tissues are often reduced to a single narrow cylinder of bicollateral bundles or to four or six narrow bundles in the cortex. Most of the species lack secondary thickening and their stems are succulent and brittle (*e.g.*, *Rhizanthella*). Lignification is generally confined to a narrow ring of xylem vessels; phloem is present in very small amounts mainly as parenchyma (Leake, 1994).

Leaves

In fully myco-heterotrophic orchids, the nutrients are obtained solely from fungi, so leaves no longer serve a useful function. Thus, leaves are reduced to widely spaced achlorophyllous scales on the inflorescence axis. Occasionally, leaves are present only on underground rhizomes or tubers or even totally absent. The vascular supply to the leaf scales is mostly reduced to a single trace or may be absent. Stomata are generally absent, but some species retain rudimentary stomata on their leaves and shoots (Leake, 1994).

Seeds

Most species of myco-heterotrophic orchids have extremely small seeds, termed as dust seeds (Arditti and Ghani, 2000). A reduction in seed size and complexity is one of the most significant modifications in mycoheterotrophic orchids. The reduction in seed size is coupled with a reduction of endosperm and a lack of differentiation of the embryo at the maturity, *e.g.*, a single capsule of the mycoheterotrophic orchid *Galeola altissima* contains about 18,000 seeds (Arditti and Ghani, 2000). However, not all orchids produce large number of seeds per capsule, *e.g.*, *Rhizanthella gardneri* produces 20-25 seeds only (George and Cooke, 1981). As seeds are small and reserve less, germination depends on colonization by a mycorrhizal fungus (Eriksson and Kainulainen, 2011).

Reproductive Strategies of Myco-heterotrophs

A very little is known about the reproductive strategies adopted by mycoheterotrophic orchids. Bidartondo (2005) in his review on mycoheterotroph biology hypothesized that mycoheterotrophic plants specialized on fungi, will infrequently be specialized towards pollinators due to the evolutionary instability for specializing on two interactions. Since mycoheterotrophic plants already engage in exclusive specialized symbiotic association with fungi in one aspect of their life history, and it would be an evolutionarily unstable strategy to engage in additional compulsory associations. Hence, mycoheterotrophs are likely to evolve reproductive traits free from highly specialized symbiotic associations, with characteristics including a generalist pollination syndrome, high occurrence of autogamous self- pollination and resource allocation away from metabolically exorbitant reproductive structures, such as large attractive flowers, instead dedicating resources to production of a vast number of seeds. Production of a large number of tiny seeds could be a reproductive strategy aimed at increasing the likelihood of at least a few offsprings locating a suitable host.

Since myco-heterotrophs are found growing under shady forests, it has also been hypothesized that myco-heterotrophs will converge on floral characters to attract pollinators more common in understory forests. This could potentially lead to a myco-heterotroph floral syndrome, consisting of small white flowers, with a scent attractive to fungus gnats, as seen in *Neottia cordata*, and tight synchrony of blooming period among co-occurring population members (Ackerman and Mesler, 1979).

According to Dressler (1981) and Leake (1994), limited carbon supply, patchy distributions, and a restriction to habitats with few pollinators, may lead to a reliance on autogamy among mycoheterotrophs. It has also been shown that variations in abiotic factors may have severe impact upon myco- heterotroph reproduction. Removal of the forest canopy and change of litter com-position can severely affect mycoheterotroph reproductive effort, with observed declines in the reproductive output of mycoheterotrophs possibly corresponding to unfavorable shifts in abiotic factors affecting the composition of mycorrhizal communities and/or the vitality of obligate fungal associates (Luoma, 1987; Moola and Vasseur, 2004).

Techniques to Study Orchid Mycorrhiza

Molecular Approach

Earlier, knowledge about orchid mycorrhiza has been procured from *in vitro* isolation of fungi. This has allowed the identification of basic fungi and conducting *in vitro* seed germination experiments with some root isolated fungi as both can be cultured axenically, at least in the early stages of fully autotrophic orchids (Clements, 1988; Warcup, 1971). However, there has been difficulties in accurately identifying the isolated fungal partner. However, isolation often provides mostly contaminants or endophytes (*i.e*. fungi that for all or part of their life cycle inhabit living plant tissues but do not form pelotons nor cause any obvious disease symptoms; Wilson, 1995).

In the recent years, fungal taxonomy is studied, especially by isolating fungal DNA from host roots and sequencing the nuclear ribosomal DNA (Seiffert, 2009). The fungal partners of orchid mycorrhiza can be more accurately identified directly from orchid protocorms, roots, tubers and rhizomes (Bougoure *et al.,* 2005; Martos *et al.,* 2009; Swarts *et al.,* 2010). PCR amplification of colonized orchid tissues using fungus-specific primers is commonly used (Dearnaley and Bougoure, 2010; Dearnaley and Le Brocque, 2006). Sequencing of the internal transcribed spacer (ITS) of the nuclear ribosomal DNA after PCR amplification using a variety of primer combinations (Gardes and Bruns, 1993; White *et al.,* 1990) is now a popular and reliable method for identifying orchid mycobionts. Jacquemyn *et al.* (2010) and Lievens *et al.* (2010) introduced DNA array technologies for the identification of orchid fungal partners in which oligonucleotides were prepared from a preliminary exploration of fungal diversity in a limited number of individuals (Lievens *et al.,* 2010), and the array was successfully used to investigate the fungal partners of three closely related *Orchis* species and their hybrids (Jacquemyn *et al.,* 2011). This method allows swift and efficient handling of numerous samples, especially as compared to the cloning of PCR products.

Stable and Radioactive Isotopes

There is an indirect approach to evaluate the mode of nutrition of an individual orchid is mass spectrometric analysis of natural C and N isotope abundances (Abadie *et al.,* 2006; Bidartondo *et al.,* 2004; Julou *et al.,* 2005; Martos *et al.,* 2009; Ogura-Tsujita *et al.,* 2009; Zimmer *et al.,* 2007). Stable isotopes also provide access to trace elements for which no natural

radioisotopes exist (*e.g*., nitrogen and oxygen) (Schimel, 1993). Fully mycoheterotrophic orchids have been identified to have ^{13}C signatures similar to those of their mycorrhizal partners (Gebauer and Meyer, 2003; Trudell *et al.,* 2003) whereas similar/higher ^{15}N abundance than their mycorrhizal fungi, suggesting thereby a limited trend to ^{15}N accumulation along the food chain (Trudell *et al.,* 2003). Partial mycoheterotrophs showed stable isotope signatures intermediate between fully mycoheterotroph and autotrophic species (Abadie *et al.,* 2006; Julou *et al.,* 2005). As expected, some fully autotrophic orchids such as *Goodyera* species have even lower amounts of these natural isotopes as autotrophic orchids are least dependent on nutrient acquisition from fungi (Bidartondo *et al.,* 2004; Gebauer and Meyer, 2003).

The movements of isotopically labelled compounds can be traced from fungal partner to orchid mycorrhizas. Although they only provide snapshot views of the metabolism at the time of pulse, they also allow to track the exchanges between symbionts. The first demonstration of movement of ^{14}C labelled photosynthates from tree species to the fully MH orchid *Corallorhiza trifida* via ECM fungi was demonstrated by McKendrick *et al.* (2000). Bougoure *et al.* (2010) provided the flow of ^{13}C-labelled carbon from *Melaleuca scalena* to the fully mycoheterotrophic orchid *Rhizanthella gardneri* via an ECM fungal conduit. *R. gardneri* also obtained nitrogen from its fungal partner, indicated by adding $^{13}C + ^{15}N$-labelled glycine to hyphae and surrounding soil. Labelling experiments by Cameron *et al.* (2006, 2007, 2008) demonstrated that the fully autotrophic orchid *Goodyera repens* obtains carbon, nitrogen, and phosphorous from its fungal partner. Notably, *G. repens* also transfers significant amounts of photosynthates (likely greater than 3 % of its photosynthetic carbon) back to its *Ceratobasidium* mycobiont, which is the first direct demonstration of a net carbon flow from orchid to fungi (Cameron *et al.,* 2006, 2008). Recently, Ercole *et al.* (2015) investigated temporal variations in the mycorrhizal fungi and nitrogen stable isotope natural abundance in adult plants of *Anacamptis morio* (a wintergreen meadow). They observed that irrespective of differences in the seasonal environmental conditions, plant phenological stages, and the associated fungi, the isotopic content in mycorrhizal *A. morio* remains quite constant over time.

Other Approaches

The gene expression studies of ECM and AM associations with mycoheterotrophs have largely been neglected. Watkinson and Welbaum (2003) studied gene expression in the mycorrhizal association of *Cypripedium parviflorum* var. *pubescens* via differential mRNA display. A trehalose phosphate phosphatase was down-regulated in the association, showing changes to orchid carbohydrate transport. Dearnaley (2007) speculated that modern gene expression techniques such as *in situ* hybridization, microarrays and RT-PCR may provide additional understanding of the molecular functioning of orchid mycorrhiza. Specifically, whole genome sequencing and transcript profiling of orchid mycorrhizal fungi, both free-living and in plants, may disclose the fungal genes that are up-regulated in the mutualistic association (Martin *et al.,* 2008).

Study of fungal symbionts in mycorrhiza with the help of electron microscopy provides a fair idea of ultrastructural details (Kottke *et al.,* 2010; Martos *et al.,* 2009, 2012; Pereira *et al.,* 2003; Schatz *et al.,* 2010; Selosse *et al.,* 2004; Suarez *et al.,* 2008). First, characters of the fungal cell wall as well as septal structure, *e.g*. dolipore and parenthesomes, allow a distinction of the three major mycorrhizal taxa encompassed under the name *Rhizoctonia* (Moore, 1987). It has helped to confirm how some unexpected taxa form pelotons and thus are mycorrhizal. Atractiellomycetes, members of the rust lineage (Pucciniomycotina), are mycorrhizal in some neotropical orchids was supported by molecular approaches (Kottke *et al.*, 2010). Selosse *et al.* (2004) verified molecular identification of ascomyceteous *Tuber* spp. as the main mycorrhizal partners in *Epipactis microphylla* by using transmission electron microscopy to check for the presence of Woronin bodies in pelotons and immunogold reactions using antibodies specifically raised against a truffle phospholipase A2, interestingly, basidiomycetes that were found by molecular means were never seen by microscopy. Immunolabelling transmission electron microscopy has been used to demonstrate pectin deposition in the interfacial matrix around *Ceratobasidium* hyphae, but not *Russula* hyphae, in adjacent mycorrhizal root cells of *Limodorum abortivum*, highlighting an orchid's capability to react differently to different fungal partners (Paduano *et al.,* 2011). Eventually, Huynh *et al.* (2004) used scanning electron microscopy imaging of stems and protocorms to determine the most effective fungal isolates for conservation of the threatened orchid *Caladenia formosa*.

Threats

Myco-heterotrophs prefer regions that have been free from disturbance in recent history (Cheek and Williams, 1999; Taylor and Roberts, 2011). The major threat for the survival of myco-heterotrophic plants is habitat destruction. This is the unavoidable result of the

expansion of human populations and anthropogenic activities. Habitat destruction is the primary cause of the loss of biodiversity in terrestrial ecosystems (Pimm and Raven, 2000). Ecosystems can suffer from anthropogenic activities causing pollution. Pollution that impacts plant and fungal diversity is commonly caused by pesticides, sewage, fertilizers from agricultural fields, industrial chemicals and wastes, emissions from factories and automobiles, and sediment deposits from eroded hillsides (Relyea, 2005). Herbivory may also have a negative impact on reproductive success of orchids (Klooster and Culley, 2009; Taylor and Roberts, 2011). Since, introduction of herbivores into myco-heterotrophic orchid habitats can cause potential harm to local myco-heterotrophs, rare and endangered species of myco-heterotrophs may also suffer from overenthusiastic botanists, who collect materials and trample populations during collection trips (Taylor and Roberts, 2011). The global climate change also has severe impact on the existence of myco-heterotrophs. The emission of greenhouse gases (GHGs) has been constantly increasing over the past 100 years. Scientific evidence says that the increased levels of GHGs produced via anthropogenic activities have already affected the world's climate and ecology and these effects will possibly increase in the future (Primack, 2008) and this may be especially harmful for montane forests and their associated myco-heterotrophs (Foster, 2001; Pounds *et al.,* 1999).

Strategies for Conservation of Myco-heterotrophic Orchids

The best and most straightforward approach to conserve the myco-heterophic plants is to protect the habitats where they grow. Conservation of rare and endangered orchids can be supported by establishment of new populations. Seed germination of fully mycoheterotrophic orchids has been achieved by burying seed packages near ectomycorrhizal trees (Bidartondo, 2005; Bidartondo and Bruns, 2001; McKendrick *et al.,* 2000), showing the possibility of re-introducing myco-heterotrophs into existing suitable habitats. A dependence of myco-heterotrophic orchids on narrowly specific interactions with fungi and pollinators may predispose many orchids to become threatened (Bonnardeaux *et al.,* 2007; Dearnaley, 2007; Swarts *et al.,* 2010). Moreover, the anthropogenic activities such as vegetation clearing, altered fire regimes, herbivores introduction and global climate change have further led to the decline in the populations of many rare orchids (Brundett, 2007) for which suitable measures should be taken to conserve the symbionts. The areas of their natural occurrence should be declared as strictly protected areas and human interventions should be strongly prohibited. For the majority of orchid mycorrhiza, protection of the uppermost organic layer is important, as this location is the key habitat for *Rhizoctonia* associates (Brundrett *et al.*, 2003). Regular monitoring of orchid associated fungi is an essential management procedure. This can be done by seasonal observations of fungal fruiting bodies for some associate orchid species. *Ex situ* conservation by germinating the seeds of threatened mycoheterotrophic orchids is a common approach (Stewart and Kane, 2007; Zettler *et al.,* 2007). *Ex vitro* approach where seed is sown in pot soil inoculated with the appropriate fungal partner has an additional advantage in that seedling may form associations with other microorganism present in the medium (Wright *et al.,* 2009). Batty e*t al.* (2001) reported that by immersing the orchid mycorrhizal fungi inoculum in liquid nitrogen or by encapsulation of both seeds and fungi in alginate beads with low temperature (Sommerville *et al.*, 2008) may assist in conservation strategies.

Conclusion

Myco-heterotrophic orchids do not possess any direct economic importance; they are neither useful for consumption nor for pharmaceutical purposes with only one exception *i.e.*, *Gastrodia elata* used in Chinese traditional medicinal system (Xu and Guo, 2000). However, the presence of myco-heterotrophs in forest ecosystems may offer an indirect economical value through recreational services for mankind. Apart from this value, myco-heterotrophic plants offer a unique model system to study mycorrhizal mutualism and ecological symbioses in general, which is being overlooked. Moreover, considerable advances have been made in understanding the ecology and evolution of orchid mycorrhiza in the recent years, but considerable knowledge gaps still exist which need to be studied.

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VALUE ADDITION IN ORCHIDS

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Abstract

Value addition in floriculture increases the economic value and consumer appeal of any floral commodity. In floriculture, value addition is made through genetical changes, processing or diversification. Orchid is a highly diversified flower crop and indigenous species of *Aerides, Bulbophyllum, Calanthe, Coelogyne, Cymbidium, Paphiopedilum, Rhynchostylis, Renanthera* and *Vanda* are used as breeding materials. They are adapted to diversified climate and grow as epiphytes, terrestrials and as lithophytes; these are grown organically with locally available resources by the growers. Many of these can be grown on rocks and logs for placing in the landscape. Hybrids of *Aranda, Cattleya, Cymbidium, Dendrobium, Mokara, Oncidium, Paphiopedilum, Phalaenopsis, Renantanda, Vanda* etc. with different colour and forms are used as cut flowers, and as floral displays and exhibits. Tribal people of NorthEastern hill region use wild orchids for a variety of folk medicine as these plants are rich in alkaloids, flavonoids, glycosides, carbohydrates and other phytochemicals. Fragrant orchids including *Aerides multiflora, A. odorata, Cattleya maxima, Coelogyne cristata, C. ochracea, Dendrobium chrysotoxum, Rhynchostylis retusa* and *Zygopetalum intermedium* are delightful in outdoor living areas. Leaves, tubers and pseudobulbs of different species are used for edible purposes. Vanilla- a major spice crop and source of vanillin comes from *Vanilla planifolia*. *Anoectochilus* leaves are used as vegetables in Indonesia and Malaysia. Pseudobulbs of *Cymbidium madidum* and *Dendrobium speciosum* and tubers of *Microtis uniflora* and *Caladenia carnea* are also eaten. Miniature cymbidiums can be used as value added packed items. Bright flowers of orchid genera like *Cattleya, Cymbidium, Dendrobium, Paphiopedilum, Pholidota* etc. can be used for drying. Among orchids, *Cymbidium, Dendrobium,* and *Phalaenopsis* are excellent for wedding counter-pieces.

Introduction

VALUE ADDITION is the way taken to increase the value of a raw product anytime between harvesting and sales of the final product. A typical value addition includes processing in some ways like cleaning, cutting, packaging, smoking, drying, freezing, extracting or preserving. Value added products give a higher return, open new markets, create brand recognition and add variety to a farm operation and value addition does not offer any guarantee on profitability. Careful planning and management are required to promote profitability. The key factors for the success of value added enterprises include quality products, good marketing and sufficient capital. Other factors required for value added enterprises are: a unique product; an enthusiastic promoter of the product; the right kind of labeling and packaging; aggressive marketing; a full time presence on the farm; strong agricultural or livestock knowledge; ability to cater to customers; assistance from agencies and universities; a strong relationship with the local community; safe food handling and food safety regulations; and product liability insurance.

Value addition in floriculture increases the economic value and consumer appeal of any floral commodity. In floriculture, value addition is made through genetical changes, processing or diversification. The profitability of a commodity is increased when a raw material is converted into a unique product. Although it requires more time, labour and skill but can significantly increase the net cash return of a small scale floriculture enterprise. Value addition gives high premium to the grower as well as it provides quality products for the domestic and export market. Recently, the consumption pattern is getting diversified towards value added products such as essences, perfumes and other by-products from flowers. There is an urgent need for value addition in floricultural products through processing, packaging and supply chain management to increase farm income and generate employment. The value added products from non-conventional floricultural crops like essential oils of rose, tuberose, jasmine, marigold and plant extracts used in medicines and pharmaceutical industry are unique and have export-import potentialities.

Orchids comprise one of the largest families of flowering plants with 25,000 to 35,000 species belonging to 600-800 genera and covers 6.83% of the flowering plants. They are prized for their incredible diversity in the size, shape and colour and attractiveness of their flowers and high keeping qualities even upto 6 weeks. Most of the orchids have originated from tropical humid forests of Central and South America, India, Sri Lanka, Burma, South China, Thailand, Malaysia, Philippines, New Guinea and

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Australia. Brazilian *Cattleya*, Mexican *Laelia* and Indian *Cymbidium, Dendrobium* and *Vanda* have played a major role in developing present day beautiful hybrid orchids which numbers more than 2,00,000. In the international trade, amongst the top ten cut flowers, orchids rank the sixth position and amongst orchids *Cymbidium* ranks the first position and in floricultural crops it accounts for 3% of the total cut flower production.

India is a major orchid habitat of the world and with its perfect climate, it is home to 1331 species including 400 endemics (Misra, 2007); the terrestrials are located in humus rich moist forest floors under tree shades in North Western India, Western Ghats harbour the small flowered orchids and epiphytic orchids are common in NorthEastern India which grow upto an elevation of 2000 m from sea level. Indian orchids with high ornamental values used as breeding materials are *Aerides multiflora, A. odorata, Arundina graminifolia, Arachnis* spp.*, Bulbophyllum* spp.*, Calanthe masuca, Coelogyne elata, C. flavida, C. corymbosa; Cymbidium aloifolium, C. lowianum, C. devonianum, C. hookerianum, C. lancifolium,; Dendrobium aphyllum, D. chrysanthum, D. densiflorum, D. nobile, D. farmeri, D. fimbriatum, D. jenkinsii, D. moschatum; Paphiopedilum. hirsutissimum, P. insigne, , P. spicerianum, P. venustum; Phaius wallichii, Pleione praecox, Renanthera imschootiana, Rhynchostylis retusa, Thunia alba, Vanda cristata, Vanda coerulea* and *Vanda coerulescens* (Singh, 1990).

Orchids and Their Various Uses

Orchids are found in nearly every environment in the world. Epiphytic orchids like *Aerides, Aranda, Aranthera, Bulbophyllum, Calanthe, Cattleya, Coelogyne, Dendrobium, Laelia, Phalaenopsis, Thunia,* with thick leaves and succulent stems have CAM and are drought tolerant with higher water use efficiency. Rhizomatous orchids like *Eulophia, Habenaria, etc.* require terrestrial climate. Each orchid genus has different requirements for potting medium, collected from locally available organic sources. It is very important to have the suitable medium for each type of orchid, depending on whether it is terrestrial or epiphytic. Growing media commonly include fir bark, coconut husk, sphagnum moss, tree fern fibre, coco peat, saw dust and perlite, and more frequently, it is a mixture of two or three of these materials. All orchids potted in a typical bark medium need to be repotted every 18 to 24 months, depending on the requirement of the individual plant.

Orchid scaping is the use of orchids permanently planted into specially prepared beds or attached to trees, shrubs or rocks in the appropriate spot in the garden. Combined with other traditional ornamentals such as palms, ferns, flowering perennials, shrubs, trees and herbs *etc*., it is easy to create some of the most interesting and beautiful gardens imaginable, depending upon the cost involvement and micro-climatic factors. Many orchids can be grown on rocks and logs for placing in the landscape. They are attached to either cut wooden logs, coconut logs or living trees and shrubs. Once the orchids are established, they will attach to the trees and logs (Teoh, 2005). In order to create the visual impact in landscaping, the orchids should be planted in a single bed of one type and of one colour. If somebody has only one or two plants of a type, it is advisable to grow these in pots. Almost all spider orchids (*Arachnis* and their intergeneric hybrids, terete and semi-terete vandas, *Phaius tankervilliae*, *Calanthe* spp., and Lady Slippers) perform well, if they are grown on the ground in full sun with liberal watering and fertilization. Sloping or flat ground with good drainage provides the ideal location for orchid beds.

With a view to developing an orchidscape, gardener should be aware of the flowering period of each orchid. Some gardeners enjoy seasonal burst of colour. For them, cymbidiums and dendrobiums which flower from winter to spring should be the first choice (Friend, 2004). Winter flowering orchids include *Bulbophyllum hirtum, B. putidum, Cymbidium lowianum, C. mastersii, Eria bambusifolia, Paphiopedilum fairrieanum, P. insigne, P. spicerianum, Pleione maculata,* and *P. praecox.* Spring flowering orchids include *Ascocentrum ampullaceum, Calanthe plantaginea, Coelogyne cristata, Cymbidium devonianum, C. eburneum, Paphiopedilum hirsutissimum, P. villosum, Phalaenopsis lobbii, Pleione humilis.* Summer flowering orchids include *Coelogyne corymbosa, C. cristata, C. nitida, C. ochracea, Cymbidium aloifolium, Dendrobium fimbriatum, D. heterocarpum, D. nobile, Pleione. mannii, P. hookeriana, Phaius flavus, P. tankervilliae, Renanthera imschootiana, Rhynchostylis retusa, Spathoglottis plicata, Vanda coerulea, V. cristata, V. stangeana,* and *V. tessellata.* In Balcony gardens, lithophytic orchids can be grown by attaching them in free standing rocks or to the balcony's masonry walls. Genera suitable for shady location may include *Bulbophyllum, Coelogyne, Eria, Maxillaria*, some oncidiums*, Sarchochilus* hybrids, *Phalaenopsis* and *Cattleya* hybrids. According to Taylor (2009), an orchid tree is a variation on mounting orchids except the placement of many orchids on a branch or branches to give a completely natural look. It is used in those areas of the country where orchids are grown outdoors, most of the year. Usually, the larger plants are

attached to the bottom and the smallest on the upper portions for aesthetic reasons and to provide extra weight at the bottom to balance the weight of the structure. It is better to select those plants which require similar light, temperature and humidity conditions. Another factor that has to be considered is flowering times to get different colours on the tree throughout the year. The chosen plants are mounted on the tree with sphagnum moss and fishing wire. Proper misting and maintenance of humidity are essential for a month to establish the plants on the structure.

Potted orchids last for longer than cut flowers, their shelf life being three weeks to four months depending upon species and hybrids (Nagrare and Ram Pal, 2008). Tall growing monopodial orchids are best grown in large clay pots upto 30 cm in diameter. Terrestrial and semi-terrestrial plants like *Cymbidium* and *Paphiopedilum* perform better in deep pots. Orchid plants, as a rule are to be grown near one another to aid a microclimate higher in humidity. Basket culture is useful for those orchids like *Arachnis*, *Rhynchostylis,* and *Vanda* with pendent flower spikes and long dangling roots. Clay pots are the best suitable for terrestrial orchids. Plastic pots are used for epiphytes. Slabs or logs of tree fern are effective for cool growing orchids. Important orchid genera used as potted plants in the international market are *Ascocenda, Brassia, Cattleya, Cymbidium, Dendrobium, Epidendrum, Miltonia, Oncidium, Paphiopedilum, Phalaenopsis,* and *Vanda* (Lopez and Runkle, 2005).

Several local species of *Ascocentrum, Calanthe, Cymbidium, Dendrobium, Paphiopedilum* and *Vanda, etc.* are in great demand in international market for breeding materials (Bose and Bhattacharjee, 1980; Kumar and Sheela, 2007).

Orchid hybrids of *Aranda, Cattleya, Cymbidium, Dendrobium, Mokara, Oncidium, Paphiopedilum, Phalaenopsis, Renantanda, Vanda etc.,* with different colour and forms are used as cut flowers, floral displays and as exhibits (Bhattacharjee and De, 2005; De, 2011; De *et al.,* 2013).

Table 1. Common varieties and hybrids under different genera of orchids.

Sr. No.	Genus	Hybrids/Varieties
1	*Aeridovanda*	Doctor Poyck, Vieng Ping, 'Bensiri', 'Noreen', 'Early Bird', 'Shiv Sidhu', 'New Dawn', Harrison Luke Somsri Sunlight'
2	*Aranda*	Ang Hee Seng, Logtakjep, Bertha Braga, Christine, City of Singapore, Deborah, Federal Beauty, Hee Nui, Hilda Galistan, Iskandar of Johor, Kooi Choo, Lucy Laycock, Gaw Bon Chan, Majula Rimau, Mandai Gardens, Merry Maggie, Myrna Braga, Peter Ewart, Sweet Honey, Tan Mei Ying, Tan Theng Suan, Wong Bee Yeok, Chao Praya Beauty, Thailand Sunspot
3	*Arachnis*	Ishbel, Maggie Oei, 'Maroon Maggie', Bartha Braga'
4.	*Ascocenda*	Apinantat Red Berry, Pralor Tuyen, Pak-Kred, Bangkok, Surin, Karnada, Crownfox, Sundancer, Laksi 'Red Ruby',Guo Chia Long 'Spotty', Fuchs Angel frost
5.	*Cattleya* and allied genera	Lovely Bangkok, Admiration, Bob Belts, General Patton, Joyce Hannington, Little angel, Margaret Stewart, Nillie Roberts, Pearl Harbour, Primma Donna, Queen Sirkhit 'Diamond Crown', Secret Love, Ladda Belle 'Pink Pearl', Maikai, Pastoral, Robert, Prism Palette 'Tricolour Magic', Chinese Beauty Orchid Queen, Chia Lin New City, Ahmad Seikhi
6.	*Cymbidium*	Levis Duke Bella Vista, Madrid Forest King, Sparkle Late Green, Angelica December Gold, Sleeping Nymph, Pine Clash Moon Venus, Soul Hunt, Dr. H. C. Aurora, Susan Highes, Tia Gaig Suther Land, Miss Sanders, Amesbury, Kenny Wine, Red Star, Red Princess, Show Girl , Jungfrau 'Snow Queen', Jungfrau 'Dos Pueblos', Lilian Stewart 'Coronation', Lilian Stewart 'Party Dress', Orkney 'Pink Heather', Ensikhan 'Alpha Orient', Fire Storm Blaze, Bob marlin Lucky
7.	*Dendrobium*	Emma White, Thongchai Gold, July, Eruka, Sonia-17, Sonia-28, Kasem White, Madam Pompadour, Bangok Blue, Ann, Gold Twist,Candy Stripe Pink, Genting Blue, Bengal Beauty, Sakura Pink, Candy Stripe, Burana Charming, Blue Fairy, Channel, Nette White
8.	*Mokara*	Walter Oumae 'Seksan', Thailand, Sayan, Walter Oumae 'Royal Sapphire', Susan 'Orange', Walter Oumae 'Calypso' , Eng Ling, Madame Panne, Mak Chin On, Bangok Gold, Bibi, Chao Praya Gold, Chark Kuan Orange, Chark Kuan Pink, Chark Kuan Rose, Chark Kuan Super, Dinah Shore, Kelvin Red, Kelvin Orange, Luenberger Gold, Margaret Thatcher, Pink Star, Sayan, Sayang Pink, Walter Oumae, WTO, Jiti, Happy Beauty, Salaya Gold
9.	*Odontoglossum*	Carroll, Ismene, Cynthia Hill, Mayapan, Quito, Italian Job

Table 1. Common varieties and hybrids under different genera of orchids (contd.).

Sr. No.	Genus	Hybrids/Varieties
10.	*Oncidium*	Aloha Iwanga Dogasima, Goldiana, Gower Ramsey, Golden Shower, Sum Lai Who Jungle Queen, Taka H & R, Sharry Baby Sweet Fragrance AM/AOS, Golden Glow, Popki Red, Irine Gleason Red, Vision Brownish Red, Catherine Wilson x New Calidonia Brownish Red, Robson Orchid Glad
11.	*Paphiopedilum*	Niveum, Concolor, *P. rothschildianum* (3 to 5 flowers), *P. sanderianum* (3 to 5 flowers), Prince Edward of York, Michel Kooppwitz, Saint Swithin, Mount Toro, Sorcerers Apprentice, Grande, Don Wimber, Elizabeth March, Hanne Popow, Jason Fischer, Living Fire
12.	*Phalaenopsis*	Taisuco Crane, Taisuco Kochdian, Cygnus, Yukimai, Sogo Musadian, White Dream, Florida Snow, Nobby's Pink Lady, Minho Valentine, Minho King Beauty, New Cinderella, Taisuco Firebird, Sogo Smith, Carol Campbell, Emil Giles, Brother Lawrence, Taipei Gold, Golden Bells, Sogo Managers, Brother Passat, Be Glad, Cassandra, Vilind, Carmelas Pixie, Zuma's Pixie, Timothy Christopher, Be Tris, Quevedo, Strawberry, Detroit, Maki Watnabe, Kaleiodoscope
13.	*Renanthera*	Brookie Chandler, Manila T-Orchids, Kilauea, Mok Yark-Seng, Poipu, Tom Thumb, Datin Blanche, Red Leopard, '20th WOC Singapore-2011', 'Bart Motes'
14.	*Renantanda*	'Forever Yvonne', 'Inspiration Ng Teng Fong', 'Ladda Glow'; 'Polyetheramine Singapore', 'Momon Shija', 'Paul Gripp', 'Science Arts', 'Memoria Charles Darwin', 'Prof. G.J. Sharma', 'Kebisana Shija', 'Mary Motes', 'Kofi Annan'
14.	*Rhynchovanda*	'Wilton Hill', 'Jammie Harper', 'Apichart', 'Noo Noi', 'Peter Draper', ' Brighton's Albino', 'Prairie Lady'
15.	*Vanda*	Annette Jones, Antonio Real, Golamcos Blue Magic, Fuch's Charmer, Jimmy Millers RF Orchids, Keree Delight, Memoria Lyle Swanson, Motes Indigo x Merrillii, Motes Honeybun, Motes Primerose, Miss Joaquim,V. Rothschidiana, VTMA –Red, Pink, White, Vasco, Johnny Miller,Veerawan, Roberts Delight, Rasriprai, Pat Delight, Pakchong Blue, Mimi Plammer, Manuvade, Lumpini Red, Kultana Gold x Thongchai Gold, Fuchs Delight, Charles Goodfellow, Pine River, Adisak, Doctor Anek, John Club, Bill Sutton, Ellen Noa, Emily Notley, Evening Glow, Honomu, Honolulu, Hilo Blue
16.	*Vascostylis*	Paragon Joy x Kasems Delight, Precious, Veeraphool, Crown Fox 'Red Yen', Aroon Fairy, Viboon Velvet, Chao Praya Lime', 'Lanna Rosy', 'Jeans Delight', 'Bay Sapphire', 'Spring Hill'

Tribal people use wild orchids for a variety of folk medicine as orchids are rich in alkaloids, flavonoids, glycosides, carbohydrates and other phytochemicals (cf. Pathak *et al.,* 2010; Rao, 2004). Some of the common medicinal orchids are mentioned in Table 2.

Many medicinal orchids are rich in alkaloids. Experimental evidences have reported on the isolation of a number of alkaloids like anthocyanins, stilbenoids and triterpenoids from orchids. Orchinol, hircinol, cypripedin, jibantine, nidemin and loroglossin have been reported from orchids. Some of phytochemicals isolated from orchids along with active ingredient are listed in Table 3.

Fragrant orchids are delightful in the outdoor living areas. *Brassavola* species are perfumed at night and the Australian native dendrobiums perfume the air on warm spring mornings. Other aromatic orchids are *Aerides multiflora, A. odorata, Aeranthes, Bulbophyllum odoratissimum, Cattleya maxima, Coelogyne cristata, C. ochracea, Cymbidium densifolium, Dendrobium nobile, D. chrysotoxum, Epidendrum cristatum, E. floribundum, E. nocturnum, Phaius tankervilliae, Rhynchostylis retusa, Vanda cristata, V. tesselata* and*, Zygopetalum intermedium*.

Leaves, tubers and pseudobulbs of different species are used for edible purposes. Vanilla- a major spice crop and source of vanillin comes from *Vanilla planifolia*. *Anoectochilus* leaves are used as vegetables in Indonesia and Malaysia. Pseudobulbs of *Cymbidium madidum* and *Dendrobium speciosum* and tubers of *Microtis uniflora* and *Caladenia carnea* are eaten. The popular beverage called as *Faham* or *Madagascar Tea* on the islands of Mauritius and Madagascar is prepared from orchid *Jumellea fragrans*. The tubers from the orchid genera like *Acianthus*, *Dipodium, Glossodia, Lyperanthus, Prasophyllum* and *Thelymitra* have been used as food by the inhabitants of Australia. In Africa, the tubers of *Cynorchis, Disa, Eulophia, Habenaria* and *Satyrium* are used as food or to extract juice from them. Roots, tubers or rhizomes of

Table 2. Some of the common medicinal orchids.

Species	Part(s) Used	Uses
Acampe papillosa	Roots	Used for curing rheumatism, sciatica and uterine diseases
Aerides multiflora	Tubers	Used as anti-bacterial
A. odorata	Fruits, leaves	The ground fruit is used for healing wounds; juice of leaves is used to heal boils in ear and nose
Anoectochilus formosum	Tubers	Used for curing hepatitis, hypertension, cancer
Arundina graminifolia	Stems	Bulbous stems are used to heal cracks
Bletilla striata	Pseudobulbs	Used as anti-bacterial, anti-inflammatory, demulcent, skin styptic
Calanthe discolor	Whole plant	Used for hair restoring
Cymbidium aloifolium	Whole plant	Ground plant is used to cure chronic illness, weakness of eyes, vertigo and paralysis
C. aloifolium	Rhizomes	Salep is used as nutrient and demulcent and as emetic and purgative
C. ensifolium	Rhizomes and Flowers	Used for curing eye sores
C. giganteum	Leaf juice	Used for blood clotting
C. longifolium	Pseudobulbs	Used as emetic and demulcent
Dendrobium chrysanthum	Leaves	Used as antipyretic, Immuno regulatory and for curing skin diseases
D. densiflorum	Leaves	Leaves are crushed into paste with salt and applied on fractured area to set bones
D. loddigesii	Leaves	Used as stomach tonic
D. moschatum	Leaves	Leaf juice is used as ear drops
D. nobile	Stems	Fresh and dried stems are used in preparation of Chinese drugs for longevity and as aphrodisiac, stomachic and analgesic
Habenaria acuminata	Roots	Roots are used as tonic
H. edgeworthii	Leaves and roots	Used for curing blood diseases
H. intermedia	Leaves and roots	Used for curing blood diseases
H. pectinata	Leaves and tubers	Used for curing arthritis
H. repens	Tubers	Used as aphrodisiac
Malaxis acuminata	Pseudobulbs	Used as tonic and as a cure for tuberculosis, burning sensation, fever and also for enhancing sperm production
Orchis laxiflora	Bulbs	Used for curing diarrhoea, bronchitis, convalescence
Pholidota chinensis	Pseudobulbs	Used for curing scrofula, toothache and stomachache
P. imbricata	Pseudobulbs	Psedubulbs are mixed with mustard oil and applied on joints for curing rheumatic pain
Rhynchostylis retusa	Roots	Roots are effective against rheumatism, asthma, tuberculosis, cramps, epilepsy, vertigo, kidney stone, menstrual disorder
Vanda coerulea	Leaves	Leaf juice is used against diarrhoea, dysentery and external application for skin diseases
V. cristata	Leaves	Leaves are used as tonic and expectorant
V. spathulata	Flowers	Used for curing asthma
V. teres	Leaves	Leaf paste to reduce temperature in fever
V. tessellata	Whole Plant	Used for curing fever, arthritis, rheumatism and bronchitis

Eulophia, Gastrodia, Habenaria, Orchis, Pholidota, Platanthera and *Spiranthes* are used as food in Asia. Tubers of *Disa engleriana, D. robusta* and *D. zambica, Habenaria clavata, Satyrium ambylosacco, S. buchananii* and *S. carsonii* are used as foods in Malaysia. In Bhutan, the inflorescence or the flowers and pseudobulbs of *Cymbidium* spp. are eaten.

Cilindra is a gift of a glass flute containing a flowering mini *Cymbidium* and Stylish setting is a festive packaging for special occasions like Birthday.

People of Assam and Arunachal Pradesh use *Aerides odorata*, *Papilionanthe teres, Rhynchostylis retusa, Vanda roxburghii,* and many *Dendrobium* species in

Table 3. Orchids and phytochemicals.

Species	Phytochemical class	Phytochemical(s)
Aerides crispum	Phenanthropyran	Aeridin
Agrostophyllum brevipes	Triterpenoid	Agrostophyllinol
A. callosum	Triterpenoid	Isoagrostophyllol
		StilbenoidsOrchinol, 6-methoxycoelonin, imbricatin, flaccidin, oxoflaccidin, oxoflaccidin, isooxoflaccidin, flaccidinin, agrostophyllin, callosin, callosinin, callosumin, callosuminin, callosumidin
Anoectochilus formosanus	Glycoside	Kinsenoside
Arundina graminifolia	Stilbenoids	Arundinan
Bulbophyllum gymnopus	Phenanthrene	Gymopsin
Cypripedium calceolus, C. pubescens	1-4 phenanthrenequinone	Cypripedin
Dendrobium macraei	Alkaloid	Jebantine
D. moschatum	Phenanthrene	Rotundatin and moscatin
D. nobile	Bibenzyl	Gigantol Bibenzyl Moscatilin Alkaloid Dendrobine
Dracula chimaera	Anthocyanins	
Eulophia nuda	Phenanthrene	Nudol
Nidema boothi	Triterpenoid	Nidemin
Orchis latifolia	Glucoside	Loroglossin
Vanda roxburghii	Glycoside	Melianin

their religious and cultural festivals. In Assam, the flowering spike of *Rhynchostylis retusa* known as *Kopou Phul* is used by the girls to adorn their hair during the spring festival. The flowers of some other orchids like *Coelogyne nitida* and *Vanda roxburghii* are also used to adorn hair of girls of Assam and Arunachal Pradesh in local festivals. The flowers of *Papilionanthe teres* are offered to Lord Buddha and spirits by the Khamtis and other Tai ethnics of Assam and Arunachal Pradesh. In Kameng district of Arunachal Pradesh, *Dendrobium hookerianum*, *D. nobile* and *D. gibsonii* are considered as the symbol of purity and sanctity by the local people. Monpas consider the flowers of *Cymbidium grandiflorum* important for holy worship. The young naga women of Manipur wore the orange flowers of *Dendrobium densiflorum* behind their ears. Similarly, the flowers of *Vanda coerulea* are used by the women of Manipur in hair during the autumn puja festival. In several countries, orchid species and hybrids are used as National Flowers. For example, *Vanda* Miss Joaquim in Singapore, *Peristeria elata* in Panama and *Lycaste skinneri* var. *alba* in Guatemala. Orchids are depicted on stamps of several countries like Venezuela, USA, New Zealand, Australia, Indonesia, India, Singapore, Japan, Russia, Thailand, Malaysia and many others (Bhattacharjee and Das, 2008).

As orchid flowers are highly attractive, delicate and available in variety of colours, they can also be preserved by drying for their use in flower arrangement and dried flower craft. These can be dried best using silica gel for microwave drying or by freeze drying. Drying orchids is a challenging task as these flowers are considered difficult to be preserved. Dried orchids are used for different purposes such as the dried orchids, for use in vases and baskets and sometimes in shadow boxes. Bright flowers of orchid genera like *Cattleya, Cymbidium, Dendrobium, Paphiopedilum* and *Pholidota etc*. can be used for drying.

As the orchids symbolize wealth, beauty and social status, orchid flower arrangements are used for good table decorations and venue decorations during weddings. Amongst orchids, *Cymbidium, Dendrobium* and *Phalaenopsis* are excellent for wedding counter-pieces. An arch decorated with chic white silk combined with white orchids can be considered as an

admirable orchid flower arrangement. In home, they can be displayed in three ways *i.e.,* single flower vases, plants in pots and traditional mixed flower arrangements. In Philippines and New Guinea, the stem of some Dendrobium species is used to make baskets and bracelets. In some tribes, *Cattleya labiata* var. *autumnalis* sap is used as glue for musical instruments. In Central America, the schomburgkias empty pseudobulbs are used to make horn.

Conclusion

The cut-flower industry is one of the higher industries in many developing and underdeveloped countries. The orchids are marketed both as potted plants and as cut-flowers. In the past few years, the orchid trade has increased both in volume and value throughout the world. In floricultural crops, orchids account for 3% of the total cut-flower production. As the orchids symbolize wealth, beauty and social status, the use of orchid flower arrangements has increased tremendously for good table decorations and venue decorations during weddings and other functions. Besides this aspect, orchids are also used by local populations for curing a variety of ailments. An orchid grower should be very careful while selecting the potential species to be grown in a particular region, their perfect growth conditions and suitable potting substrate for the suitable growth and production of these floriculturally and medicinally important species.

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ONTOGENY OF MICROSPORANGIUM AND DEVELOPMENT OF MALE GAMETOPHYTE IN *PERISTYLUS SPIRALIS* A. RICH. (ORCHIDACEAE)

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Abstract

The anther in *Peristylus spiralis* A. Rich was dithecous and tetrasporangiate. Its wall development conformed to the monocotyledonous type. Each archesporial cell developed into a block of sporogenous cells which ultimately organized into massulae. The anther wall was 4-layered. The endothecial cells developed ring like tangential thickenings on the inner walls. Tapetal cells were uninucleate and of dual origin. The microspore tetrads were tetrahedral, decussate, linear and T-shaped. Pollen was shed at 2-celled stage.

Introduction

THE EMBRYOLOGY of Orchidaceae has attracted the attention of several investigators from time to time because of their extreme specialization in exhibiting great diversity in development of male and female gametophytes, suspensor and embryo apart from their vegetative and floral organs as shown by the most comprehensive embryological works of Abe (1972a,b), Levitte (1901), Schnarf (1931), Swamy (1949a,b), Wirth and Withner (1959), and Veyret (1974). These investigators, in addition to their own observations have also given reviews of previous embryological works. Some of the recent embryological works in the area include those of Bhanwra *et al.* (2006), Fredrikson (1990, 1991), Gurudeva (2009, 2010, 2011a,b, 2012, 2014), Govindappa and Karanth (1980), Gurudeva and Govindappa, (2008), Krishna Swamy *et al.* (2003, 2005), Mohana Rao and Sood, (1979a,b, 1986, 1987), Kant and Hossain (2010), Sood (1985a,b, 1986, 1988, 1989, 1992), and Sood and Mohana Rao (1986a,b). The genus *Peristylus* Blume (sub-tribe: Orchidinae; tribe: Orchideae; sub-family: Orchidoideae of Dressler and Dadson, 1960) comprises of 70 species distributed in Indo-Malayan regions. In India, the genus is represented by 28 species and 2 varieties, 6 of which occur in Karnataka (Ananda Rao and Sridhar, 2007) but the embryological studies in the genus are meager. Swamy (1949a) studied the development of male and female gametophyte in *Peristylus spiralis* and *P. stocksii*. The present communication deals with the detailed study on the mode of wall layer development, nature of endothecial thickenings and derivation of massulae from archesporial cells in *Peristylus spiralis*.

Material and Methods

Peristylus spiralis A. Rich. is a terrestrial leafy herb with small oblong tubers. The *stem* is erect, slender and often covered with basal sheaths and the *leaves* are 3 – 5 in number. They are linear-lanceolate, acute, spirally arranged at the base of the stem (Fig. 1). The *inflorescence* is spirally twisted spike. The *flowers* are greenish white and arise in the axil of small bracts. The sepal and petals are sub-equal. The *lip* is variable, longer than the sepals, cuneate and 3-cleft to about

Figs. 1-2. *Peristylus spiralis*: 1, Flowering plant with tubers;2, Close-up of inflorescence.

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the middle. The *median lobe* usually shorter, broader and curved. *Spur* is a minute globose sac. *Column* is short. *Ovary* is inferior and pale green (Fig. 2).

The flower buds were collected at different stages of development from Bhagamandala, Talacauvery, Kodagu district (Karnataka, India) during September to October, These were fixed in formalin-acetic-alcohol and stored in 70% ethanol following a thorough wash in running water. Conventional micro-techniques were followed. The serial transverse and longitudinal sections at 10-12μm were stained with Heidenhain's iron-alum and haematoxylin. Erythrosin in clove oil was used as counter stain. Mature anthers were selected and placed in a watch glass treated with 1N HCL and gently warmed over the flame. The treated anthers were macerated with crystal violet and mounted in glycerine. Drawings were made using Camera Lucida and Meopta microscope. Photomicrographs were taken by using Olympus-CH20i microscope with built in analogue camera (CM-1.4MP). Computer images were captured using AV-digitiser having Grand VCD-200 captured guard.

Results

Ontogeny of Microsporangium

A very young anther in transection was two lobed. Each lobe lodged two rows of densely protoplasmic hypodermal archesporial cells (Figs. 3, 4). The location of each row was the site of a microsporangium. Periclinal division of the archesporial cells occured early to delimit the primary parietal layer from the primary sporogenous layer. Primary sporogenous cells underwent both anticlinal and periclinal divisions and organized into a block of sporogenous cells, each block representing the future pollen massula (Fig. 5). After a period of anticlinal divisions, the primary parietal cells divided periclinally to produce two layers of cells (Fig. 6). The outer parietal layer directly developed into endothecium. The inner parietal layer divided periclinally to give rise to the middle layer and the glandular parietal tapetum (Fig. 7). Meanwhile, cells of the connective adjoining the sporogenous tissue acquired dense cytoplasm and organized into a complete sheath of connective tapetum. As a result, the entire tapetal layer around the sporogenous tissue is of dual origin (Fig. 8). The wall layers of microsporangium consists of epidermis, endothecium, middle layer and tapetum.

Microsporogensis and Pollen Development

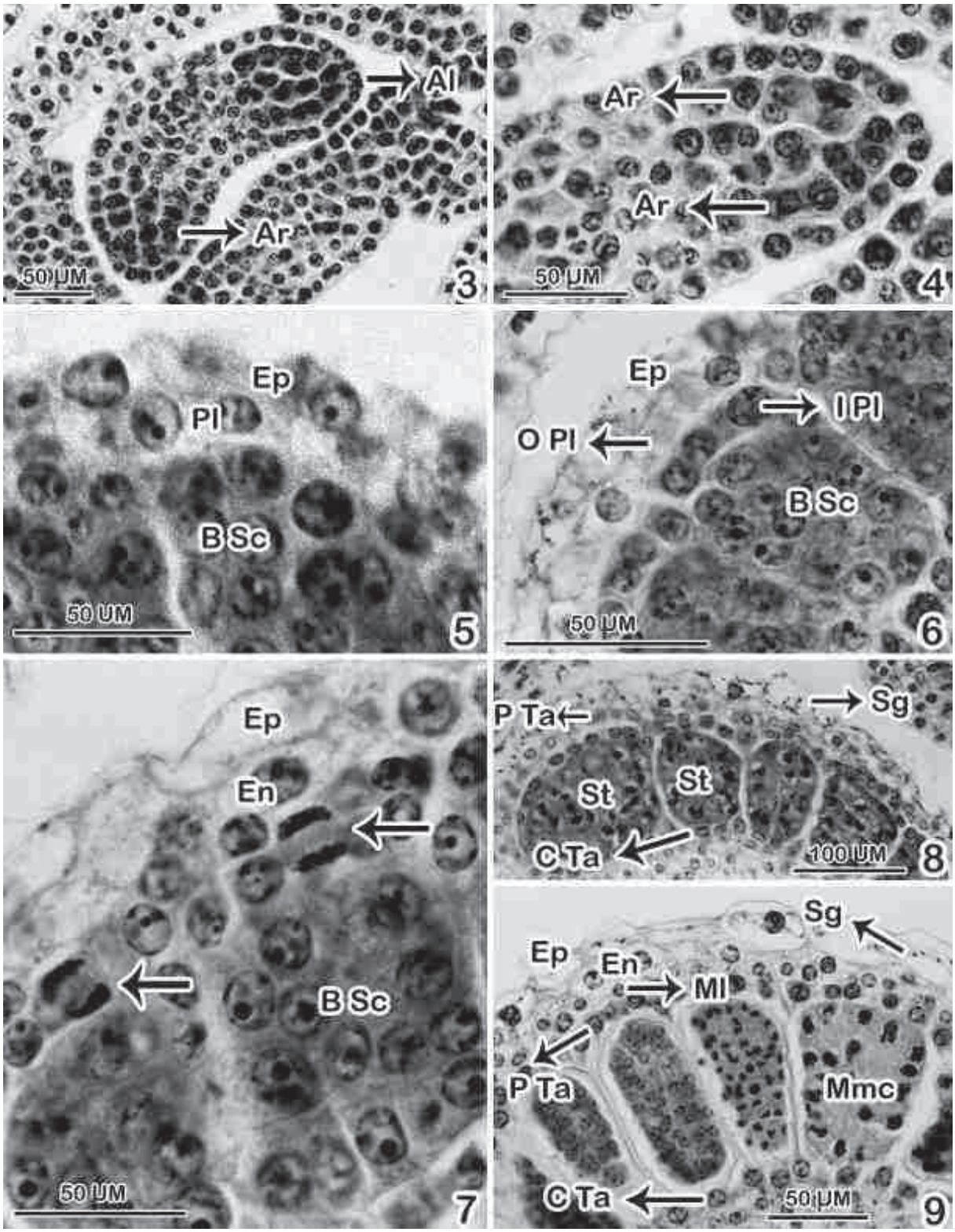
Sporogenous cells in each block differentiated into microspore mother cells (Fig. 9). Meiotic divisions occured in the microspore mother cells. The first nuclear division was not followed by a wall (Figs.10-12). The resulting dyad nuclei divided simultaneously and gave rise to four microspores. The orientation of the spindles of the dividing dyad nuclei varied considerably (Figs. 13, 14). After the simultaneous quadric-partition of the mother cells, tetrahedral, rhomboidal, T-shaped and linear microspore tetrads were formed within a massula (Figs.15-18). The spores of the tetrad did not separate apart, so also the tetrads of a massula. The nuclei of the microspores of the tetrads divided synchronously to render each one of them as bi-celled. The orientation of the spindles of the dividing nuclei especially in the tetrahedral and rhomboidal tetrads were always disposed along the proximal and distal axis (Figs.19-21). The smaller densely protoplasmic generative cell was always cut off towards the distal end adjoining the spore coat (Figs. 22, 23). The generative cell then separated itself from the spore coat and entered into the cytoplasm of the vegetative cell in the microspores of all the types of tetrads (Figs. 24-27). By this time, the pollen massulae were fully covered by a coat of sporopollenin and these appeared as independent structures within the microsporangium.

During the subsequent development, the tapetal cells became conspicuous, remained uninucleate and provided nourishment to the spore mother cells, microspores and pollen grains, while other layers extended laterally. Finally, the large epidermal cells accumulated starch grains. The cells of the endothecium acquired ring-like thickenings, one per cell and tangentially disposed on the inner surface of the cell wall. The tapetum and middle layers got absorbed and the endothecium and epidermis were left when pollen massulae were fully organized and ready for release (Figs. 28-30).

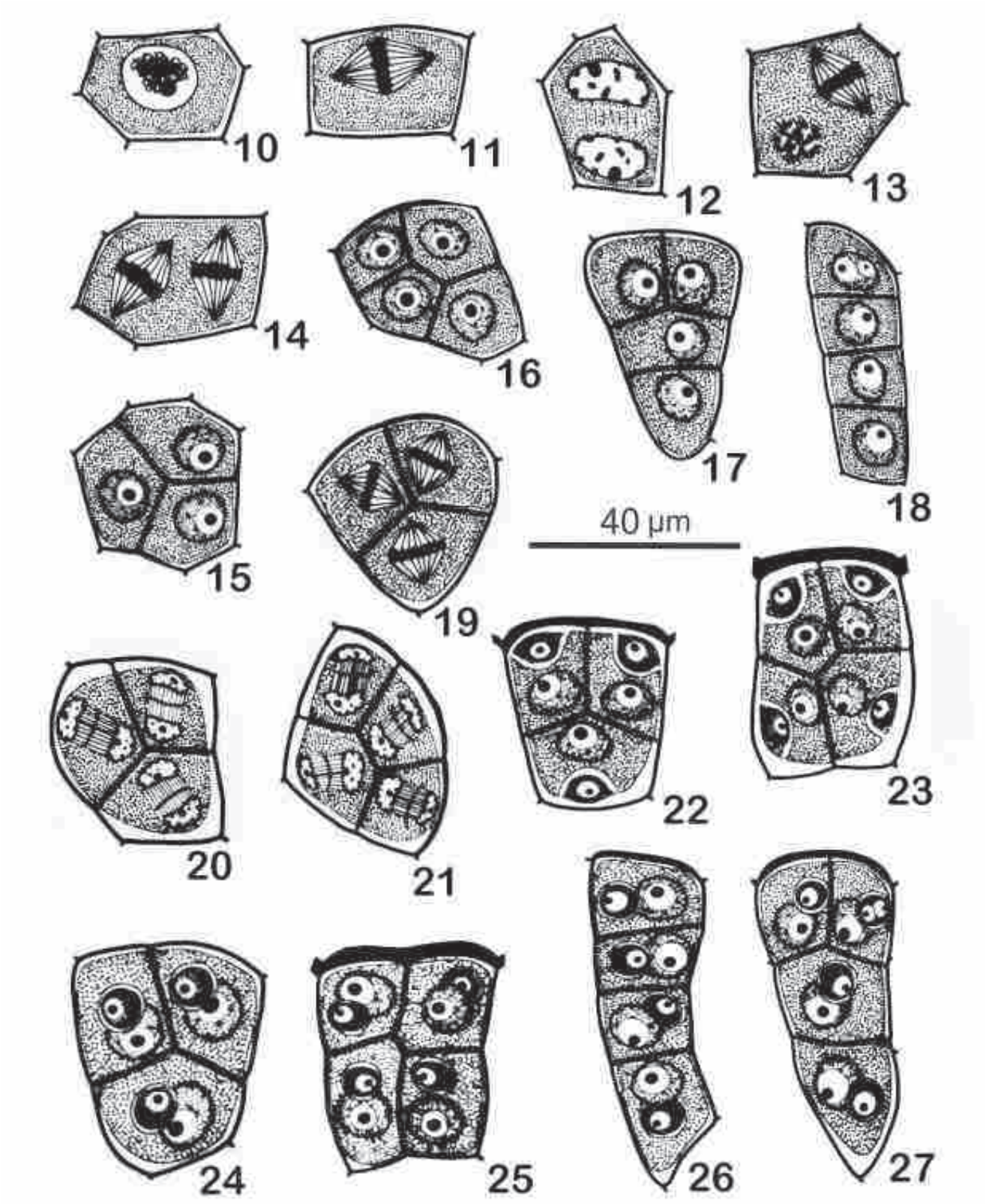
By this time, pollen massulae were fully organized, the group of smaller thin-walled cells belonging to the separating layer (connective cells) between the adjacent microsporangia and the cells located in the sub-epidermal region at the junction of the sporangial walls, were broken down. Aided by the endothecial thickenings, a common opening was created between the adjacent microsporangia, permitting the exit of the pollen massulae (Figs. 31-35).

Discussion

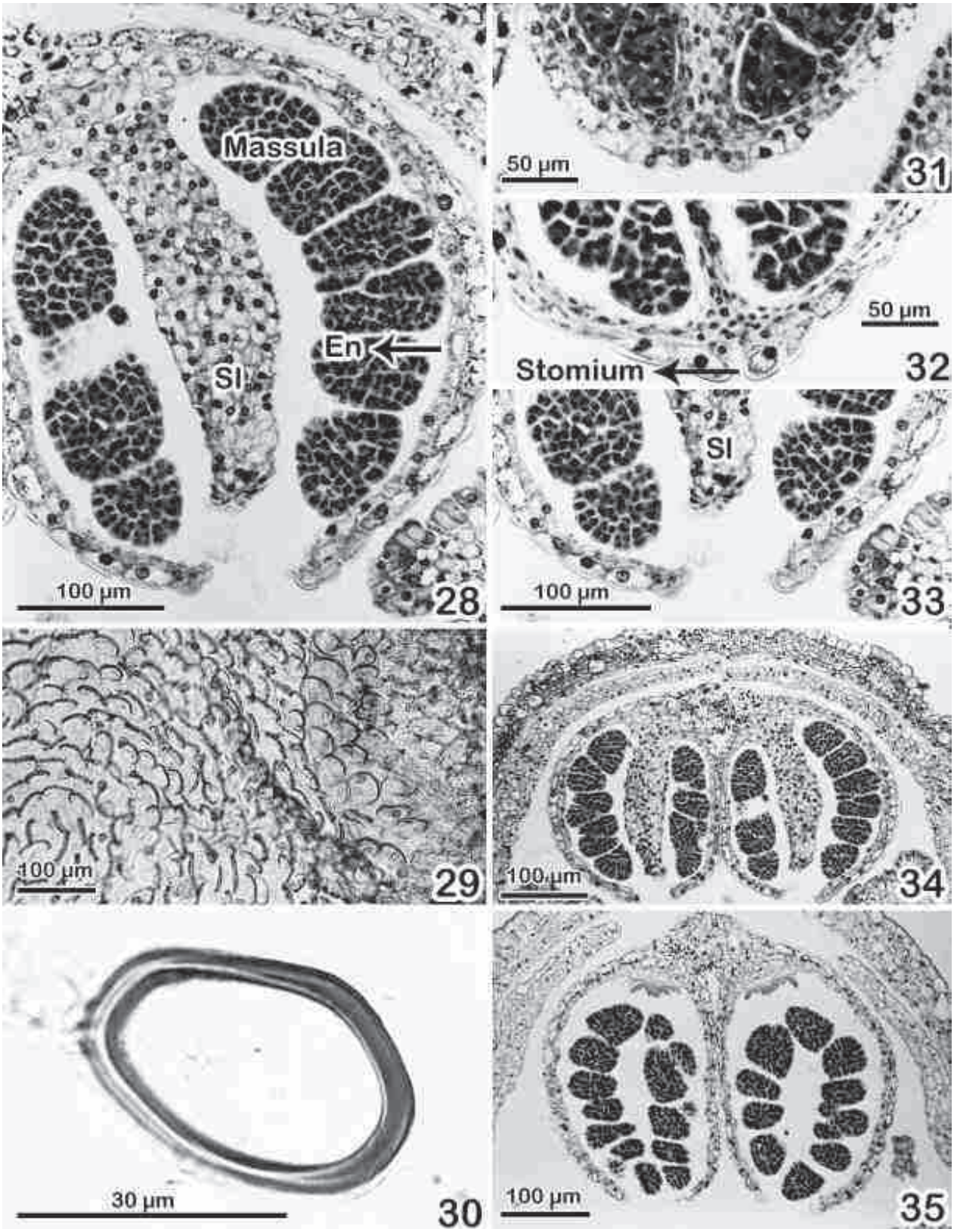
The anther was dithecous and tetrasporangiate. Similar nature of anther has been recorded in most of the orchids (Gurudeva, 2012; Krishna Swamy, *et al.*, 2003; Sood, 1985a, 1989, 1992). The mode of



Figs. 3-9. Ontogeny of microsporangium in *Peristylus spiralis*: 3-4. T.S. of very young anther showing archesporial layers; 5, Portion of microsporangium showing epidermis, primary parietal layer and block of sporogenous cells; 6, Portion of microsporangium showing outer parietal and inner parietal layers; note blocks of sporogenous cells; 7, Portion of microsporangium showing division in inner parietal cells, indicated by the arrows; 8-9, Portion of microsporangium showing wall layers and microspore mother cells, note starch grains in the epidermal cells. Abbreviations: Al, Anther lobe; Ar, Archesporial cells; B Sc, Block of sporogenous cell; C Ta, Connective tapetum; En, Endothecium; Ep, Epidermis; I Pl, Inner parietal layer; Ml, Middle layer; MMC, Microspore mother cells; Pl, Primary parietal layer; P Ta, Parietal tapetum; Sg, Strach grains; St, Sporogenous tissue.



Figs.10-27. Microsporogenesis and development of pollen in *Peristylus spiralis*: 10-12, Meiosis-I in the microspore mother cells; 13-14, Simultaneous division of dyad nucleus; 15, Three microspores in a tetrahedral tetrad; 16, Rhomboidal tetrad; 17, T-shaped tetrad; 18, Linear tetrad; 19-21. Divisions of microspores in the tetrad; note proximal and distal orientation of nuclear spindles of the dividing nuclei; 22-27, Migration of parietal disposed generative cell in the pollen tetrad.



Figs. 28-35. Dehiscence of microsporangium in *Peristylus spiralis*: 28, Mature microsporangium showing tangentially disposed endothecial thickenings; note the starch grains in the epidermal cells and massulae in the sporangium; 29, Whole mount of endothecial layer; 30, Oval shaped thickening detached from the endothecial cell; 31-35, Show the formation of stomium and stages of microsporangial dehiscence. Abbreviations: En, Endothecium; Sl, Separating layer.

organization of the anther wall conforms to the monocotyledonous type (Davis, 1966). A similar method of wall development has been recorded in *Habenaria edgeworthii, H. elisabethae, H. galeandra, H. intermedia,* and *Neottia listeroides* (Sood, 1984, 1985b, 1986), *Oreorchis foliosa* (Mohana Rao and Sood, 1987), *Epipactis helleborine* and *E. veratrifolia* (Vij and Sharma, 1987). It is very likely that in others where the type of organization has not been studied so far, could also be of similar pattern. In the presently investigated species, the sporangial wall comprised four wall layers namely epidermis, endothecium, middle layer and the tapetum. Similar number of wall layers has been recorded earlier in most of the investigated taxa (Cocucci, 1964; Gurudeva, 2012; Mohana Rao and Rao, 1983, 1984; Sood, 1986; Swamy, 1949a).

Presently, the epidermis was always single-layered, its cells were generally larger in size and tangentially extended and was basically protective in function and remained persistent even at anthesis. A similar observation has been made in *Aa achalensis* (Cocucci, 1964), and *Neottia listeroides, Microstylis cylindrostachya* and *Habenaria* species (Sood, 1984, 1985a, 1986). Epidermis showed the presence of starch grains which indicated that this layer was concerned with nutrition besides its usual function of protection. Nutritive role of epidermis has also been recorded in *Zeuxine longilabris* (Karanth, *et al.,* 1979), *Epipogium roseum* (Govindappa and Karanth, 1981) and *Habenaria diphylla* (Gurudeva, 2012). As this behaviour of the epidermis of the microsporangial wall has not been recorded so far in any of the angiosperms, gymnosperms and pteridophytes studied so far, appears unique.

Presently, the endothecium was single layered. At maturity, its cells acquired thickenings on the inner surface of their walls and these thickenings were ring-like, single and tangentially disposed. This type of endothecial thickenings corresponds to Type-II of Freudenstein (1991). Similar type of tangentially disposed endothecial thickenings were earlier recorded in *Habenaria diphylla* (Gurudeva, 2012) and *Habenaria clavigera* (Sharma and Vij, 1987). Different types of endothecial thickenings in orchids have been recorded by Untawale and Bhasin (1973) and were classified by Freudenstein (1991). Further it is worthwhile to investigate the exact functional role of the different kinds of thickenings, especially in connection with the opening of the anther lobe at the time of release of massulae / pollinia release.

The middle layer consists of single row of thin-walled tangentially extended cells. During microsporogenesis, when the tapetal cells become very conspicuous and active, this layer gets gradually crushed and absorbed. Similar observation has been made in *Aphyllorchis montana, Dendrobium microbulbon, Platanthera susannae,* and *Sirhookera latifolia* (Krishna Swamy *et al.,* 2003). Persistence of middle layer and acquisition of thickenings along with the endothecium and their role assisting in opening of anther lobe has been recorded in *Arundina graminifolia* (Rao, 1967), *Bromheadia finlaysoniana* (Jayanayaghy and Rao, 1966), and *Spathoglottis plicata* (Prakash and Lee-Lee, 1973).

The inner most layer of the sporangium wall was the tapetum. Because of its dual origin, it was completely surrounded by the sporogenous tissue. It was of glandular type. Similar feature has been recorded in majority of orchids (Gurudeva, 2012; Kant *et al*. 2013; Krishna Swamy *et al*., 2003; Sood and Mohana Rao, 1986a, 1986b; Sood and Sham, 1987; Swamy, 1949a). Tapetal cells remained uninucleate throughout and it is in conformity with several orchids (Krishna Swamy *et al.*, 2003; Mohana Rao and Sood, 1987; Sood, 1985a,b; Sood and Mohana Rao, 1988). Finally the tapetal layer breaks down leaving its remnants within the confines of the locule. In addition to the nutritional role, it is generally believed that tapetal cells play a role in exine formation by secreting sporopollenin precursors, which are then polymerised during maturation of the pollen in angiosperms (Heslop-Harrison, 1971).

The archesporial cells after producing a parietal layer functioned together as sporogenous tissue. Presently, in current investigation in *Peristylus spiralis,* the sporogenous cells belonging to a massula are derived from a single archesporial cell. A similar condition has been reported in *Himantoglossum hircinum* (Heusser, 1915), *Calanthe veratrifolia, Neottia ovata,* and *Orchis maculata* (Guignard, 1982), and in several species of *Habenaria* and *Peristylus* (Swamy, 1946, 1949a). The sporogenous cells enlarge and become microspore mother cells in all the species so far studied (Blackmen and Yeung, 1983; Swamy, 1949a; Wirth and Withner, 1959) including the present investigation. The microspore mother cells underwent the usual meiotic divisions and resulted in different types of microspore tetrads. Quadri-partition of microspore mother cells is simultaneous in most of the taxa investigated so far (Coccuci, 1967; Prakash and Lee-Lee, 1973; Swamy, 1941, 1946, 1949a; Vij and Sharma, 1987) including the present study. The tetrads may be arranged in different patterns. The type of microspore tetrads was dependent on the orientation in which the walls were deposited during meiotic division. The orientation of

microspores in tetrad has been described as tetrahedral, isobilateral, rhomboidal, linear and T-shaped tetrads. The location of the type of tetrads within the massulae was variable. Usually, the per cent of linear, T-shaped, isobilateral and rhomboidal tetrads are more at the periphery than at the centre of the massula, whereas tetrahedral tetrads were more common at the centre of the massula.

The microspores were with dense cytoplasm and a large centrally located nucleus. The nuclear division within the microspore tetrad was synchronous and asymmetrical in conformity with earlier records (Hagerup, 1938; Mohana Rao and Sood, 1986; Prakash and Lee-Lee, 1973). The small newly formed generative cell was initially adpressed to the wall of the microspore, later it separated itself from the microspore wall and entered into the cytoplasm of vegetative cell. The pollen grains were 2-celled when massulae were ready for pollination in all the species as studied earlier by many workers (Gurudeva, 2012; Pace, 1909; Prakash and Lee-Lee, 1973; Sood, 1986; Swamy, 1949a).

At the time of anther dehiscence, a well-developed stomium was formed at wall cells at the junction of the two adjacent microsporangia which got disorganized leading to the formation of vertical slit in each of the two anther lobes so as to facilitate carrying of the massula.

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SEED MORPHOMETRY OF SOME INDIAN ORCHIDS WITH SPECIAL REFERENCE TO THEIR INTER-RELATIONSHIPS AND ECOLOGICAL SIGNIFICANCE

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Abstract

SEM (Scanning Electron Microscope) studies on seed morphometry of nine orchid species, such as *Acampe praemorsa* (Roxb.) Blatt. & Mc. Cann., *A. rigida* (Buch.-Ham.ex J. E.Sm.) P.F. Hunt, *Calanthe triplicata* (Willem.) Ames, *Luisia zeylanica* Lindl., *Malaxis densiflora* (A. Rich.) Ktze, *Oberonia arnottiana* Wight., *O. ensiformis* (J.E. Sm.) Lindl., *Vanda testacea* (Lindl.) Reichb. f., and *V. tessellata* (Roxb.) W. J. Hook. ex Don have been carried out. The present data deals with quantitative data related to the length and width of the seed and embryo, seed and embryo volume, percentage of air space, and number of testa cells. The seed truncation character can be used to differentiate between species in the genera such as *Acampe, Calanthe, Luisia, Malaxis, Oberonia,* and *Vanda*. These data indicate that the seeds of *Calanthe* species are more truncated than those of the other studied taxa. Seeds with higher ratio of seed volume/embryo volume (more than 2.2) especially in *C. triplicata, V. tessallata,* and *V. testacea* are expected to be more buoyant than those with a lower ratio of seed volume/embryo volume. These are widely spread out species in Western Ghats of South India. The buoyancy of seeds could be attributed to the dispersal of seeds to vast areas as well as wide distribution of the species.

Introduction

THE ORCHIDACEAE is one of the largest families of flowering plants; it comprises about 779 genera and 22,500 species (Mabberley, 2008). In India, with 1331 species spreading over 184 genera, it represents second largest flowering plant family and contributes about 10% of Indian Flora (Kumar and Manilal,1994). Orchid seeds are light in weight and the tiniest amongst the seeds produced by flowering plants and these are non-endospermic, vary considerably in their size, morphology, colour, and minute details. In majority of orchid species, seed size shows variation from 300–800 μm (Molvray and Kores, 1995). The taxonomic significance of the seed characteristics was first reported by Clifford and Smith (1969). Besides, serving as taxonomic markers, the morphological characters of seeds can be used to deduce phylogenetic relationship (Barthlott, 1976) and to identify their involvement in hybrid genotypes (Arditti *et al.,* 1979).

Seed morphology has got importance in delineation of species within the genus and also delineation of subgeneric groups (Augustine *et al.,* 2001; Larry, 1995; Mathews and Levins 1986; Ness, 1989; Pathak *et al*., 2011; Verma *et al*. 2014; Vij *et al*.,1992). Molvary and Kores (1995) also reported that the orchid seed varies in shape from filiform to fusiform, clavate to ellipsoidal and often prominently winged. Barthlott and Ziegler (1981) worked elaborately on the seed coat structure of orchids and recognized 20 different seed types by taking varying seed characteristics.

In South India, about 250 species spreading to 70 genera have been reported (Abraham and Vatsala, 1981). Except for a few detailed reports (Augustine *et al*., 2001; Pathak *et al*., 2011; Swamy *et al*., 2007; Verma *et al*. 2014; Vij *et al*., 1992), not much work has been done on seeds of Indian orchids. The present investigation deals with the Scanning Electron Microscopic (SEM) studies on seed characters of nine orchid species belonging to six orchid genera *i.e., Acampe, Calanthe, Luisia, Malaxis, Oberonia,* and *Vanda.*

Materials and Methods

Seeds of nine orchid species belonging to subfamily Epidendroideae were collected from different parts of Eastern and Western Ghats of India (Table 1). The mature capsules were freshly collected during 2010-2012. Seeds were separated from capsules and collected in petri dishes. Optical photomicroscope (Motic 2.0, 5 Megapixel) was used to measure the length and width of seeds.

The seeds of all the above species were fixed in 2.5% glutaraldehyde prepared in 0.2 M cacodylate buffer (pH 7.2) and kept in room temperature for two hours; the seed samples were then dehydrated in graded ethyl alcohol: acetone series. Subsequently, these were

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Table 1. *List of species presently investigated for Scanning Electron Microscope (SEM) studies.

Species	Place of collection and elevation	Habitat & host tree	Accession Number
Sub family - Epidendroideae			
Tribe - Malaxideae			
Malaxis densiflora (A.Ri.ch.) O. Kuntze.	Pallode (KE), 900 m	Te	ANUH 1010
Oberonia arnottiana Wight.	Paderu (AP), 910 m	Epi & *Proteum serratum*	ANUH 1011
O. ensiformis Lindl.	Paderu (AP), 910 m	Epi & *Pterocarpus morsupium*	ANUH 1012
Tribe - Arethuseae			
Subtribe - Bletiinae			
Calanthe triplicata Lindl.	Pallode (KE), 900 m	Epi & *Oozenia ozenensis*	ANUH 1017
Tribe- Vandeae			
Subtribe - Aeridinae			
Acampe praemorsa Blatt. & Mc. C.	Chintapalli (AP), 839 m	Epi & *Terminalia chebula*	ANUH 1018
A. rigida Lindl.	TBGRI, Pallode (KE), 900 m	Epi & *Albezia lebbeck*	ANUH 1019
Luisia zeylanica Lindl.	Giddalur (AP), 300 m	Epi & *Terminalia alata*	ANUH 1020
Vanda testacea (Ldl.) Reich. F.	Lothugadda (AP), 750 m	Epi & *Artocarpus heterophyllus*	ANUH 1021
V. tessellata Hk. F.	Lothugadda (AP), 750 m	Epi & *Artocorpus heterophyllus*	ANUH 1022

*Arranged according to Dressler (1993)

Epi, Epiphyte; Te, Terrestrial; AP, Andhra Pradesh; KE, Kerala; TN, Tamil Nadu; TBGRI, Tropical Botanical Garden and Research Institute.

dried in critical point dryer. After the critical drying, these samples were mounted on to copper stubs and were gold coated for five min. The processed specimens were examined and photographed on a

Table 2. Seed characters and quantitative data.

Taxa	Time of fruiting	Colour	Length (mm)	Width (mm)	L/W	Seed volume mm^3x10^{-3}	Average length of tests cells (mm)	Average width of Testa cells (mm)	Average no. of testa cells
Malaxis densiflora	Mar-Jun	White	0.3289 ± 0.0497	0.0985 ± 000983	3.33	0.0008355 0.355 mm^3x10^{-3}	37.81	13.69	11.62
Oberonia arnotiana	Sept-Oct	Yellow	0.27398 ± 0.004986	0.09012 ± 0.004733	3.03	0.0005805 0.5605 mm^3x10^{-3}	105.03	17.88	3.62
O. ensiformis .	Sep-Oct	Light yellow	0.2657 ± 0.00546	0.08009 ± 0.00434	3.31	0.,000443 0.443 mm^3x10^{-3}	107.5	20.91	3.79
Calanthe triplicata	Apr-May	White	0.9474 ± 0.1701	0.0992 ± 0.0227	9.55	0.002440 2.440 mm^3x10^{-3}	140.54	31.18	9.87
Acampe praemorsa	Mar-Jun	Light brown	0.1847 ± 0.06906	0.06906 ± 0.00345	2.67	0.0002306 (0.2306 $mm^3x10^{-3)}$	68.56	11.19	3.66
Acampe rigida	Mar-Jun	Light brown	0.2402 ± 0.003910	0.0633 ± 0.00452	3.79	0.0002520 (0.2520 $mm^3x10^{-3)}$	79.22	13.24	5.42
Luisia zeylanica	Jun-Jul	Yellow	0.2545 ± 001553	0.07445 ± 0.003838	3.39	0.00037045 (0.37045 mm^3x10^{-3}	84.52	12.29	3.1
Vanda testacea	Mar-Apr	Light yellow	0.2185 ± 0.0344	007232 ± 0.0004432	4.87	0.00029855 (0.2985 mm^3x10^{-3}	47.82	13.91	4.42
V. tessellata	Apr-May	Yellow	0.1892 ± 0.021051	0.06829 ± 0.000453	2.77	0.0002308 (0.2308 $mm^3x10^{-3)}$	69.50	11.06	4.81

HITACHI, S3000 N Model Scanning Electron Microscope in IICT (Indian Institute of Chemical Technology, Hyderabad, India). Under light microscope with micrometer, at the longest and widest axis of the seed, the width and length of seeds were measured clearly. Seeds exhibited different forms, therefore, seed volumes were calculated using the formula Vs = 2 [(Ws/2)^2 (1/2Ls) (1.047)], where Vs = seed volume, Ws/2 = half of seed width, Ls = seed length, 1.047 = ð/3 (Arditti *et al*., 1980). Orchid embryos were elliptical in cross section and therefore their volume was calculated by using the formula Ve = 4/3 LeWe^2, where Ve = embryo volume, Le = half of the embryo length, and We = half of the embryo width. Percentage of airspace was calculated by using the formula, seed volume – embryo volume/seed volume × 100. Standard deviation was also calculated for each character of seed and embryo.

Results and Discussion

SEED CHARACTERS

Seed Colour

The colour of the seeds in all investigated species was pale yellow to yellow and light brown to white.

Seed Shape

Scanning Electron Microscope (SEM) photographs showed the fine details of the *M. densiflora* seeds. Seeds were quadrilateral-shaped with blunt ends (Fig. 1A, B) with an ellipsoidal embryo (Fig. 1A). The seeds were with opening at the base, *i.e*., at the chalazal or suspensor end (Fig. 1B). The seeds of *O. arnottiana* were short and spindle shaped with blunt ends (Fig. 1 E, F, G, H). In *O. ensiformis* also, seeds were spindle shaped but with a bulged central part having ellipsoidal embryo (Fig. 2A, B). The seeds were with openings at chalazal end (Figs. 1 G H; 2 C, D). In *C. triplicata,* seeds were filamentous shaped with visible embryo located in the centre (Fig 2 I, J, K). The SEM studies revealed that the seeds of *A. praemorsa were* ovoid (Fig. 2E, F, G) or spathulate; whereas in *A. rigida,* these are fusiform with slight curvature. All seeds were with an ellipsoidal embryo with blunt ends (Fig. 2F). The seeds of *V. tessellata* and *V. testacea* were spindle shaped or oblong (Figs. 2 O, P, Q). Testa cells were elongated and longitudinally oriented in *V. tessellata*; in case of *V. testacea,* testa cells were spirally arranged, giving a characteristic rope-like appearance to seeds (Fig. 2 O, P, Q, R).

Length/Width (L/W) Ratio of Seed

Length/width ratio of seeds gives some interesting information on relative degree of truncation of orchid seeds (Arditi *et al*., 1980; Augustine *et al*., 2001). The maximum L/W ratio was observed in *Calanthe triplicata* (9.55) whereas minimum L/W ratio was observed in *A. praemorsa* (2.67). The L/W ratio in other investigated taxa was, 4.87 in *V. testaceae,* 3.79 in *A. rigida*, 3.39 in *L. zeylanica,* 3.31 in *O. ensiformis,* and 2.77 in *V. testellata.* The present data is in agreement with studies of Vij *et al*. (1992) and Swamy *et al.* (2004). The seed truncation character can be used to differentiate between species in the genera such as *Acampe, Calanthe, Luisia, Malaxis, Oberonia,* and *Vanda*. The present data indicate that the seeds of *Calanthe* species are more truncated than those of the other studied taxa.

Seed Volume

In the present study, seed volume ranged from 0.2306 mm^3 × 10^{-3} to 2.44 mm^3 × 10^{-3} (Table 2). The highest seed volume was observed in *Calanthe triplicata* (2.44 mm^3 × 10^{-3}) followed by *M. densiflora, O. arnottiana, O. ensiformis*, followed by *L. zeylanica*. In *V. testaceae* and *V. tessellata,* the seeds were of lesser volume and small sized. In the species of *Bulbophyllum* and *Cymbidium,* the higher seed volume is the result of long width to some extent than length of testa (Augustine *et al.,* 2001; Swamy *et al.,* 2004). Healey *et al.,* (1980) and Augustine *et al*. (2001) were justified in selectively using seed morphometry to find out phylogenetic relationship in orchids.

Average Number of Testa Cells

The average number of testa cells in the long axis of the seeds was 11.2 in *Malaxis densiflora* followed by *A. rigida, C. triplicata, L. zeylanica, O. ensiformis, O. arnottiana, V. tessellata,* and *V. testacea* (Table 2). The least number of testa cells were found in *L. zeylanica i.e.,* 3.1.

The longest testa cell was observed in *C. triplicata* (140.54 μm) and the testa cell with greatest width was also observed in *C. triplicata* (31.18 μm). The testa cells of smallest width were found in *V. tessellata* (11.06 μm).

Vij *et al.* (1992) categorized the orchid seed into three types based on the length of testa cells; those that are greater than 200 μm were categorized as long ones, less than 200 μm to 100 μm as intermediate, and below 100 μm as short ones. The presently investigated taxa namely *O. ensiformis, O. arnottiana,* and *C. triplicata* are the group with intermediate cells and other studied species are the group with short testa cells because of short length of their testa cells.

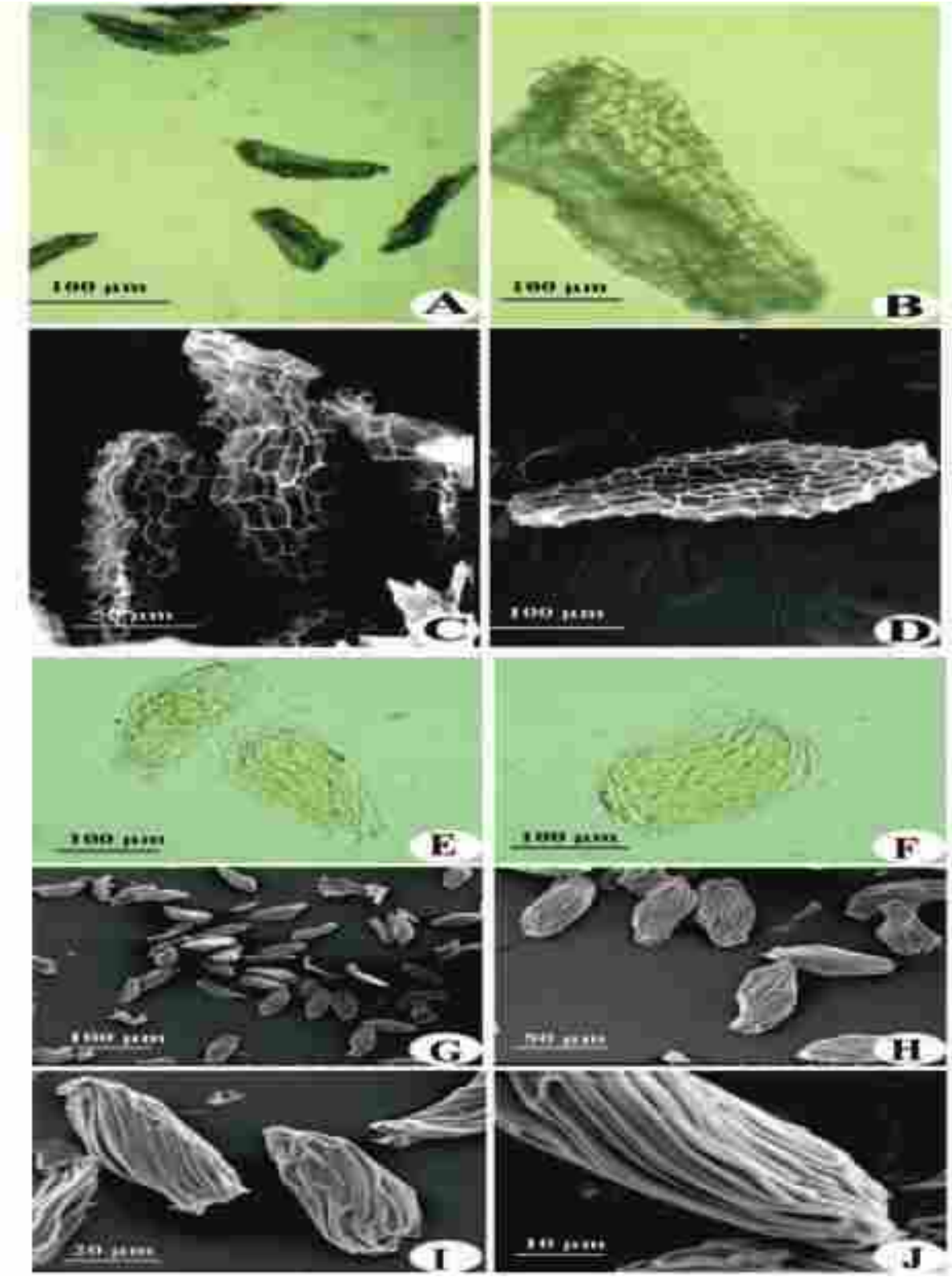

Fig. 1. Light microscopic and scanning electron microscopic (SEM) photographs of *Malaxis densiflora*. (A-D) and *Oberonia arnottiana* (E-J): A, Few seeds with embryo under the light microscope; B, Transparent seed under the light microscope; C, A few seeds under SEM; D, A seed under SEM; E, Few seeds under the light microscope with embryos; F, Seed under the light microscope with embryo; G, Few seeds under SEM; H, Enlarged view of seeds under SEM; I, Enlarged view of seed with high magnification under SEM; J, Part of the testa under high magnification of SEM.

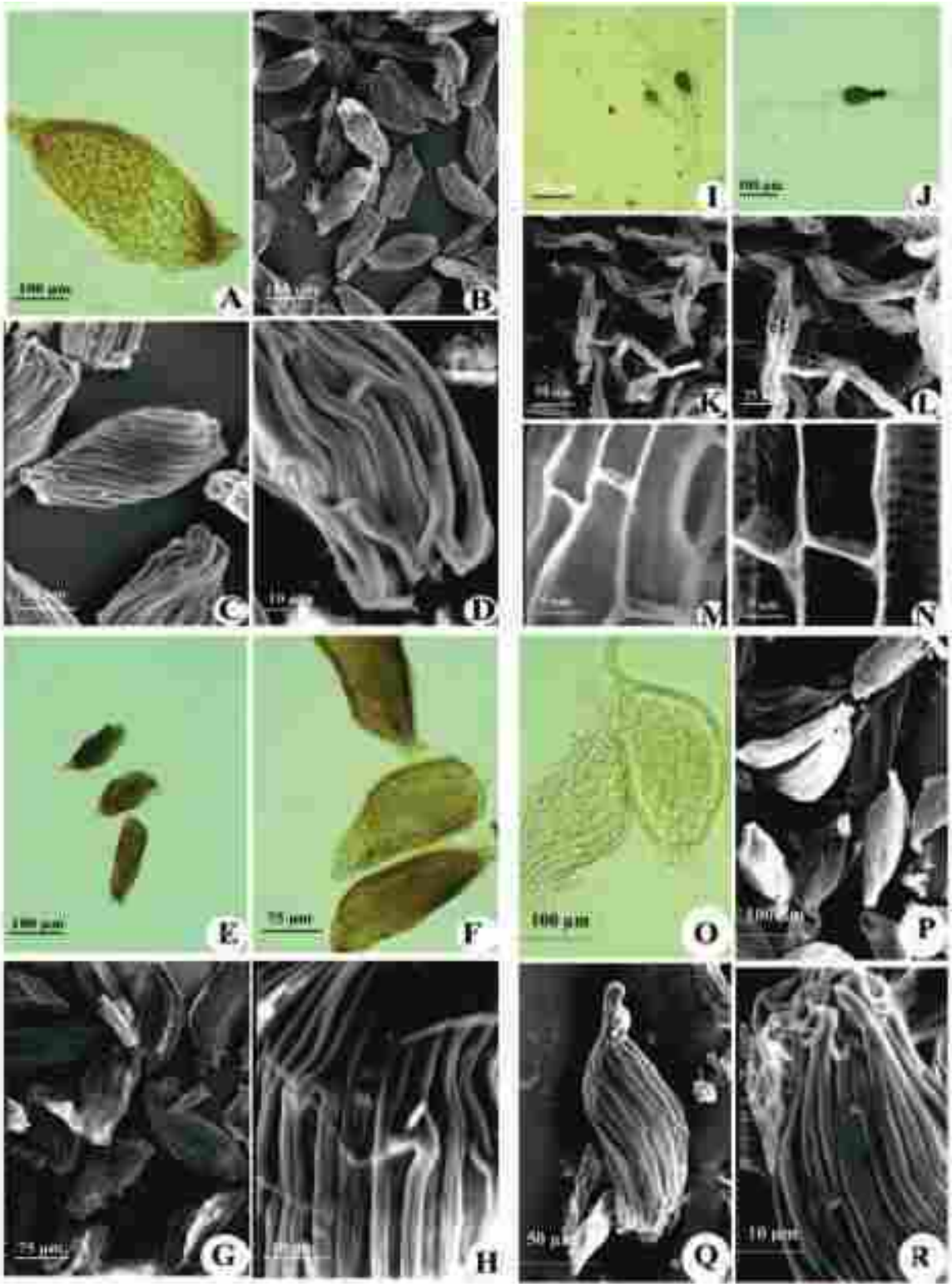

Fig. 2. Light microscopic and SEM photographs of *Oberonia ensiformis* (A-D), *Acampe praemorsa* (E-H*), Calanthe triplicata* (I-N*), and Vanda testacea* (O-R): A, A seed under the light microscope with embryo; B, A few seeds under SEM; C, Enlarged view of seeds under SEM; D, Part of the testa under SEM; E, Few seeds with embryo under the light microscope; F, Seeds under the high power of light microscope; G, Seeds under SEM; H, Testa cells; I, Seeds under the light microscope; J, Enlarged view of a seed with embryo under light microscope; K, A few seeds under SEM; L, A few seeds under SEM; M, Testa cells with transverse walls under high magnification of SEM; N, Testa cells with pores, transverse walls under high magnification of SEM; O, Seeds with embryos under the light microscope; P, Few seeds under SEM; Q, Seed under SEM; R, Chalazal pore of seed under SEM.

Table 3. Embryo characters and quantitative data.

Taxa	Colour	Length (mm)	Width (mm)	L/W	Embryo Volume mm^3x10^{-3}	Seed volume to embryo volume	Airspace (%)
Malaxis densiflora	White	0.1621 ± 0.002952	0.0628 ± 0.02501	2.58	0.0003339 (0.3339 mm^3x10^{-3})	2.50	60.50
Oberonia arnottiana	Yellow	0.09975 ± 0.00769	0.00937	1.20	0.00003505 (0.03505 mm^3x10^{-3})	1.65	39.62
O. ensiformis	Light yellow	0.09689 ± 0.01744	0.07394 ± 0.007629	1.31	0.0002756 (0.2756 mm^3x10^{-3})	1.60	38.19
Calanthe triplicata	White	0.1413 ± 0.0591	0.07513 ± 0.0251	1.88	0.00041661 (0.4166 mm^3x10^{-3})	5.85	42.92
Acampe praemorsa	Light brown	0.1073 ± 0.00295	0.0515 ± 0.00654	2.08	0.0001486 (01486 mm^3x10^{-3})	1.55	35.53
A. rigida	Light brown	0.1703 ± 0.02150	0.04215 ± 0.002150	4.09	0.0001579 (0.1579 mm^3x10^{-3})	1.59	37.34
Luisia zeylanica	Yellow	0.1212 ± 0.01217	0.05333 ± 0.00321	2.27	0.0001791 (0.179 mm^3x10^{-3})	2.06	51.63
Vanda testacea	Light yellow	0.1250 ± 0.0150	0.0452 ± 0.00264	2.76	0.0001334 (0.1334 mm^3x10^{-3})	2.23	55.31
V. tessellata	Yellow	0.1452 ± 0.001829	0.0340 ± 0.01252	4.26	0.000087734 (008773 mm^3x10^{-3})	2.63	62.00

EMBRYO CHARACTERS

The colour of the embryo in the presently investigated taxa varied from, light yellow to yellow and white to brown. In seeds, the embryos generally occupied a very small portion. According to Augusteine *et al.,* (2001), orchid embryos occupied small portion in seeds but in *Bulbophyllum* embryos, it occupied a large portion in the seed and the maximum embryo length and width was observed in *A. rigida* is (0.1703).

In this present investigation, maximum L/W ratio was found in *V. tessellata* (4.26) followed by *A. rigida* (4.09) and minimum in *O. arnottiana* (1.20). L/W ratio of embryos in *A. praemorsa, L. zeylanica, M. densiflora* and *V. testacea r*anged from 2.08 to 2.76 (Table. 3). According to Healey *et al*. (1980), the volume of embryo differs from genus to genus. In the present investigation, highest seed volume was observed in *C. triplicata* (0.4166$mm^3 \times 10^{-3}$) and minimum in *V. tessellata* (0.08773 mm^3x10^{-3}) (Table. 3).

Seed Volume to Embryo Volume (Vs/Ve) Ratio

Some observations of seed volume to embryo volume ratio are very interesting in present investigation. This value was maximum in *C. triplicata* (5.85) followed by *V. tessellata, M. densiflora,* and *V. testacea.* (Table 3). According to Arditti *et al.* (1980), the species showing greater variation in seed and embryo volumes and percentage of air space could survive amongst their different populations.. Young seeds have small undifferentiated embryos where as the mature seeds from the dehisced capsules have embryos of a larger volume.

Air Space

In the present investigation, the seeds with maximum percentage of airspace were noticed in *C. triplicata* (82.92%) followed by *V. tessellata, M. densiflora, V. testacea* and *L. zeylanica.* (Table 3). These orchids with more airspace are said to be widely distributed in Eastern and Western Ghats whereas *A. praemorsa, O. ensiformis,* and *O. arnottiana* with low air space are restricted and endemic to Southern India.

From the above data, direct correlation has been drawn between the seed/embryo volume ratio, the percentage of air space and the buoyancy of the seeds. The seeds having larger Vs/Ve ratio are expected to be more lighter than those with smaller ratio (Arditti *et al*., 1979; Garg *et al.,* 1992). The buoyancy of seeds could be attributed to the distribution of seeds to vast areas.

Ecological Significance

Seeds with higher ratio of seed volume/embryo volume (more than 2.2) especially in *C. triplicata, V. tessellata,* and *V. testacea* are expected to be more buoyant than those with a lower ratio of seed volume/embryo volume. These are widely spread out species in Western Ghats of South India. Higher percentage of

airspace was also noticed in these orchid taxa. In general, the dust- like minute seeds are suitable for long distance dispersal by wind. Many scientists [Clifford and Smith (1969); Pathak *et al*., (2011); Rasmussen (1995); Swamy *et al.,* (2004); Verma *et* al., (2014; Vij *et al.,* (1992)] opined that the seed size also has direct correlation with plant habit (epiphytes with smaller seeds than the terrestrials). In the other studied orchid taxa such as *Acampe rigida, A. praemorsa, O. arnottiana, O. ensiformis*, its value was less than three and air space was also reduced indicating thereby that their distribution is restricted (localized) to Western and Eastern Ghats of Southern India.

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EVOLUTION AND CONTRIBUTION OF MADS-BOX GENES IN RELATION TO FLORAL DIVERSITY IN ORCHIDS

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Abstract

Orchid flowers with myriad shapes, sizes and colors have always fascinated the scientists, growers, entrepreneurs, and common men. This peculiar floral organ identity control is attributed to MADS-box genes which form the foundation of the much accepted ABCDE model of orchid floral organ identity specification. In angiospermic ABC model, the transcriptional factors encoded by Class-B floral homeotic genes, encode petal and stamen identity of flowers. These have two ancient clades: *DEF (DEFICIENS)*-like and *GLO (GLOBOSA)*-like genes with one copy each. However, in orchids four copies of *DEF*-like floral homeotic genes are present as a result of two rounds of duplication, and subsequent sub-functionalisation followed by neo-functionalisation (ancient gene Clade 1, 2, 3 and 4). A combinatorial differential expression of genes belonging to these clades is responsible for formation of specialised labellum (inner median perianth) and petaloid whorls (outer tepals and lateral inner tepals). Multiple studies have time and again refined and given inputs to resolve the complexities in orchid floral morphogenetic networks. Orchid Code remains a tested, validated and till date, widely accepted model that determines orchid perianth organ identity and puts light on evolutionary track of lip-development in orchids. However, still much needs to be learnt in the coming years to trace the underlying molecular and genetic mechanisms controlling the floral development in orchids.

Introduction

ORCHIDS HAVE always been tremendously fascinating, to some extent mysterious, to scientists, growers, entrepreneurs, and common men. These were once referred to as *gorgeous floral parasites* blazing on tree tops (Fitz-Gerald, 1906). Orchidaceae is one of the largest angiosperm family in terms of number of accepted species which counts more than 25,000, distributed in around 880 genera (Cameron *et al.*, 1999; Chase *et al.*, 2003; Gorniak *et al.*, 2010; Hsu *et al.*, 2015; Mondragón-Palomino and Theißen, 2011; Roberts and Dixon, 2008), representing approximately 10 per cent of angiosperms. Owing to tremendous diversity, distribution and specialised set of floral traits, that include zygomorphy, presence of perianth organs *i.e.*, three outer tepals (petal-like, ornamental, brightly coloured sepals to attract insects), two inner lateral tepals (petals) and one highly modified inner median tepal (labellum, main attracting organ) (Gravendeel and Dirks-Mulder, 2015), this plant family has managed to capture the imagination of people worldwide, for hundreds of years (Albert and Carretero-Paulet, 2015; Rudall and Bateman, 2002).

Owing to multiple architectural innovations in flowers, orchids are often considered to be epitome of plant evolution. In a typical orchid flower, stamens and pistil are partly/completely fused to form gynostegium, stamens are on abaxial side, pollen grains are coherently present in masses as pollinia, labellum is present opposite to fertile stamens, flowers are resupinate, and thousands of small seeds are produced per ovary (Roberts and Dixon, 2008). These features provide special advantage to these fascinating flowers to form unusual relationships with pollinators and achieve reproductive assurance. Extreme novelties/ synapomorphies have been associated with orchid floral forms, some of which include bilateral symmetric flowers due to enormous modifications in perianth, formation of gynostegium by fusion of pistil and stamens, and resupination of pedicel (Rudall and Bateman, 2002). The existence and appearance of labellum is thought to be a novel device to attract pollinators. In addition, frequent variations in colour, texture, ridges, outgrowths, blotches, wax and nectar glands, fragrance and position of stamens and stigma are leading to newer and specialized pollinator interactions by constant coupling and de-coupling processes within pollination networks (Madan *et al.*, 2013). Due to massive investment in floral display, orchids have been successful in attracting pollinators from diverse guild, including beetles, butterflies, ants, wasps, moths, bees, flies, geckos as well as birds. For sustenance in these networks and for ensuring effective pollination, diverse ecological adaptations have been adopted by orchids that include food and sexual deceit, mutualism, exclusive pollination guilds, and association with non-rewarding magnet species (Roberts and Dixon, 2008).

Orchid Evolution

After the dominance of angiosperms on earth, Late Cretaceous (76-84 mya, Million years ago) marks the evolution of orchids and their radiation occurred 33-

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57 mya which was in congruence with that of the insects (Ramirez *et al.*, 2007), rendering these as one of the most advanced of the families among angiosperms, consisting of 5 sub-families i.e., Apostasioideae (most basal), Vanillioideae, Cypripedioideae, Orchidoideae and Epidendroideae. Interestingly, it is an amazingly remarkable fact that no orchid can persist without its associate pollinators and mycorrhizal fungi. Vivid and diverse mechanisms of mimicking and temporarily trapping pollinators, epiphytism, succulent and leafless body-plan (Albert and Carretero-Paulet, 2015) are some highly sophisticated floral organizational features that open the doors to discovery of newer morphogenetic networks because of exceptionally high speciation rates in orchids (Gill, 1989). Unfortunately, the unusual set of interspecific interactions with mycorrhizal fungi as well as pollinators, exceptionally unconventional nuclear genome, are some traits that are yet to be ascertained holistically (Albert and Carretero-Paulet, 2015; Bronstein *et al.*, 2014).

It is well-established by now that Orchidaceae display unparalleled diversity in terms of floral, vegetative and physiological adaptations and is therefore, of tremendous horticultural importance (Albert and Carretero-Paulet, 2015). The specialised floral perianth acts as a selection advantage, especially to rewardless orchids that constitute one-third of the family and are scattered throughout its many unrelated clades. Exemption of rewards for pollinators may turn into a disadvantage as gradually the pollinator may learn to avoid such flowers. But orchids still continue to evolve and tempt and dupe the pollinators. Mechanisms behind evolution of such extra-ordinary strategies are hidden in the floral genetic code (Gravendeel and Dirks-Mulder, 2015). Also, it becomes imperative to decode such evolutionary mysteries because evolution is trending towards increased orchid specialisation by reduction in number of pollinator species per orchid species, thus making orchids more dependent on their corresponding pollinators and not the vice-versa (Roberts and Dixon, 2008).

The Orchid Code: Genetic Basis of Floral Structure

ABCDE model of floral organ identity in orchids is an extension of ABC model that is applicable to rest of the angiosperms. According to the latter, A class of genes alone code for the formation of sepals, Class A and B together code for petals, Class B and C for stamens, while Class C alone codes for carpels. Class A and C are mutually repressive functionally (Theissen *et al.*, 2000). Later, ABCD model was put forward, based on studies on *Petunia*, wherein Class D specified ovule development (Angenent and Colombo, 1996). Class B floral organ identity genes that specify petal and stamen identity encode MADS domain transcription factors (Mondragon-Palomino and Theißen, 2011). Two ancestral clades of these B Class genes include *DEFICIENS*-like (*DEF*) and *GLOBOSA*-like (*GLO*) genes, single copies of which are present in angiosperms, as revealed in *Antirrhinum majus* and *Arabidopsis thaliana* (Zahn *et al.*, 2005). However in case of orchids, *DEF*-like genes underwent two rounds of duplication to produce four gene copies whose functions later diverged to produce various specialized tepals in orchids. This led to emergence the ABCDE model of orchid perianth identity also well known as the Orchid Code which is rigorously tested and can be applied to most orchids (Mondragion-Palomino and Theißen, 2007, 2008, 2009). According to this code, Class A and E MADS-box proteins specify sepals, and Class A, B and E control petals, stamens are controlled by Class B, C and E gene activity, carpels by Class C and E, and ovules by Class C, D and E gene expression. And the quartet model says that a unique combinatorial result of activation and silencing of genes coding four *DEF*-like MADS-box proteins specify the fate of each whorl (Gravendeel and Dirks-Mulder, 2015; Mondragon-Palomino and Theißen, 2007). These combinatorial protein-protein interactions form multimeric regulatory complexes which specifically recognize cis-regulatory elements of target genes. This further stimulates or represses these target genes to form a specific organ. More recently, Su *et al.* (2013) reported modified molecular model of flower development based on functional analysis of gene expression profiles in *Phalaenopsis aphrodite* and identified floral organ specific genes and reported that Classes A and B in this species have novel functions due to evolutionary diversification and display differential expression patterns.

Infact , the body plan of orchid flower is decided in a founder cell where the combinatorial activity of homeotic selection genes is initiated. These encode MIKC-type MADS-box domain proteins to specify and dictate the expression of all the genes encoding the proteins required for identity, formation and development of each floral organ. Once these proteins are expressed, their differential combination forms distinct whorls as outer tepals, inner lateral tepals and inner median labellum (Mondragon-Palomino and Theißen, 2007; 2011). B Class MADS domain proteins underwent first round of duplication to produce a lineage of two sister clades encoding DEF-like proteins and GLO-like proteins (Kramer *et al.*, 1998), whose representatives are APETALLA (AP3) and PISTILLATA

Fig. 1. Evolution of B-Class *DEF*-like MADS-box genes by duplication and functional diversification giving rise to four clades specifying unique perianth of orchids (Based on Mondragón-Palomino and Theißen, 2007).

(PI), respectively. These undergo obligate heterodimerisation for petal development. Resultant four orchid clades are the drivers to regulate their own expression and that of downstream target genes. These genes later complex with Class A and E proteins to give rise to perianth initials. Outer tepals are determined by heterotropic expression of Clade 1 and 2 genes. While, Clade 1, 2 and 3 are responsible for formation of lateral inner tepals and expression of clade 1, 2 and 4 forms labellum. In short, Clade 1 and 2 are expressed in all the tepals and are considered to be responsible for petalloid nature of the tepals. Identity to inner tepal is provided by Clade 3 and that of labellum is designed by Clade 4. This combinatorial expression is the essence of beautiful orchid floral architecture (Mondragon-Palomino and Theißen, 2007) (Fig. 1).

Molecular foundation of orchid lip development lies in MADS-box gene family. According to Wagner (2008), during the entire course of evolution, gene duplications substantially facilitate evolutionary innovations and novelties in plant structures by enhancing the chances of mutational robustness. Therefore, orchids offer multiple avenues in molecular developmental and physiological research (Albert and Carretero-Paulet, 2015).

Evolution of the Code

From an evolutionary perspective, the most basal orchid sub-family Apostasoideae is considered least diverse, without a pronounced lip. Dating studies revealed that diversification and speciation of orchids was triggered by lip evolution, due to duplication of deeply rooted *DEF*-like MADS-box genes around 60-70 mya and subsequent speciation by adoption of new diverse functions by the newly formed gene copies or orthologs (Chang *et al.*, 2010; Gravendeel and Dirks-Mulder, 2015; Hsu and Yang, 2002; Kim *et al.*, 2007; Mondragon-Palomino and Theißen, 2007; Tsai *et al.*, 2004, 2005; Xu *et al.*, 2006). However, *GLO*-like gene exists as a conserved single copy, not contributing to tepal distinction (Kim *et al.*, 2007; Ramirez *et al.*, 2007).

The striking floral morphological novelties in orchids are a result of lineage specific expansions and contractions in MADS-box gene subfamilies that underlie protein functional diversification and generate unique regulatory interaction networks (Albert and Carretero-Paulet, 2015).

Modularization of orchid perianths forming dramatically different floral structure, leading to floral diversification, is the basis of evolutionary developmental biology (evo-devo) of orchids. This makes orchids a well suited system to link evolutionary and phylogenetic development with morphological speciation (Lu *et al.*, 2007). ABCDE model persists in scientific community as the most dominant concept determining bipartite perianth and labellum in orchids.

Evolutionarily, the genes belonging to Clade 1 and 2 follow the ancestral pattern of gene expression because these are expressed in all the perianth organs, while those belonging to Clade 3 and 4 are the derived states. In details, these four clades are two pairs of sister clades where the paralogs, even after duplication, retained their regulatory elements and are still controlled by similar upstream factors (Mondragon-Palomino and Theißen , 2011). On expression in their separate domains, these genes respond independently to natural selection, leading to evolutionary divergence of initially identical structures (Mondragon-Palomino and Theißen, 2007). Orchids have pushed the limits of evolution in a number of ways; therefore, an understanding of these limits may reveal the factors behind the key innovations exclusive to orchids. Certainly, with advent of evo-devo molecular approach, the complexities of floral development in orchids have been simplified to a greater extent (Aceto and Gaudio, 2011).

Conclusion

This highly specialized code bridges the gaps between the orchid diversification and phylogenetic basis. It would provide a rational framework not only in understanding the evolution and function of floral ontogeny genes, but it also gives identity to the appearance and diversification of such enigmatic floral innovations and evolution of arms-race concept (Mondragon-Palomino and Theißen, 2007). This code has managed to resolve uncertainties regarding evolutionary ancestries of orchid floral architecture (Tsai *et al.*, 2014). Time and again revisions, refinements and inputs have been added to the basic Orchid Code, but its applicability is widely tested in diverse genera across the five sub-families and is accepted validly. Mondragon-Palomino and Theißen (2011) performed expression based experiments to establish that organ identity is not defined as a simple ON and OFF pattern of *DEF*-like genes, instead it is due to the distinct mRNA levels from combinatorial expression of each of the four copies of *DEF*-like genes. Similarly, Hsu *et al.* (2015) comprehensively linked petal identity to gene expression and explained the concept of 'Perianth Code' in highly specialized orchids, owing to the tissue specific expression of *AP3* (B Class) and *AGL6* (E Class) that are duplicated MADS-box gene

copies. According to Perianth Code, there exists a competition MADS-box gene protein L-complex, to promote lip expression and SP-complex, to promote petal expression. These competitive/antagonistic protein interactions have been validated by FRET analysis and also using virus-induced gene-silencing. Down-regulation of *OAGL6* gene in L-complex resulted in conversion of lips to petal-like structures in *Onicidium* and *Phalaenopsis* orchid mutants (Gravendeel and Dirks-Mulder, 2015). Such detailed and comprehensive studies to elucidate floral developmental steps are much needed in the coming years.

Future Prospects

Detailed studies validate the fact that this unusual plant family can serve as a well-tested and accepted model system and will be successful to address radical questions, especially those related to interspecific interactions, physiological adaptations and evolutionary ecology, including evolutionary arms race with pollinators (Albert and Carretero-Paulet, 2015; Gravendeel and Dirks-Mulder, 2015; Mondragon-Palomino and Theißen, 2007). Molecular and genome based studies have provided ample evidence regarding this exceptionally different model for perianth formation and is still a potentially alluring area of future researches (Bronstein *et al.,* 2014). A renewed interest has been developed amongst the researchers associated with studying evo-devo studies based on floral ontogeny. Exposing the intricacies at different levels of orchid floral development may help in a better understanding of the process of natural selection that played an important role in radiation of orchids. Expression based studies are still a nascent area which needs to be taken up to sort out the mysteries behind floral innovations and associated biological diversity (Mondragon-Palomino and Theißen, 2007). During the past decade, there is an increasing focus observed towards efforts in ascertaining and resolving the evolutionary mysteries and ecological novelties in orchids using rigorous and advanced phylogenetic methods and molecular techniques (Mondragon-Palomino, 2013; Tsai *et al.*, 2014).

Highly specialized adaptations have acted as boon, as well as proved to be a bane, towards a clearer understanding of molecular basis of orchid floral ontogeny. Because orchids have long life-cycle, large genome size, and inefficient transformation system, extensive studies on classes A, C, D and E are still in infancy. Development of an exclusive and specialized gynostegial structure and ovule development provide tremendous opportunities to address the evolutionary queries because these form the potential areas accessible to many researchers and may lead to new discoveries of genetic variants in terms of floral architecture (Hsiao *et al.*, 2011). Sequence data and other genomics tools (transformation and virus-induced gene silencing) may lead to a better understanding of more promising areas such as that of reverse genetics (Lu *et al.*, 2007). However, still much needs to be learnt to trace the underlying molecular and genetic mechanisms controlling the floral development in orchids as also indicated earlier by Albert and Carretero-Paulet (2015).

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REGENERATION OF *EULOPHIA DABIA* THROUGH RHIZOME EXPLANTS AND FLOWERING: A STUDY *IN VITRO*

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Abstract

Eulophia dabia D. Don (Hochr.) is a rhizomatous ground growing orchid; its seeds were collected from Mullanpur near Chandigarh and germinated *in vitro* using three different nutrient media (PDA, M, and MS). The seedlings thus obtained after 32 wks of culturing were used as the source for the rhizomatous explants (*ca.* 5-7mm long). The efficacy of these explants was assessed on M (Mitra *et al.,* 1976) medium and its combinations with various growth regulators for regeneration *in vitro*. The basal medium supported shoot bud initiation in 55% cultures in 5.75 ± 0.50 wks and the shoots with 2-3 leaves and roots were formed. BAP at 0.5 mgl^{-1} and 1mgl^{-1} favored regeneration via shoot bud formation; the former combination proved useful for inducing rooting in these while the latter combination proved inhibitory to rooting. NAA at 0.5mgl^{-1} also induced regeneration via shoot bud formation within 6.25 ± 0.50 wks whereas its increased concentration (1mgl^{-1}), however, proved inhibitory. BAP (1mgl^{-1}) in combination with NAA (0.5mgl^{-1}) proved the best and plantlets with 2-3 leaves and 3-4 roots were obtained in 4 wks. TDZ in the medium invariably induced multiple shoot buds formation; its concentration at 0.1 and 1mgl^{-1} also induced regeneration via PLBs formation. *In vitro* flowering was induced in combination containing NAA (0.5mgl^{-1}) and TDZ (0.5, 1mgl^{-1}).

Introduction

EULOPHIA DABIA (D. Don) Hochr. (=*E. campestris* Lindl.) is an Indian orchid species met within an altitude of 300-360 m. It dwells on sandy soils near and along the water embankments and its distribution extends from the plains of North India, southward to Deccan and Eastwards to Sikkim and Bengal. *E. dabia* tubers yield *Salep* which is useful as a tonic and aphrodisiac. The tubers are extensively collected for their rejuvenating and curative properties and are used in Ayurvedic formulations as appetizer, tonic, aphrodisiac and blood purifier and to cure stomachache (cf. Pathak *et al*., 2010). Chauhan (1990) also indicated its use in curing purulent cough and paralytic strokes. Consequently, its natural populations are succumbing to commercial collection pressures. The situation is further compounded by the destruction of its natural habitats due to rapid urbanization. The present paper reports the *in vitro* regeneration potential of rhizome explants and the aim has been to develop a reproducible micropropagation system for the species.

Materials and Methods

Seed Germination and Rhizome Explant Preparation

Mature seeds from dehisced capsules (*pods*) of *Eulophia dabia* (D. Don) Hochr. were collected on sterilized filter paper and surface sterilized with 30%(v/v) Sodium hypochlorite (0.7%) solution with Teepol as the wetting agent for 30 min and then thoroughly and repeatedly rinsed with sterilized distilled water. Sterilized seeds were sown on PDA (Potato Dextrose Agar), M (Mitra *et al*., 1976) and MS (Murashige and Skoog, 1962) media with 20 gl^{-1} sucrose and 9gl^{-1} agar in test tubes, each containing 25ml of medium. AC (Activated charcoal) at 0.2% was also used in some of the experiments. Rhizome segments (5-7mm) procured from 32 wks-old axenic seedlings, were inoculated on agar-gelled basal M medium and its various combinations with BAP (0.5, 1mgl^{-1}), NAA (0. 5, 1mgl^{-1}) and TDZ (0.1, 0.5, 1mgl^{-1}) at different concentrations.

Culture Media and Culture Conditions

The pH of nutrient media was adjusted to 5.6 prior to autoclaving at 121 ºC at 1kg cm^{-2} for 20 min. The cultures were maintained under a 12-hr photoperiod of 30 μmol m^{-2} s^{-1} light intensity and a temperature of 25 ± 2ºC, and observed regularly. The problem of phenolic exudates was overcome by frequent subculturing on fresh nutrient media.

Statistical Analysis

All the experimental manipulations were carried out under aseptic conditions and for each experiment at least 4 replicates were used and experiments were repeated thrice. The data was analyzed statistically using one-way analysis of variance (ANOVA), and the

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data means ± SE of at least three different experiments were represented and compared using Duncan's multiple range test with the level of significance set at 5%.

Results

In the basal medium, the explants (55.00 ± 5.78%) regenerated via shoot buds at the nodal region within 5.75 ± 0.50 wks (Fig.1). These differentiated 2-3 leaves in 10 wks and 1st root within 11wks (Fig.2) respectively. The roots became tuberous in 14 wks (Fig.3) and healthy plantlets were obtained in 16 wks. The regeneration response varied with the chemical stimulus in the medium (Table 1; Figs. 1-14). BAP at 0.5 mgl^{-1} and 1mgl^{-1} favoured regeneration via shoot bud formation; the former combination proved useful for inducing rooting in these while the latter combination proved inhibitory to rooting. NAA at 0.5mgl^{-1} also induced regeneration via shoot bud formation within a 6.25 ± 0.50 wks whereas its increased concentration (1mgl^{-1}), however, proved inhibitory.

BAP (0.5mgl^{-1}) in combination with NAA (1mgl^{-1}) promoted either cell proliferations at cut ends or shoot bud formation. The callus was brief, creamish-brown and non-organogenetic (Fig.4); callusing was probably due to position effect of the explants on the donor tissue. In the combination containing BAP (1mgl^{-1}) and NAA (0.5mgl^{-1}), shoot buds (Fig.5) followed accelerated development into plantlets (Fig.6). Plantlets complete with 2-3 leaves and 3-4 roots were obtained in 4 wks and these flowered after 7 months (Fig.7). TDZ in the medium invariably induced multiple shoot buds formation (Figs. 8 and 9); its concentration at 0.1 and 1mgl^{-1} induced regeneration via PLBs formation (Fig.10) whereas at 0.5 mgl^{-1} it induced formation of non-organogenetic callus in some cultures (Fig.13). The PLBs soon differentiated into leafy shoots but root development invariably eluded in combination with TDZ at 0.5 and 1 mgl^{-1}. Incidentally, these shoots on their transfer to medium containing activated charcoal (0.1%), developed the roots and subsequently healthy plantlets (Fig.11). *In vitro* flowering was induced in combination containing TDZ (0.5, 1mgl^{-1}) within 1 year (Figs.12,14).

Discussion

Pseudobulbs and other storage organs like rhizomes and tubers are frequently used to propagate orchids *in vivo*, but the technique, often referred to as backbulb culture technique, is a time consuming preposition; it generates only a limited number of propagules and that too only during a favourable season. However, utility of such perennating structures as donor organs for micropropagating orchids is being increasingly realized. Presently, the rhizomes segments were successfully utilized for regenerating *Eulophia dabia* in accord with their similar utility in a number of orchid species (Bapat and Narayanaswamy, 1977; Bhadra and Hossain, 2003; Gayathery and Taha, 2003; Lee *et al.*, 2011; Lu *et al.*, 2001; Martin, 2003; Niimi *et al.*, 1993; Paek and Kozai, 1998; Paek and Yeung, 1991; Sheelavantmath *et al.*, 2000; Shimasaki and Uemoto, 1990; Takahashi and Kondo, 1998; Vij *et al.*, 1989; Yuki and Okubo, 2006). The regeneration response and developmental pathway was, however, markedly influenced by the chemical stimulus. In an earlier study on *E. hormusjii*

Table 1. *In vitro* regeneration through rhizome explants and flowering of *Eulophia dabia* on M (Mitra *et al*, 1976) medium and its combinations with various growth regulators.

Additives	Response (%)	Regeneration response				*In vitro* flowering	Time taken for onset of regeneration (wks)
		PLBs	Shoot Buds	Root	Callus		
-	55.00 ± 5.78^{b}	-	+	+	-	-	5.75 ± 0.50^{c}
$BAP_{(0.5)}$	45.00 ± 5.77^{a}	-	+	+	-	-	5.75 ± 0.50^{c}
$BAP_{(1.0)}$	68.75 ± 12.50^{c}	-	+	-	-	-	4.25 ± 0.50^{b}
$NAA_{(0.5)}$	47.50 ± 5.00^{b}	-	+	+	-	-	6.25 ± 0.50^{c}
$NAA_{(1.0)}$	-	-	-	-	-	-	-
$BAP_{(1.0)} + NAA_{(0.5)}$	96.25 ± 4.79^{d}	-	+	+	-	+	2.50 ± 0.58^{a}
$BAP_{(0.5)} + NAA_{(1.0)}$	93.75 ± 4.79^{d}	-	+	+	+	-	4.25 ± 0.50^{b}
$TDZ_{(0.1)}$	97.50 ± 2.89^{d}	+	+	+	-	-	2.25 ± 0.50^{a}
$TDZ_{(0.5)}$	73.75 ± 2.50^{c}	-	+	-	+	+	3.00 ± 0.00^{a}
$TDZ_{(1.0)}$	40.00 ± 8.12^{a}	+	+	-	-	+	4.50 ± 0.58^{b}

Figures in parentheses indicate the concentration of growth regulators in mgl^{-1}; Entries in column nos. 2and 5 are Mean's: same alphabetical letter in the superscript denotes that the corresponding means are in the same group using Duncan's multiple range test at 5%.

Figs. 1-14. *In vitro* regeneration of *Eulophia dabia* rhizome explant culture; 1, Explant with shoot bud (M); 2, Complete plantlet (M); 3, Tuber formation in 14 weeks (M); 4, Callusing of explant ($M + BAP_{0.5} + NAA_{1.0}$); 5, Shoot bud development ($M + BAP_{1.0} + NAA_{0.5}$); 6, Multiple shoots ($M + BAP_{1.0} + NAA_{0.5}$); 7, *In vitro* flowering ($M + BAP_{1.0} + NAA_{0.5}$); 8, Multiple shoot bud formation at the cut ends of the explant ($M + TDZ_{0.1}$); 9, Multiple shoot buds and PLBs formation ($M + TDZ_{0.1}$); 10, PLBs multiplication ($M + TDZ_{1.0}$); 11, Healthy plantlets ($M + TDZ_{1.0} + AC$); 12, Development of floral buds ($M + TDZ_{1.0}$); 13. Non-organogenetic callus formation ($M + TDZ_{0.5}$); 14, *In vitro* flowering ($M + TDZ_{0.5}$).

(Vij *et al.,* 1989), presence of organic growth supplement (Peptone/Yeast Extract) in the nutrient medium was obligatory for shoot bud development and callusing was invariably eluded. Presently, however, in *E. dabia*, the rhizome explants regenerated via PLBs/ shoot buds and non-organogenetic callus was also generated. The formation of non-organogenetic callus similar to our studies was, however, earlier reported in excised rhizomatous segments of *Spathoglottis plicata* (Bapat and Narayanaswamy, 1977). Vij *et al.* (1989) reported that NAA (1mgl^{-1}) in combination with YE and KN (1mgl^{-1}) proved beneficial for development of shoot bud and subsequent development of plantlets. Presently, however, NAA (0.5mgl^{-1}) with BAP (1mgl^{-1}) proved the best combination for regeneration and subsequent plantlet development. Acording to Paek and Yeung (1991), shoot formation in *Cymbidium* species using rhizome segments appears to be regulated by the auxin/cytokinin ratio; higher auxin/ cytokinin ratio in the culture medium generally enhances the rapid growth of the rhizome while a lower auxin/cytokinin ratio promotes shoot formation. The present results in *Eulophia dabia* also conform to this tendency as *Eulophia* is closely related to *Cymbidium* belonging to tribe Cymbidieae. TDZ exhibits strong cytokinin activity, promoting development of multiple shoots and PLBs and eluding rooting at high concentrations (Ernst, 1994; Chang and Chang, 2000).Presently, *In vitro* flowering was induced in combination containing NAA (0.5mgl^{-1}) /or TDZ (0.5, 1mgl^{-1}). Chang and Chang (2003) also reported that it promotes flowering at higher concentrations. Hence, our present results of TDZ in the nutrient medium confirm the earlier findings on TDZ activity. In plant tissue culture, AC is widely used to stimulate rooting of micropropagated shoots since it can adsorb both inhibitory substances and cytokinins in the medium (Luo *et al*., 2008). The inhibitory effect of TDZ at 0.5 and 1mgl^{-1}on rooting in the presently studied species was counteracted by shifting the plantlets to AC containing medium.

Present studies indicated that M medium containing BAP (1mgl^{-1}) in combination with NAA (0.5mgl^{-1}) proved the best and plantlets with 2-3 leaves and 3-4 roots obtained in 4 wks, flowered after 7 months. All these results suggest that *Eulophia dabia* rhizome explants could be successfully used for its propagation and *ex situ* conservation.

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FOLIAR ANATOMY IN SOME SPECIES OF *BULBOPHYLLUM* THOU.

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Abstract

The present paper deals with foliar anatomy of ten species of *Bulbophyllum i.e., Bulbophyllum affine, B. careyanum, B. polyrhizum, B. reptans, B. retusiusculum, B. rufinum, B. scabratum, B. secundum, B. trichocephalum,* and *B. xylophyllum* from NorthEast India. The aim has been to understand the inter-specific leaf anatomical variations within the genus *Bulbophyllum* and their divergent adaptations. The leaves varied in thickness from 332.5 μm (*B. reptans*) to 4180 μm (*B. xylophyllum*) Cuticle was most well developed in *B. xylophyllum* (31.25 μm) and least so in *B. scabratum* (7.26 μm). Epidermis thickness was maximum in *B. polyrhizum* (121.25 μm) and minimum in *B. secundum* (17.66 μm). Water storage cells were present in all the species. The leaves were invariably hypostomatic with polygonal epidermal cells. The most common stomatal type observed was floating type. Guard cells were cuticularised in *B. retusiusculum*, *B. secundum* and *B. trichocephalum.* The stomatal apparatus area was largest in *B. affine* (3996.63 μm^2) and smallest in *B. xylophyllum* (145.23 μm^2). Stomatal density was highest in *B. secundum* (51.15 mm^{-2}) and lowest in *B.affine* (6 mm^{-2}). These data suggest the drought tolerant nature of all the presently studied species. An attempt has also been made for the formation of identification key, based on anatomical features, with a view to helping in their identification under vegetative state.

Introduction

BULBOPHYLLUM IS an epiphytic genus of orchids with nearly 1900 species distributed all over the world. In India, it is represented by 80 species (Misra, 2007), majority of which are NorthEast Indian in distribution. The genus exhibits wide distribution amplitude extending from tropical to temperate countries. It stands characterised by having creeping rhizomes with pseudobulbs that carry one or two leaves, rarely three. Dunbar-Co *et al*. (2009) observed that the effect of environment on plants and their adaptation to the environment is exhibited by leaf traits. The presence of xeromorphic features in plants is an indication of aridity (Haworth and McElwain, 2008).

Owing to the great variation and heterogeneity of shapes (morphology), variations in the anatomy are also expected. In the present paper, leaf and dermal anatomy of ten species of the genus *Bulbophyllum* Thou. *i.e.*, *Bulbophyllum affine* Lindl., *B. careyanum* (Hook.) Spreng.*, B. polyrhizum* Lindl.*, B. reptans* (Lindl.) Lindl., *B. retusiusculum* Reichb. f.*, B. rufinum* Reichb. f., *B. scabratum* Reichb. f.*, B. secundum* Hook. f.*, B. trichocephalum* (Schltr.) Tang & Wang and *B. xylophyllum* Par. & Reichb. f. were studied with a view to understanding their significance in devising cultural tactics under green house conditions. An identification key based on anatomical features for easy identification under vegetative condition was also prepared.

Materials and Methods

Plant Material

The plants for the present study were collected from various districts of Manipur (Table 1) and grown in the Orchidarium of the Centre for Orchid Gene Conservation of Eastern Himalayan Region at Hengbung. Approximately after a year, the flowering plants were critically studied for taxonomical and anatomical characters.

Preparation of Leaf Sections

For histological observations, mature leaves were collected and free hand transverse sections were made with a sharp razor blade. The thin sections were stained with safranin and mounted on glass slides. The sections were examined and photographed under $\times$100 and $\times$400 of a light microscope (Model-CX31, Olympus Corp. Japan). Selected parameters like thickness of cuticle, epidermis, mesophyll and leaf were measured at the midpoint of each transverse section with a standardised ocular micrometer scale.

The adaxial and abaxial epidermis of middle leaf parts of mature leaves were peeled from fresh leaves and photographed under $\times$100 and $\times$400 of a light

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microscope. Digital images were manually analysed with Adobe Photoshop 7.0. The length and width of stomata were recorded. Stomatal apparatus area (A_s) and stomatal density (d) were also recorded. Stomatal apparatus area (A_s) was calculated using,

A_s = ¼ x ð x l x w (Shelley and David, 2001)
Stomatal density (d) was also calculated using the standard formula,

d = Number of stomata in one grid/Number of grids × Area of one grid square.

For leaf histological observations, 10 leaves from 10 different individuals were examined for each species.

Statistical Analysis

Statistical analysis was carried out using One way Analysis of Variance (ANOVA) followed by Duncan's Post Hoc test. Results were presented as a value ± standard deviation (SD). Significant levels were defined at $p < 0.05$ as analyzed by ANOVA.

Results

Foliar Anatomy (Fig.1A-E)

The leaf section in all the species studied were V-shaped in outline. Cuticle was present on both the adaxial and abaxial surfaces and it was smooth in all the species except that of in *B. polyrhizum* and *B. reptans* where, it was slightly ridged. Adaxial cuticle

Table 1. Ecological traits in presently studied species of *Bulbophyllum.*

	Source			
Species	Locality	Altitude (m)	Flowering	Distribution in India
Bulbophyllum affine	Tamenglong (Dailong, Longku)	1029 –1179	June	Eastern and Western Himalayas
	Senapati (Willong Khunou, Sadim Pukhri)	800–1485		
	Ukhrul (Kamjong)	1598		
B. careyanum	Tamenglong (Longku, Longchum, Dailong Rangan)	403–1422	Feb-March	Arunachal Pradesh, Meghalaya, Sikkim,
	Senapati (Sadim Pukhri)	1485		Manipur, Uttar Pradesh and West Bengal
	Chandel (Kwatha)	470		
B. polyrhizum	Chandel (Kwatha) Ukhrul (Kamjong)	402 1480	April	Sikkim, Darjeeling, Manipur and Western Himalaya
B. reptans	Tamenglong (Longku)	1303	Oct-Dec	Arunachal Pradesh, Manipur, Meghalaya,
	Senapati (Mao)	2390		Sikkim and Western Himalaya
B. retusiusculum	Senapati (Willong, Khunou, Mao)	1028-2390	August	Nagaland and Manipur
B. rufinum	Senapati (Hengbung)	1298	Sept-Oct	Manipur
B. scabratum	Tamenglong (Longku)	1303	April	Arunachal Pradesh, Manipur, Meghalaya, Darjeeling and Garhwal Himalaya
B. secundum	Senapati (Sadim)	1512	June-Aug	Nagaland, Manipur and Sikkim
B. trichocephalum	Ukhrul (Kamjong)	1460	August	Sikkim, Manipur and Meghalaya
B. xylophyllum	Chandel (Kwatha)	490	January	Manipur and Meghalaya

was thicker than the abaxial one in all the species (Table 2), with the exception of *B. affine* (CT_{ad} 22.5 μm and CT_{ab} 26.25 μm) *and B. trichocephalum* (CT_{ad} 14.38 μm and CT_{ab} 15.63 μm). Cuticular ledges were visible in *B. rufinum, B. scabratum, B. secundum,* and *B. xylophyllum*. Epidermis was single-layered in all the species and it was slightly modified above the mid-rib in *B. affine*, *B. careyanum, B. rufinum, B. scabratum, B. secundum, and B. xylophyllum.* Epidermis was followed by hypodermis in all the species except in *B. polyrhizum, B. reptans,* and *B. rufinum*. Hypodermis was composed of thin-walled water-storage cells with pitted or banded thickenings. Vascular bundles in all the species were collateral and arranged in one row with the median bundle being the largest and lateral bundles smaller with the exception of *B. secundum* where, the lateral bundles were larger than the median bundle. Both xylem and phloem in all the species were bounded by fibrous caps except in case of *B. polyrhizum*. Phloem cap was more prominent in most of the species. Mesophyll was modified into spongy and palisade cells except in *B. polyrhizum*. Multicellular glandular hairs within epidermal crypt were observed on both the adaxial and abaxial surfaces in all the species. Mesophyll tissue was 17-20 layers in *B. affine*, 8-12 in *B. careyanum*, 4-7 in *B. polyrhizum*, 6-12 in *B. reptans* and *B. retusiusculum*, 11-15 in *B. rufinum*, 7-10 in *B. scabratum*, 6-9 in *B. secundum*, 10-14 in *B. Trichocephalum,* and 14-20 in *B. xylophyllum*.

Dermal Anatomy (Fig.2A-E)

Stomata were observed only on the abaxial surface in all the species. Size of stomata and stomatal apparatus area was largest in *B. affine* (71.75 × 70.5 μm & 3996.63 μm^2) and smallest in *B. xylophyllum* (15.25 × 12.25 μm & 145.23 μm^2)*;* see Table 2. Epidermal cells were polygonal. Stomata were very small and sunken in *B. polyrhizum* and *B. xylophyllum* and slightly sunken in *B. affine.* Wax-secreting cells were found on both the adaxial and abaxial surfaces. Guard cells were with chloroplasts in all the species and cuticularised in *B. retusiusculum, B. Secundum,* and *B. trichocephalum*. Stomatal clustering was observed in all the species. Three stomatal types were observed: Foating type was the most common and observed in seven of the species studied (*B. affine, B. careyanum, B. polyrhizum, B. reptans, B. rufinum, B. scabratum* and *B. xylophyllum*), Cyclocytic type was found in *B. retusiusculum* and *B. Trichocephalum*, and Tetracytic type was found in *B. secundum*.

One way Analysis of Variance (ANOVA) followed by Duncan's Post Hoc test values were statistically significant at $p < 0.05$ ($n = 10$).

Based on the various anatomical characters, an attempt has been made to form a key for identification of the species studied under vegetative state which is as follows:

Identification Key

1a. Cuticular ledges present	2
1b. Cuticular ledges absent	5
2a. Sunken stomata present	*B. xylophyllum*
2b. Sunken stomata absent	3
3a. Guard cells cuticularised	*B. secundum*
3b. Guard cells not cuticularised	4
4a. Hypodermis present	*B. scabratum*
4b. Hypodermis absent	*B. rufinum*
5a. Abaxial cuticle thicker than adaxial	6
5b. Abaxial cuticle thinner than adaxial	7
6a. Guard cells cuticularised	*B. trichocephalum*
6b. Guard cells not cuticularised	*B. affine*
7a. Phloem cap absent	*B. polyrhizum*
7b. Phloem cap present	8
8a. Hypodermis absent	*B. reptans*
8b. Hypodermis present	9
9a. Guard cells cuticularised	*B. retusiusculum*
9b. Guard cells not cuticularised	*B. careyanum*

Discussion

Foliar Anatomy

Amongst the ten species studied, it was observed that leaf thickness was maximum in *B. xylophyllum* (4180 μm) and minimum in *B. reptans* (332.5 μm). Cuticle was found on both sides of the lamina in all the species. Thickest adaxial cuticle was observed in *B. xylophyllum* (31.25 μm) while abaxial cuticle thickness was maximum in *B. polyrhizum* (26.88 μm). *B. scabratum* showed minimum cuticle thickness; both adaxial and abaxial (Table 2). Cuticle helps in reducing water loss from the leaf interior (Mill and Schilling, 2009). Thick cuticle is usually found in plants of dry habitats (Haworth and McElwain, 2008). Adaxial epidermal thickness was maximum in *B. polyrhizum* (121.25 μm) and minimum in *B. secundum* (17.66 μm) while abaxial epidermal thickness was maximum in *B. affine* (68.75 μm) and minimum in *B. reptans* (15.84 μm). Large

Fig. 1A-E. A. *Bulbophyllum affine:* a, T.S. of leaf (×100); b, Floating stomata (×400); c, Vascular bundles with fibrous caps (arrows) (×100); B. *B. careyanum:* a, T. S. of leaf (X100); b, Floating stomata (×400); c, Water-storage cells with pitted walls (×400); C. *B. polyrhizum:* a, T. S. of leaf (×100); b, Floating stomata (×400); c, *B. careyanum*. Wax-secreting cell on upper epidermis (×400); D. *B. reptans:* a, T. S. of leaf (×100); b, Floating stomata (×400); c, Water-storage cells with banded thickenings (×400); E: *B. retusiusculum.* a, T. S. of leaf (×100); b, Cyclocytic stomata (×400); c, Multicellular glandular hair within epidermal crypt (×400); Ad, adaxial epidermis; Ab, abaxial epidermis; Cu, cuticle; ET, epidermal tissue; PT, palisade tissue; ST, spongy tissue; MVB, median vascular bundle; LVB, lateral vascular bundle; WSC, water storage cells; GC, guard cell; EC, epidermal cell; SC, subsidiary cell. Scale bars, Column 1,100 μm; Column 2,10 μm; Column 3,50 μm.

Table 2. Foliar anatomy in presently studied species of *Bulbophyllum.*

Species	LT (μm)	CT_{ad} (μm)	CT_{ab} (μm)	ET_{ad} (μm)	ET_{ab} (μm)	MT (μm)
Bulbophyllum affine	1897.50 ± 37.64^{g}	22.50 ± 3.23^{c}	26.25 25 ± 2.64^{f}	49.38 ± 8.04^{b}	68.75 ± 8.84^{g}	1820 ± 63.25^{f}
B. careyanum	977.50 ± 68.41^{e}	14.38 ± 3.02^{b}	13.13 ± 1.98^{cd}	47.5 ± 14.49^{b}	38.75 ± 2.64^{d}	803.75 ± 6.79^{d}
B. polyrhizum	463.75 ± 10.94^{c}	28.75 ± 6.04^{d}	26.88 ± 7.82^{f}	121.25 ± 14.49^{e}	63.13 ± 15.72^{g}	288.75 ± 24.62^{a}
B. reptans	332.50 ± 69.27^{a}	16.50 ± 0.03^{b}	11.22 ± 1.70^{bc}	19.47 ± 2.89^{a}	15.84 ± 2.60^{a}	295 ± 64.60^{a}
B. retusiusculum	758.75 ± 61.53^{d}	14.19 ± 2.72^{b}	9.90 ± 3.11^{abc}	23.76 ± 4.87^{a}	21.45 ± 1.74^{ab}	671.25 ± 62.37^{c}
B.rufinum	1313.77 ± 300.37^{f}	25.25 ± 4.48^{c}	24.25 ± 4.72^{e}	74.50 ± 32.36^{c}	47 ± 14.76^{e}	1159.34 ± 248.7^{e}
B.scabratum	441.25 ± 37.75^{bc}	7.26 ± 1.39^{a}	6.80 ± 1.22^{a}	22.44 ± 3.03^{a}	21.45 ± 1.74^{ab}	397.50 ± 40.74^{b}
B. secundum	346.25 ± 64.5^{ab}	10.40 ± 3.02^{a}	9.24 ± 2.36^{ab}	17.66 ± 3.12^{a}	16.01 ± 2.34^{a}	289.38 ± 49.31^{a}
B. trichocephalum	852.50 ± 53.94^{d}	14.38 ± 3.02^{b}	15.63 ± 3.29^{d}	42.50 ± 5.74^{b}	27.5 ± 4.37^{bc}	680 ± 22.97^{c}
B. xylophyllum	4180 ± 27.13^{h}	31.25 ± 5.89^{d}	25.63 ± 1.98^{f}	101.25 ± 30.45^{d}	56.25 ± 5.1^{f}	3968.75 ± 8.83^{g}

Mean ± SD (n = 10). Different letters in the same column indicate statistical difference $p < 0.05$ (ANOVA).

LT , leaf thickness; CT_{ad}, adaxial cuticle thickness; CT_{ab}, abaxial cuticle thickness; ET_{ad}, adaxial epidermis thickness; $ET_{ad,}$ abaxial epidermis thickness; MT, mesophyll thickness.

Table 3. Dermal anatomy in presently studied species of *Bulbophyllum*.

Species	L_s (μm)	W_s (μm)	A_s (μm^2)	S_a (μm)	d (mmE^2)
Bulbophyllum affine	71.75 ± 8.5^{g}	70.50 ± 8.96^{g}	3996.63 ± 853.12^{i}	40 ± 4.71^{g}	6.00 ± 0.69^{a}
B. careyanum	64.02 ± 1.7^{f}	48.18 ± 1.7^{f}	2442.38 ± 106.32^{h}	44.55 ± 1.74^{h}	15.83 ± 2.29^{b}
B. polyrhizum	57.50 ± 4.86^{e}	38.75 ± 3.78^{e}	1753 ± 257.44^{g}	39.00 ± 4.28^{g}	15.40 ± 2.41^{b}
B. reptans	54.12 ± 3.55^{e}	39.93 ± 4.52^{e}	1611.42 ± 202.99^{fg}	34.98 ± 1.7^{f}	26.4 ± 2.66^{d}
B. retusiusculum	46.53 ± 3.95^{d}	34.32 ± 1.7^{cd}	1252 ± 102.76^{de}	25.74 ± 3.41^{d}	35.83 ± 3.46^{e}
B.rufinum	48.18 ± 3.55^{d}	37.45 ± 2.47^{de}	1416.51 ± 142.77^{ef}	29.37 ± 2.56^{e}	20.15 ± 0.66^{c}
B.scabratum	40.59 ± 4.41^{c}	25.08 ± 3.55^{b}	801.01 ± 154.98^{bc}	23.43 ± 2.31^{cd}	18.75 ± 3.76^{bc}
B. secundum	32.50 ± 3.02^{b}	27.39 ± 1.77^{b}	699.28 ± 82.15^{b}	16.33 ± 1.82^{b}	51.15 ± 5.07^{h}
B. trichocephalum	37.95 ± 2.46^{c}	27.23 ± 2.83^{b}	812.55 ± 113.31^{bc}	22.77 ± 1.87^{c}	39.25 ± 3.75^{g}
B. xylophyllum	15.25 ± 2.49^{a}	12.25 ± 2.49^{a}	145.23 ± 30.61^{a}	10.25 ± 1.42^{a}	36.55 ± 4.71^{fg}

Mean ± SD (n = 10). Different letters in the same column indicate statistical difference $p < 0.05$ (ANOVA).

$L_{s,}$ stomatal length; W_s, stomatal width; A_s, stomatal apparatus area; $S_{a,}$ stomatal aperture; d, stomatal density.

Fig.2A-E. A. *Bulbophyllum rufinum*: a, T.S. of leaf (×100); b, Floating stomata (×400); c, Cuticular ledges (×400); B. *B. scabratum:* a, T. S. of leaf (×100); b, Floating stomata (×100); c, Water-storage cells with pitted walls (×400); C. *B. secundum:* a, T. S. of leaf (×100); b, Tetracytic stomata (×400); c, Hypodermis made up of water-storage cells with pitted walls (arrows) (×100); D. *B. trichocephalum:* a, T. S. of leaf (×100); b, Cyclocytic stomata (×400); c, Multicellular glandular hairs within epidermal crypt (arrows) (×100); E. *B. xylophyllum*: a, T. S. of leaf (×100); b, Floating stomata (×400); c, Water-storage cells with banded thickenings (arrows) (×100). Scale bars, Column 1,100 μm; Column 2,10 μm; Column 3, 50 μm.

epidermal cells in many orchid species serve as water-storage cells. In some species of orchids, the water stored in epidermal cells can account for up to 80% of the entire leaf volume (Pridgeon and Stern, 1982). Maximum mesophyll thickness (3968.75 μm) was observed in *B. xylophyllum* and minimum in *B. polyrhizum* (288.75 μm). In all the species studied, the median vascular bundle was larger than the lateral ones except in *B. secundum*. The presence of multicellular glandular hairs within epidermal crypt in all the species is an indication of the plant's adaptability to reduce the rate of transpiration. It also protects the plants from outer injurious agencies (Pandey, 2001).

Dermal Anatomy

Stomatal size and stomatal apparatus area was largest in *B. affine* and smallest in *B. xylophyllum* (Table3). The exchange of gases takes place through the stomata (Buckley, 2005). The distribution, size, density, morphology and behaviour of stomata are closely associated with plant transpiration (Willmer and Fricker, 1996). Under severe water scarcity, smaller stomata are more efficient than larger stomata (Aasamaa *et al.,* 2001). Maximum stomatal density was observed in *B. secundum* and minimum in *B. affine* (Table 3). Plants with lower stomatal density are usually able to tolerate a more arid environment than plants with higher stomatal density (Kebede *et al*., 1994). All the species studied were hypostomatic with polygonal epidermal cells. A distinct predominance of hypostomatic over amphistomatic leaves was shown by Lavarack (1971), Williams (1979), and Avadhani *et al*., (1982). Guard cell chloroplast was present in all the species studied. Guard cell chloroplasts can contribute to stomatal opening (Zeiger *et al.,* 2002). However, guard cell cuticularisation was observed only in *B. retusiusculum, B. secundum* and *B. trichocephalum*. The presence of wax-secreting cells in all the species help in reducing the rate of transpiration thereby, aiding in water conservation. These cells also protect the leaves from shedding rain so the leaf cells don't become overly saturated with water and burst. Hoover (1986) observed that stomatal clusters may help in conserving water in plants. The most common stomatal type observed was floating type. However, cyclocytic and tetracytic types were also observed in some of the species. Floating condition is suggested to arise when anticlinal walls between subsidiary cells in a tetracytic configuration dissolve (Singh and Singh, 1974). These data suggest that all the ten species studied are xeromorphic in nature and can tolerate long periods of drought. *B. Xylophyllum*, however, showed maximum xeromorphic features. The leaves of this orchid are very thick and has the thickest adaxial cuticle with extremely small and sunken stomata. Thus, it can be concluded that all the species studied are able to tolerate long periods of drought and efficient in water-use and these traits can be useful for conservation of these species under green house conditions.

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REVERSION OF REPRODUCTIVE PHASE TO VEGETATIVE PHASE IN THE INFLORESCENCE SEGMENTS OF *SACCOLABIUM PAPILLOSUM* LINDL. - A STUDY *IN VITRO*

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Abstract

The regeneration potential of inflorescence segments of *Saccolabium papillosum* Lindl. was tested on M (Mitra *et al.*, 1976) medium and its combinations with different growth additives. The regeneration response varied with the position of explants on the donor axis. The explants, only from upper 2/3 region with undifferentiated floral buds responded to regeneration; these followed direct and callus mediated plantlet development. The percentage of regeneration in the explants was directly proportional to the level of Plant Growth Regulators (PGRs) in the nutrient medium. The regeneration response was obligatory to the use of PGRs such as cytokinins [benzyladenine (BA), 6-furfurylaminopurine/kinetin (KN)], auxins [α-naphthalene acetic acid (NAA)] at 0.5 and 1.0 mg^{-1} and organic growth supplement, Peptone (P; at 1.0 gl^{-1}) in the nutrient pool. NAA favoured multiplication of Protocorm-like bodies (PLBs). Addition of activated charcoal promoted early plantlet development.

Introduction

THE GENUS *Saccolabium* includes nearly 40 species of epiphytic orchids and is distributed in the Indian subcontinent (from tropical India, Burma, throughout Indonesia to New Guinea) (Bose and Bhattacharjee, 1980). It derives its name from the bag-like shape of the labellum. Usually, the plants are dwarf, evergreen with leafy stems. The blooms are delicately colored and occasionally fragrant. Although, the flowers are small, they are produced in large numbers. Due to extermination of the forests, their natural populations are shrinking day by day. With a view to ameliorating their ever-declining wild populations, presently *S. papillosum* was selected for the purpose. *Saccolabium papillosum* Lindl. (=*Acampe papillosa* Lindl.) is a beautiful epiphytic orchid species with leaf opposed, 4-8 flowered, sub-corymbose racemes. It is distributed all along the tropical Himalaya at an altitude of 500-800 m (from Kumaon Eastwards to Arunachal Pradesh) and inhabits a variety of broad-leaved phorophytes, including *Mangifera indica* and *Shorea robusta.* While the ornamental potential of this species is yet to be explored, its therapeutic utility is well documented. Its roots are used as a substitute drug for Sarsaparila (Lawler, 1984). *S. papillosum* is faced with habitat destruction pressures that far exceed its natural regeneration. As a result, the species has become threatened in its natural habitats. The genus *Saccolabium* is included in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2014). Through this communication, we emphasize to conserve, propagate and multiply the species, through tissue culture techniques with a view to popularizing the species amongst amateurs, nurserymen and professionals for its cultivation, thus saving its wild populations from getting extinct.

In clonal propagation, it is immensely important to maintain genetic uniformity in *in vitro* raised progenies. In outbreeding taxa like orchids, seed raised progenies are extremely heterozygous. To maintain genetic stability of the regenerants, it is important to identify appropriate explants and their *in vitro* propagation protocols. The utility of inflorescence segments, as an effective alternative to shoot meristem for micropropagating orchids is a successful approach in this direction as this method provides opportunities to produce a large number of true to type plantlets of interest. The orchids are propagated *in vitro* by using various explants such as shoot meristem, leaves, roots, protocorms *etc.* obtained from axenic cultures but the regenerative potential of inflorescence segments as explants has been less explored as compared to the other explants. Therefore, presently an attempt was made to establish an efficient regeneration system by using inflorescence segments as explants in *S. papillosum* with a view to assessing the: i) influence of position of the explant; and ii) the effect of growth regulators individually and/or in combination on frequency and onset of regeneration and the subsequent development of regenerants into plantlets. In this paper, an efficient and reproducible single step protocol for the regeneration, multiplication and development of plantlets using inflorescence segments is reported.

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Materials and Methods

Plant Collection and their Maintenance in the Greenhouse

Saccolabium papillosum Lindl. plants were obtained from a commercial grower of Dehradun, Uttarakhand, India. The healthy plants were replanted in pots (diameter 27.5 cm × 22.4 cm) containing epiphytic substrate, *i.e.*, pieces of charcoal, brick and bark in a ratio of 1:1:1. *Sphagnum* moss covered the top surface of the potting mix. The plants were maintained in a greenhouse under natural light, 70% relative humidity and 25°C/20°C day/night temperature.

Sterilization Procedure and Culture Media

The inflorescence axis was obtained from green house grown plants. It was segmented into 1.0-1.5 cm long explants. The segments were scrubbed with a soft brush under running tap water to remove any debris. Later, these segments were washed with a dish wash detergent solution. These were swabbed with ethyl alcohol under a sterile (laminar) hood and surface sterilized with 0.1% (wv^{-1}) mercuric chloride ($HgCl_2$; Qualigens, Mumbai, India) solution containing 1–2 drops of "Teepol" as a wetting agent for 4-5 min. This was followed by 2–3 rinses with sterilized distilled water. Thereafter, the ends (1.0-2.0 mm long) were severed-off and remaining explants were inoculated in M (Mitra *et al*., 1976) medium and supplemented with growth regulators such as cytokinins [benzyladenine (BA), 6-furfurylaminopurine/kinetin (KN)] and auxins [α-naphthaleneacetic acid (NAA)] at 0.5, 1.0 mg^{-l} and organic growth supplements such as peptone (P) at 1 gl^{-l} in the medium. In a separate set of experiment, the effect of activated charcoal (AC) 2 gl^{-1} was also tested. The medium was autoclaved at 121°C at a pressure of 1.06 kg cm^{-2} for 15 min. The autoclaved medium was kept at 37°C to check any contamination in the culture medium.

Inoculation and Incubation Conditions

All inoculations were performed under aseptic conditions in a laminar airflow cabinet. The cultures were incubated at 25 ± 2°C under a 12-h photoperiod with a light intensity of 3500 lx (fluorescent tubes 40W, Philips India Ltd, Mumbai, India). Four replicates were used for each experiment and, to check the reproducibility, the experiment was repeated twice. The cultures were observed regularly under a binocular microscope (Olympus SZX10, Japan).

Statistical Analysis

One way analysis of variance was performed with respect to each response (average ± standard error against each additive as mentioned in Table 1). As ANOVA results showed the non significant difference of additives at 5% level of significance, various groups of additives showing identical/similar response were formed statistically. To this end, Tukey Test was performed at 5% level with respect to each response.

Results and Discussion

The ability of *Phalaenopsis* inflorescence segments to regenerate *in vitro* opened up exciting opportunities to use them as explants (Rotor, 1949). They have proved as an effective alternative to excised shoot meristem for micropropagating the orchids. The regeneration potential of floral buds and inflorescence segments have been tested in a few orchid species and hybrids (cf. Arditti and Ernst, 1993; Chen and Piluek, 1995; Chen *et al*., 2002; Collins and Dixon, 1992; Goh and Wang, 1990; Ichihashi, 1992, Kher *et al*., 1997; Lin, 1986; Martin *et al*., 2005; Mitsukuri *et al*., 2009; Shimasaki and Uemoto, 1991; Tokuhara and Mii, 1993; Vij *et al*., 1986,1997).

Figs. 1-6. *In vitro* inflorescence segment culture of *Saccolabium papillosum* in M medium and its various combinations with growth adjuncts: 1, PLB mediated regeneration response in $M+NAA_{(1.0)}$; 2, Multiplication of regenerants in $M+NAA_{(1.0)}$; 3, Plantlet development in $M+NAA_{(1.0)}$; 4, Compact, chlorophyllous and organogenetic callus mediated PLB development in $M+BAP_{(0.5)}$; 5, Multiplication of PLBs in $M+BAP_{(0.5)}$;6, Healthy growth of plantlets in $M+P_{(1.0)}+AC$ enriched medium.

Presently, the undifferentiated floral-buds were amenable to transformation into vegetative ones and are paralleled with the ability of floral buds to de-differentiate and assume vegetative growth as reported earlier in epiphytic orchids such as *Dendrobium crepidatum* and *Oncidium* (Lim-Ho *et al.*, 1984). In the presently investigated species, the response in the inflorescence segments was obligatory to the use of PGRs in M medium and it varied with their position on the donor axis. Incidentally, their proliferative potential was directly proportional to the level of PGRs in the nutrient medium. However, as Rotor (1949) could obtain somatic embryos on a growth regulator free medium, it appears that the hormonal requirements, during regeneration vary with the species. The results are summarized in Table 1 and illustrated in Figs.1-6.

In our cultures, the segments from lower 1/3 region of the mother axis, with well differentiated buds, necrosed within 2 wks of culture unlike their normal development into flowers in *Ascofinetia, Neostylis* and *Vascostylis* (Intuwong and Sagawa, 1973) and *Saccolabium* (Vij *et al*., 1986). Konar and Kitchlue (1982) hinted at the involvement of nutritional complexities during normal development of the flowers. In earlier study, a treatment with IAA, KN and CH/U was, however, obligatory for development of *S. calceolaris* buds into normal flowers (Vij *et al.*, 1986).

In our cultures, the segments from upper 2/3 region with undifferentiated floral buds were able to regenerate to the chemical stimulus in the nutrient pool. The explants followed both, direct and callus mediated PLB-plantlet development in auxin and cytokinins treated cultures. Auxin (NAA) favoured direct development of PLBs (protocorm-like bodies) without any intervening callus formation and its effect was more pronounced at 1mgl^{-1} when 74.25 ± 0.95% explants regenerated in its presence. The *neo-formations* (PLBs) multiplied profusely and up to 80 PLBs could be harvested within 15 wks (Figs. 1, 2). Plantlets were obtained in another 10 wks (Fig. 3). The importance of NAA in initiating *Oncidium* cultures from floral stalk cuttings has been demonstrated by Lim-Ho and Lee, 1987. The utility of growth additives in inducing / regulating plantlet regeneration has already been emphasized in *Dendrobium* (Vij *et al.,* 1981), *Oncidium* (Lim-Ho and Lee, 1987), *Phalaenopsis* (Lin, 1986; Tanaka and Sakanishi, 1980) and *Saccolabium* (Vij *et al.,* 1986). The benign effect of NAA with BAP/KN in inducing PLBs, callus and/or multiple shoots, is

Table 1. *In vitro* regeneration response of *Saccolabium papillosum* inflorescence segments in M medium and its combination with growth additives.

Additives	Regeneration (%)	Initiation of response (wks)	Number of regenerants	Plantlet development (wks)
-	-	-	-	-
AC	-	-	-	-
$BAP_{(0.5)}$	25.00 ± 0.81^{b}	2.50 ± 0.57^{ab}	6	29.25 ± 0.95^{def}
$BAP_{(0.5)}$ + AC	25.00 ± 0.81^{b}	2.00 ± 0.00^{a}	8	27.25 ± 0.95^{bcd}
$BAP_{(1.0)}$	50.00 ± 0.81^{c}	2.75 ± 0.50^{ab}	15	30.00 ± 0.81^{f}
$BAP_{(1.0)}$ + AC	50.00 ± 0.81^{c}	2.00 ± 0.00^{a}	20	26.00 ± 0.81^{bc}
$KN_{(0.5)}$	25.00 ± 0.81^{b}	2.75 ± 0.50^{ab}	4	31.00 ± 0.81^{f}
$KN_{(0.5)}$ + AC	25.00 ± 0.81^{b}	2.25 ± 0.50^{ab}	6	29.00 ± 0.81^{def}
$KN_{(1.0)}$	50.00 ± 0.81^{c}	2.75 ± 0.50^{ab}	10	30.00 ± 0.81^{ef}
$KN_{(1.0)}$ + AC	50.00 ± 0.81^{c}	2.00 ± 0.00^{a}	20	27.00 ± 0.81^{bcd}
$NAA_{(0.5)}$	50.00 ± 0.81^{c}	2.00 ± 0.81^{a}	20*	27.00 ± 0.81^{bcd}
$NAA_{(0.5)}$ + AC	50.00 ± 0.81^{c}	2.00 ± 0.00^{a}	18*	27.75 ± 0.95^{cde}
$NAA_{(1.0)}$	74.25 ± 0.95^{d}	2.00 ± 0.00^{a}	30*	25.00 ± 0.81^{ab}
$NAA_{(1.0)}$ + AC	75.00 ± 0.81^{d}	2.00 ± 0.00^{a}	30*	23.50 ± 1.29^{a}
$P_{(1.0)}$	10.00 ± 0.81^{a}	3.00 ± 0.00^{b}	60	34.75 ± 0.95^{g}
$P_{(1.0)}$ + AC	10.00 ± 0.81^{a}	3.00 ± 0.00^{b}	60	35.00 ± 0.81^{g}

* = Direct protocorm - like body (PLB) formation.

already on records in *Phalaenopsis* (Lin, 1986); *Diuris longifolia* (Collins and Dixon, 1992). Presently, the explants in BAP/KN (0.5 mgl^{-1}) treated cultures followed a callus mediated multiple PLB regeneration in 25.00 ± 0.81% explants; phenotypically, the callus was compact, chlorophyllous and organogenetic in nature (Fig.4). The PLBs rapidly multiplied in the respective medium (Fig.5). The response frequency was elevated to 50.00 ± 0.81% in combinations containing cytokinins at 1mgl^{-1} and their multiplicity was further accentuated if the combination was darkened with AC.

Presently, organic growth supplement (Peptone) was successfully utilized to transform undifferentiated floral buds into vegetative ones. Although, the regeneration percentage was impaired to 10.00 ± 0.81% in this combination, the regenerated callus mediated PLBs were observed to be highly proliferative, almost 60 daughter PLBs could be harvested within 18 wks. Morphogenetic processes leading to plantlet development, however, were delayed in the combination (34.75 ± 0.95 wks) but additional activated charcoal favoured healthy growth of plantlets (Fig.6). A perusal of literature reveals that peptone being water soluble protein hydrolysate loaded with very high amino acid content is able to promote growth of cultures. Similar beneficial effect of peptone was earlier observed in inducing protocorm multiplication in *Cymbidium macrorhizon* and *Cymbidium* species (Kusumoto and Furukuwa, 1977). Peptone is also known to have stimulated callus growth in *Phalaenopsis*, *Doritaenopsis*, and *Neofinetia* (Ichihashi and Islam, 1999). It also supported better seedling growth in *Paphiopedilum*, *Phaius*, and *Vanda* (Curtis, 1947). In *Peristeria elata,* peptone favoured early and healthy growth of seedlings (Bejoy *et al.*, 2004). Supplementation of organic growth additives in orchid culture medium is simple, practical, beneficial and a conventional method to improve media used for commercial production (Ichihashi and Islam, 1999). In the present studies, the used organic growth supplement contains amino acids, proteins, and organic compounds; it seems that any of these component(s) may be responsible for promoting growth and development of the present cultures. Hence, further studies are required to determine which factor(s) is responsible for promoting effect of these organic additives.

Conclusion

All these data suggest that *in vitro* regeneration potential of inflorescence segments is regulated by the developmental stage of the explant and the response is markedly influenced by the chemical stimulus in the nutrient pool. The technique can be profitably utilized as an effective alternative to shoot meristem in monopodial taxa as it is useful not only for cloning orchids but also for generating lesser number of somaclonal variations.

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GOODYERA BIFLORA (LINDL.) HOOK. F. (ORCHIDACEAE): A NEW RECORD FOR DARJEELING HIMALAYA OF WEST BENGAL, INDIA

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Abstract

Present paper deals with *Goodyera biflora* (Lindl.) Hook. f. (Orchidaceae) which was presently collected from Gairibas, Neora Valley, and Damsang forest of Darjeeling Himalaya of West Bengal and is reported as a new angiospermic record for the Darjeeling Himalayan region of India. An updated nomenclature, important synonyms, illustrated description, habitat, flowering and fruiting, altitudinal range, specimen examined, present status, and geographical distribution of species has also been given.

Introduction

The genus *Goodyera* was established in 1813 by Robert Brown; it comprises about 40 species widely distributed in the Northern Temperate Zone, South to Mexico and East to Madagascar, SouthEast Asia, the Pacific Islands, New Guinea, and Australia. Blume (1858), Schlechter (1911-14), and Seidenfaden (1978) revised the sectional treatment of the genus (Pearce and Cribb, 2002).

Plants small terrestrial herb; *rhizome* creeping. *Stems* erect, leafy. *Leaves* basal or clustered, sometimes reticulately patterned. *Inflorescence* terminal, few to many-flowered, racemose; *peduncle* and *rachis* often pubescent. *Flowers* small, often pubescent or glandular on outer surface. *Sepals* parallel to the floral axis or with lateral pair spreading; *dorsal sepal* forming a hood with *petals*. *Lip* unlobed, hollow or saccate at base, often setose within, narrowed to an acute apex. *Column* short; *rostellum* long, deeply cleft; *pollinia* 2, pyriform or clavate.

While working on Orchid flora of Darjeeling Himalaya, the authors came across interesting specimens of terrestrial orchid species. After critical examination and comparison with other authenticated specimens and literature, an unknown species of terrestrial Orchid was identified as *Goodyera biflora* (Lindl.) Hook. f. (Orchidaceae) which was collected from Gairibas, Neora Valley, and Damsang forest of Darjeeling Himalaya. A perusal of earlier literature (Bose and Bhattacharjee, 1999; Bruhl, 1926; Hara, 1966, 1971; Hooker, 1888-1890; King and Pantling, 1898; Ohashi, 1975; Pearce and Cribb, 2002; Pradhan, 1979; Pradhan and Pradhan, 1997) related to the Orchid Flora of Darjeeling revealed that the occurrence of this species has not been reported earlier from Darjeeling Himalayan region and hence the present collection is its first record of occurrence as *Goodyera biflora* (Lindl.) Hook. f. for Darjeeling Himalaya of West Bengal, India. The newly collected specimens were processed and mounted on standard herbarium sheets as per Jain and Rao (1977) and have been deposited in the Herbarium, Cluny Women's College, Kalimpong for future references. A detailed taxonomic account of the species along with a photograph, habitat, altitudinal range, present availability status, local distribution within Darjeeling, and geographical distribution is provided here to authenticate the new record and facilitate its easy identification.

Species Description

Goodyera biflora (Lindl.) Hook. f., Fl. Brit. India 6: 114. 1890. *Georchis biflora* Lindl., Gen. Sp. Orchid. 496. 1840. *Epipactis biflora* (Lindl.) Eaton, Proc. Biol. Soc. Wash. 21: 63. 1908. (Figs. 1-2).

Plant terrestrial herb, 6-11 cm tall. *Stem* decumbent, erect, stout. *Leaves* 2, 2.4-3 × 1.6-2.5 cm, ovate-cordate, obtuse, dark bluish-green and reticulated with white nerved on upper surface, petiolate. *Inflorescence* 2-7 flowered, terminal racemes with hairy rachis. *Flower* 2.3-2.5 cm across, white, hairy; *floral bracts* longer than ovary. Sepals 2.3-2.5 × 0.4-0.6 cm, narrowly lanceolate; *dorsal sepal* recurved at the apex, forming a hood over the *column* with *petals*; lateral pair shorter, strongly reflexed, connate at base. *Petals* 2.3-2.5 × 0.4-0.5 cm, linear-lanceolate, falcate. *Lip* shorter than sepals and petals, white with yellow tinge, saccate at base. *Anther* ovate; *pollinia* 2.

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Flowering and Fruiting

Fig. 1. *Goodyera biflora* (Lindl.) Hook. f. (young inflorescence).

Fig. 2. *Goodyera biflora* (Lindl.) Hook. f. (Herbarium specimen).

July–September.

Habitat

Terrestrial on shady places.

Distribution

India (Darjeeling, North West India), Nepal

Locality and Specimen Examined

Gairibas forest 2800 m, of Darjeeling Sub-Division of Darjeeling Himalaya (Border area of Nepal and India), dated 31. 07. 2010, Rajendra *et al.* 1377 (West Bengal, India).

Altitudinal Range

1900 –2850 m.

Present Availability Status

Rare in natural habitat.

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IN VITRO PROPAGATION OF *PAPHIOPEDILUM SPICERIANUM* (REICHB. F.) PFITZ. – A RARE AND ENDANGERED ORCHID SPECIES FROM NORTHEAST INDIA

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Abstract

Presently, an attempt was made to propagate *Paphiopedilum spicerianum* (Reichb. f.) Pfitz. *in* vitro using seed, leaf and shoot tip as explants. Murashige and Skoog (MS) medium with and without growth additives was used as nutritional recipe. The seeds procured from green capsules failed to respond, whereas, young leaf and shoot tip explants showed regeneration response via callusing. The formation and development of the callus was found to be extremely slow and poor. Though regeneration occurred on the leaf explants in MS medium supplemented with the combination of BAP (0.5 mgl^{-1}) + IAA (0.1 mgl^{-1}) + IBA (0.1 mgl^{-1}); KN (3.5 mgl^{-1}) + IBA (0.7 mgl^{-1}); BAP (2.5 mgl^{-1}) + 2, 4-D (0.5 mgl^{-1}), the size and weight of the callus varied with the growth stimulus (Fig. 2 A-L). In case of shoot tip explants, the regeneration occurred via callus formation in medium supplemented with only TDZ at different concentrations (*i.e.*, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35 mgl^{-1}). The initiation and development of the callus was favored with increase in the concentration of TDZ; the best results were obtained in medium with TDZ at 0.20 mgl^{-1}. Leaf formation was reported in IAA (0.2 mgl^{-1}) supplemented medium and subsequently complete plantlets were successfully produced in this endangered orchid species.

Introduction

ORCHIDS CONSTITUTING an interesting group of flowering plants with beautiful flowers are economically important. They are grown almost all over the world mainly for cut-flower and pot-plant production. According to the IUCN Action plan (1999), orchids are identified as amongst the world's most diverse and widely distributed plants (cf. Sibin *et al.*, 2014). All the orchid species are protected under Wild Life (Protection) Act, 1972, and treated as Protected species under CITES (Convention on International Trade in Endangered Species of Wild Flora and Fauna) (Hegde, 2012).

Paphiopedilum commonly known as Slipper orchid with over 70 species is native to South and SouthEast Asia (Udomdee *et al.,* 2012). *Paphiopedilum spicerianum* (Rchb. f.) Pfitz., an endangered terrestrial species which flowers during November to January (Fig. 1), is in great demand because of unique beauty of its flowers. It is a terrestrial herb with small stem, leathery leaves, and very attractive colourful flowers developing individually; each flower is with a snow white upper sepal with a pink central stripe and a similarly coloured staminodium. It is an endangered plant species of Indian sub Himalayan region (Nayar and Sastry, 1987) and it is protected under the Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES; CITES, 2013) (cf. ENVIS Centre on Floral Diversity, 2015). Kataki (1984) and Kataki *et al*. (1984) have mentioned that this orchid species is rare and endemic due to over collections as well as disturbance of its natural habitats. Due to the destruction of the natural habitat and anthropogenic pressure, its populations are very less in the natural habitats. Therefore, there is an urgent need for some conservation measures. *In vitro* propagation is one of the important advanced conservation initiatives which help to increase the population of a particular plant species within a particular period of time. This technique has been successfully used in many species by earlier researchers (Kaur and Pathak, 2014; Pathak and Vij, 2001, 2007; Pathak *et al*., 1992, 2001, 2005, 2011, 2012; Vij and Pathak, 1988, 1990; Vij *et al.,* 1994, 1995). This *in vitro* technique for rapid and mass propagation offers possibilities for 'recovery' of the endangered species, thus reducing the risk of extinction (Nadeem *et al*., 2000).

The presently selected *Paphiopedilum spicerianum* (Rchb. f.) Pfitz. is a fast disappearing species found in the Cachar District; it was earlier rediscovered by the research group under Prof. B. K. Dutta from Nimata Pahar (Borail Wild Life Sanctuary) during the year 2005 (Bhattacharjee *et al*., 2005). However, after repeated surveys during the present work (2010-2013), no specimen of this plant could be collected from the recorded wild habitat. Subsequently, we procured two

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Fig.1. *Paphiopedilum spicerianum* (Rchb.f.) Pfitz.: plant in bloom.

specimens of the said plant from the Botanical Survey of India (Eastern Regional Centre), Shillong. Presently, an attempt was made to propagate the species using seeds, leaves and shoot tips as explants, so that it may be disseminated to its wild habitat for conservation purpose.

Materials and Methods

Experimental Site

In the present work, experiments were carried out in the Sreedhar Apex Biotech, Bagbahar, Cachar, Assam.

Sample Collection

Two specimens of the orchid species were collected from the Botanical Survey of India (Eastern Regional Centre), Shillong and they were cultivated in pots under the green house conditions of the Department of Ecology and Environmental Science, Assam University, Silchar, Assam.

Explant Source

Shoot tips, leaves and seeds from green capsules (pods) were used as explants in the experiments and these were collected from the plant specimens growing in the green house of the Department of Ecology and Environmental Science, Assam University, Silchar.

Sterilization

The explants were thoroughly washed under slow running tap water for 15 min., washed in tween 80 (1 drop in 200 ml sterile distilled water), and subsequently rinsed 3-4 times with sterile distilled water (SDW) inside the Bio safety cabinet. Subsequently different explants were treated with 70% alcohol for varied time duration (leaves and shoot tips for 30 sec and the green capsules for 1 min), and rinsed in SDW.

Culture Medium

Murashige and Skoog (1962, MS) medium [readymade dehydrated medium (HIMEDIA)] was used for the *in vitro* propagation of *Paphiopedilum spicerianum* (Rchb. f.) Pfitz. The medium was supplemented with different concentration of IAA (Indole-3-acetic acid; 0.1-1 mgl^{-1}), IBA (Indole-3-butyric acid; 0.1-1 mgl^{-1}), BAP (6-benzyl-amino-purine; 0.5-5 mgl^{-1}), 2.4-D (2,4-dichloro-phenoxy-acetic acid; 0.1-1 mgl^{-1}), Kinetin (KN; 0.5-5 mgl^{-1}), and TDZ (Thiadiazuron; 0.1-0.4 mgl^{-1}) individually or in different combinations.

The pH of the medium was adjusted between 5.6 and 5.8. Agar was dissolved by boiling the mixture and about 20 and 50 ml medium was dispensed into each culture tube and flask respectively. After preparation, the culture medium was autoclaved at 121^{0}C for 20 min at 15 lb/sq. inch pressure. Then the medium was allowed to cool and kept under Bio safety cabinet for 42 hrs. If no contamination was observed, then the replicated media were used for inoculation.

Inoculation and Culture

The sterilized explants were prepared for culturing in varied ways: i) The leaf explants were prepared by aseptically removing the entire mid rib of the leaves. The resulting strips of the leaf were cut into small pieces (10 mm^2), and these explants were placed on the sterile tissue paper to dry, followed by their inoculation on MS medium and its different combinations; ii) the sterilized capsules were cut open longitudinally with a sharp sterilized surgical blade and subsequently the powdery mass of yellowish seeds was inoculated on the surface of the culture media with the help of a long spatula.

All these operations were done aseptically in a Bio-safety cabinet. All the culture vessels were kept at 25 $\pm$2 ^{0}C under 16 hour light and 8 hours dark period by white fluorescent tubes with an intensity of 1000 Lx. After every 15 days interval, explants were transferred into fresh medium for better growth.

Growth Parameters

The growth parameters taken for observation were days required for callus formation and weight of the callus.

Results and Discussion

The response of different explants used in the present experiment for *in vitro* propagation of *Paphiopedilum spicerianum* (Reichb. f.) Pfitz. varied with the nutritional recipe used. The seeds are

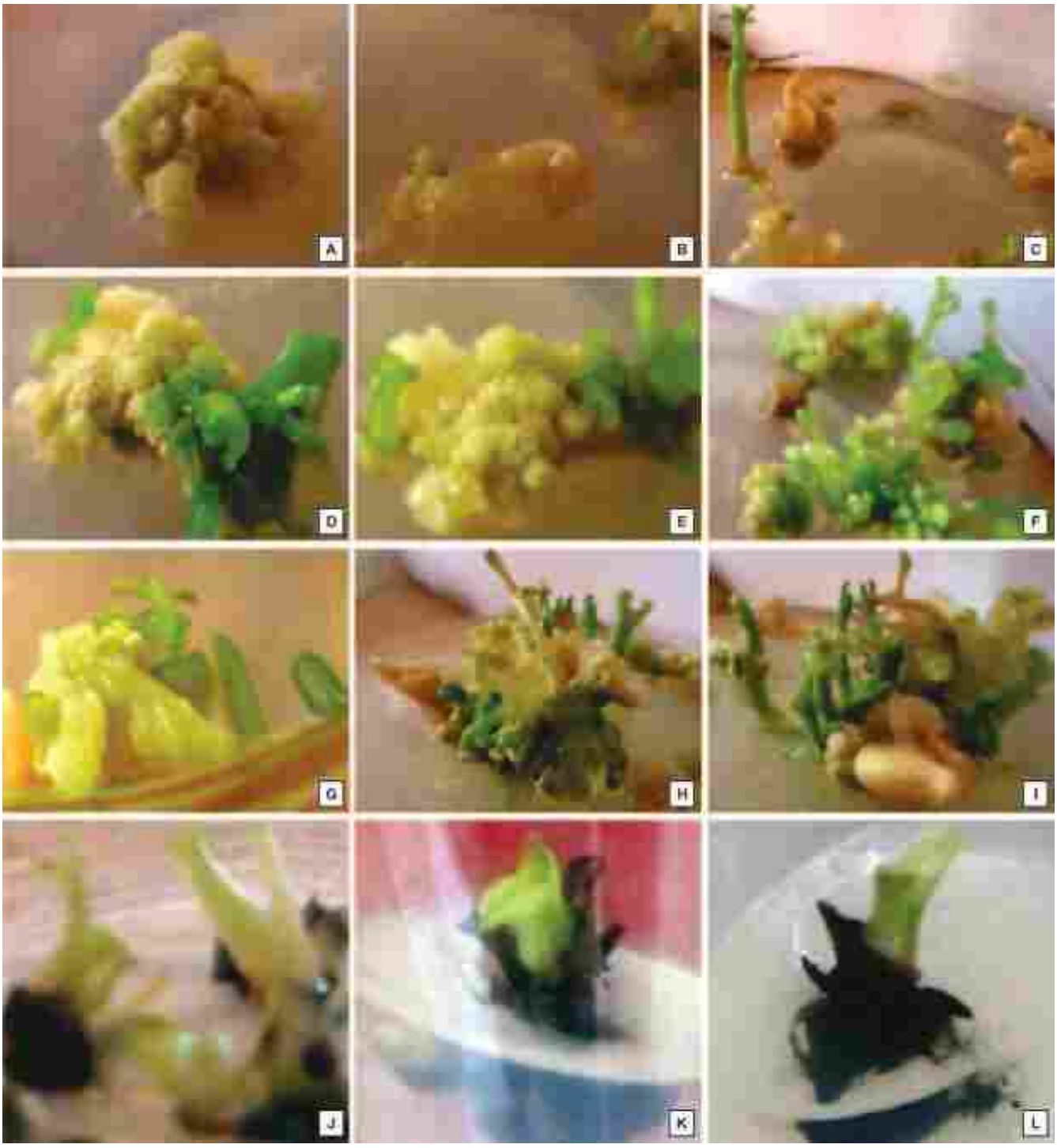

Fig.2.: *Paphiopedilum spicerianum* (Reichb. f.) Pfitz. *in vitro* propagation in MS Medium with Thiadiazuran (mgl^{-1}): A, B, C, Callus formation; D, E, F, Initiation of multiplication; G, H, I, Multiple shoot formation; J, Leaf initiation; K and L, development of leaf.

commonly used for *in vitro* propagation of *Paphiopedilum* orchids for the large scale production as the multiplication rate from the shoot tip derived explants is very low. Presently, the seeds obtained from undehisced capsules invariably failed to respond. According to Arditti and Ernst (1993), *Paphiopedilum* orchids have stringent requirements for seed germination. But little is known about their specific requirements.

Although young leaf and shoot tip explants had shown regeneration response via callusing, the formation and development of the callus was found to be extremely

slow and poor. It was reported earlier that the slow growth and low multiplication rate have been the important limiting factors of the *in vitro* culture of slipper orchids (Thongpukdee *et al*., 2013). However, presently the shoot tip explants gave better results as compared to young leaf explants. Though regeneration occurred on the leaf explants in MS medium supplemented with the combination of BAP (0.5 mgl^{-1}) + IAA (0.1 mgl^{-1}) + IBA (0.1 mgl^{-1}); KN (3.5 mgl^{-1}) + IBA (0.7 mgl^{-1}); BAP (2.5 mgl^{-1}) + 2, 4-D (0.5 mgl^{-1}), the size and weight of the callus varied with the growth stimulus (Fig. 2 A-L).

In case of shoot tip explants, the regeneration occurred via callus formation in medium supplemented with only TDZ at different concentrations (*i.e*., 0.10, 0.15, 0.20, 0.25, 0.30, 0.35 mgl^{-1}). The initiation and development of the callus was favored with increase in the concentration of TDZ; the best results were obtained in medium with TDZ at 0.20 mgl^{-1}. The success of micropropagation of *Paphiopedilum* orchids through direct shoot regeneration depends on the optimization of culture media to a large extent (Udomdee, 2012), MS and MS modified medium is generally used in the *in vitro* propagation of *Paphiopedilum* orchid species (Chen *et al*. 2002; Chen *et al*, 2004; Hong *et al.,* 2008). Although some previous literature suggested that TDZ inhibits the shoot proliferation and rooting (Huang *et al*., 2001), but Chen *et al.* (2002), reported that 0.45 μM TDZ and 4.52 μM 2,4-D supplemented in MS modified half strength medium enhanced the percentage of explants produced in the newly regenerated shoots from the stem nodal explants. Stewart and Button (1975) used shoot apex of *Paphiopedilum* to induce callus, while Chen *et al.* (2002, 2004) also reported that induction of multiple shoots could be achieved from the stem and leaf explants of *P. philippinense* (hybrids PH59 and PH60) cultured on MS medium supplemented with 4.52 µM 2,4-dichlorophenoxy acetic acid (2,4-D) and 4.54 ıM Thiadiazuran (TDZ). Lin *et al.* (2000) reported that the TDZ induced calli from the seed derived protocorm of *Paphiopedilum* hybrid orchid. TDZ seems to play a crucial role in the dedifferentiation of the orchid explants. Chang and Chang (1998) showed that presence of TDZ in the MS basal medium was essential to obtain the long term totipotent callus culture.

The time duration for the callus formation was found to vary with the explants and concentration of the plant growth regulator(s) used in the medium. The callus formation was observed within 34 days of inoculation from the shoot tip, but it took 48 days from the young leaf explants. The multiple micro shoots were transferred to MS medium with the addition of TDZ (0.2 mgl^{-1}), coconut water (40 mll^{-1}) and different plant growth regulator(s) (*i.e*., IBA, IAA, BAP and KN) at different concentrations. The cultures were kept in culture room at 18 ^{0}C. The leaf formation was observed only in IAA (0.2 mgl^{-1}) supplemented medium. Leaf-like structure were also observed in medium containing BAP (0.3 mgl^{-1}), the micro shoots, however, could not survive and died after 22 days of their transfer in the culture medium.

Though *Paphiopedilum* orchids are propagated through the division of axillary buds from the mother plants. It is time consuming, extremely unproductive and unreliable for commercialization or conservation purposes (Liao *et al*., 2011; Ng and Saleh, 2011). Nhut *et al*. (2007) reported that TDZ was the most effective than BA for the shoot induction in *Paphiopedilum delentii*. Shoot proliferation from leaf tissue is very common in ferns and dicotyledons, but it is very less in monocotyledons (Xiong and Wu, 2003). An appropriate medium, quality and quantity of plant growth regulators are important factors during seed culture and regeneration of leaf and stem nodal explants (Pathak *et al.,* 2001, 2012; Vij and Pathak, 1990; Vij *et al.,* 1994), in orchids and *Paphiopedilum* Delrosi, in particular (Thongpukdee *et al*., 2013).

Conclusion

Presently, shoot tip and leaf explants were successfully used for regeneration in *Paphiopedilum spicerianum* (Rchb.f.) Pfitz. and the present study strongly supports that this *in vitro* propagation technique may help in mass propagation and conservation of this endangered orchid species.

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EFFECTS OF PLANT GROWTH REGULATORS AND EXPLANTS ON PROPAGATION OF A MONOPODIAL AND SYMPODIAL ORCHID: A STUDY *IN VITRO*

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Abstract

In vitro culture response of different plant growth regulators was assessed in two indigenous orchid species namely, *Dendrobium aphyllum* and *Rhynchostylis retusa* of two different growth groups *i.e.*, sympodial (*D. aphyllum*) and monopodial (*R. retusa*) orchid for optimum callus induction and plantlet regeneration. Leaf, nodal and inter-nodal segments of *in vitro* grown seedlings of both the species were cultured on MS (Murashige and Skoog, 1962) and PM (Phytamax Sigma Chemical Co., USA) media supplemented with different concentrations and combinations of plant growth regulators (PGRs). The inter-nodal segments of both the species and leaf segments of *D. aphyllum* failed to response to any of the PGR combinations, whereas, leaf and nodal segments of *R. retusa* and nodal segments of *D. aphyllum* gave positive response producing different types of calli as well as multiple shoot buds depending on nutritional stimulus. In subsequent subcultures, the callus tissues underwent differentiation *via* PLB formation on a broad spectrum of PGR supplemented media. The nodal explants of both the species produced multiple shoot buds. Thus both embryogenesis and organogenesis took place and PGR combinations played an important role in the differentiation of the tissues. High concentration of auxins and low concentration of cytokinins was proved to be effective for differentiation in *R. retusa,* such effects, however, could not be observed in *D. aphyllum*. The well-rooted *in vitro* plantlets were transferred to pots containing a potting mixture composed of saw dust, coconut coir, and coal pieces with ~90% survival in outside environment.

Introduction

DUE TO ruthless collection by increasing orchid lovers, over-exploitation for medicinal purposes, deforestation for urbanization, destruction of habitats by reclamation, shifting cultivation, killing of pollinators, and unauthorized trade has led to reduction in natural populations of the members of the family Orchidaceae, which is one of the largest and most diverse of all flowering plant families (Dressler, 1993; Hágsater and Dumont, 1996; Koopowitz and Hawkins, 2012; Seaton *et al.*, 2013; Swarts and Dixon, 2009). Meanwhile many orchid species have become extinct and many others are on the verge of becoming rare and endangered. Considering the present status of orchids, the family Orchidaceae as a whole was included in the CITES (Convention on International Trade in Endangered Species) Appendix II, hence, mass propagation, eco-rehabilitation and conservation of orchids are utmost necessary. Application of *in vitro* techniques might be the best solution for mass propagation and conservation of this versatile group of plants. Although some techniques have already been devised for propagation and conservation of orchids, further perfection of the protocols is still required for specific orchids. The major obstacles for mass propagation of orchids for commercial purposes as well as conservation are: 1) non availability of efficient and reliable protocols for seed germination; 2) poor understanding of early seedling growth and development; 3) obligate mycorrhizal association for natural seed germination; 4) selection of suitable explants for micropropagation, scaling-up and automation of the techniques; 5) very slow and laborious vegetative propagation; 6) species specificity to culture medium; 7) limited germination success under controlled laboratory conditions of many rare and endangered orchids species, and 8) high mortality of *in vitro* seedlings during transplantation. Modern biotechnological approaches such as tissue culture, production of synthetic seeds, cryopreservation are routinely used for mass propagation, genetic improvement as well as conservation of plant germplasm. Establishment of simple, reliable, economical, rapidly multiplying and highly reproducible protocol is very important for commercial cultivation and conservation of orchids. Development and deployment of new technologies is very important for improving rapid and mass propagation and conservation of orchids. The modification of traditional tissue culture medium by adding specific plant growth regulators (PGRs), different complex additives (peptone, yeast extract, banana pulp *etc*.), automation of plant production through adapting bioreactor system and culture conditions are required for development of efficient germination and micropropagation protocols. Recently, orchids have become the center of attention of new areas of research, including genetic

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engineering, functional genomics, proteomics, and metabolomics, all of which require standardized micropropagation techniques. The successful application of the new approaches will help in further improvement of orchids and orchid products. Such research is very important in the context of conservation of plant biodiversity.

Based on the growth pattern, orchids have been classified into two major groups *viz*., i) Monopodial, and ii) Sympodial. The first group is characterized by a single unbranched axis of growth. On the other hand, multibranching rhizome or stem with axillary shoots exhibit the latter group. These two different growth patterns may be the result of differences in inherent endogenous hormonal levels and their functions. The application of exogenous plant growth regulators plays an important role on tissue differentiation and it depends on the nature of species, explant and, concentration and combinations of PGRs. Considering these, the present investigation was undertaken with a view to studying 1) the responses of a monopodial and a sympodial orchid to tissue culture; 2) explant-PGR interaction in the process of organogenesis and embryogenesis, and 3) establishment of simple, reliable, economical, rapidly multiplying and highly reproducible protocol for *Dendrobium aphyllum* and *Rhynchostylis retusa.*

Materials and Methods

Explants, Nutrient Media and Culture Conditions

Four months old undehisced green capsules of *Dendrobium aphyllum* and *Rhynchostylis retusa* were collected from the hilly forest of Cox-S-Bazar district (200 m above mean sea level) of Bangladesh. Two different nutrient media namely, MS (Murashige and Skoog, 1962) and PM (Phytamax®, Catalog No. P-6793, Sigma Chemical Co., USA) supplemented with 2–3% (w/v) sucrose and with or without peptone (2.0 gl^{-1}) were used for seed culture. Nodal, inter nodal and leaf segments (0.5–1.0 cm in size) of *in vitro* raised seedlings were used for further experiments. MS or PM medium fortified with different concentrations and combinations of auxins *i.e*., IAA (0.5 – 2.5 mgl^{-1}), NAA (1.0–2.5 mgl^{-1}), Picloram (pic; 0.5–2.0 mgl^{-1}) and Cytokinins *i.e*., Kinetin (0.5 – 2.5 mgl^{-1}), and Zeatin (ZN; 1.0 – 1.5 mgl^{-1}). The pH of the medium was adjusted at 5.8 before autoclaving at 121°C at 117 kPa for 20 min. Different types of glass vessels including test tubes (1.5 × 15 cm), culture bottles, conical flasks (100–150 cc) were used. Culture vessels with inoculated explants were maintained in a culture room where a cycle of 14/10 h light-dark at 60 $mmolm^{-2}s^{-1}$ provided by cool white fluorescent lamps (Philips Truelight 36w/86 65001 K B7, Philips, India), and 60% RH at 25 ± 2°C. Regular subculturing was done at 20–25 days interval.

Establishment of Axenic Culture

The capsules were rubbed with a hair brush under running tap water to remove dust particles and then surface sterilized by 0.1% (w/v) $HgCl_2$ solution for 10 min with occasional agitation and washed thoroughly with sterile distilled water. Finally, the capsules were dipped in 70% ethanol for 1 min. followed by flaming for 1–2 sec. The surface sterilized capsules were placed on a sterile filter paper and cut longitudinally with a sterile surgical blade and the seeds were cultured on the surface of the agar-gelled medium. All the operations were performed in a laminar air-flow cabinet. When seeds germinated and protocorms came out, these were taken out aseptically from the culture vessels and the masses of protocorms were sub-cultured to fresh culture media for further growth. The nodal, inter nodal and leaf segments of *in vitro* grown seedlings (5-6 cm in size) were used for micropropagation as well as study the effects of different PGRs on *in vitro* morphogenesis of the two different growth groups *i.e*., monopodial and sympodial orchids. The callus, protocorm-like bodies (PLBs) or shoot buds developed from the cultured explants were subcultured regularly to fresh nutrient media.

Rooting and Transplantation of Seedlings

Seedlings grown in *in vitro* culture conditions exhibited fewer roots, which may not support successful acclimatization on their transfer to *ex vitro* conditions. On the other hand, shoot buds that produced from nodal explants did not produce any roots. Thus, for induction of stout root system these were grown on different rooting media made up of half strength PM medium supplemented with IAA (0.5–1.0 mgl^{-1}). The well-rooted plantlets were taken out from the culture vessels and washed thoroughly under running tap water for removal of agar medium attached to the root surface and transferred to pots containing a potting mixture of saw dust, coconut coir, and coal pieces at 1:1:2 (w/w).

Data Collection and Statistical Analysis

The experiments were designed following Complete Randomize Block Design (CRD). Five replicates were taken per treatment. The effects of different PGRs in induction of callus, shoot buds, PLBs and roots in the *in vitro* experiments were tested applying Duncan's multiple range test ($P > 0.5$) in one way ANOVA. The

statistical analyses were performed using the Statistica ver. 7 (Statsoft, Tulsa, USA). The experiments were repeated thrice.

Results and Discussion

Germination of Seeds

The seeds of *D. aphyllum* and *R. retusa* germinated on both MS and PM media. Maximum seed germination (97%) of *D. aphyllum* was recorded in PM medium (Fig. 1A) whereas seeds of *R. retusa* preferred MS medium for best seed germination (95%) (Fig. 1B). Species-specific media for seed germination have been reported in orchids (cf. Arditti and Ernst, 1984; Pathak *et al.,* 2001). Different nutritional recipes have been suggested by workers in various orchid species such as *Acampe papillosa* (Piri *et al.,* 2013), *Aerides multiflora* (Pathak *et al.,* 2005), *Aerides multiflora*, *Rhynchostylis retusa*, *Saccolabium calceolare* and *Vanda testacea* (Vij *et al*., 1981), *Cymbidium aloifolium* (Hossain *et al*., 2009), *Cymbidium elegans* (Sharma and Tandon, 1990), *Cymbidium giganteum* (Hossain *et al*., 2010), *Cymbidium iridioides* (Jamir *et al*., 2002), *Cymbidium macrorhizon* (Vij and Pathak, 1988), *C. pendulum* (Pathak and Vij, 2007), *Dactylorhiza hatagirea* (Vij *et al.,* 1995), *Dendrobium aphyllum* (Hossain *et al.,* 2013) *Dendrobium chrysanthum* (Anuprapha and Pathak, 2012), *Dendrobium farmeri*, *D. primulium*, *D. moschatum*, and *D. fimbriatum* var. *oculatum* (Devi *et al*., 1990), and *Gastrochilus calceolaris* (Pathak *et al*., 2011), *Goodyera biflora* (Pathak *et al.,* 1992)*, Satyrium nepalense* (Chauhan *et al.,* 2010), and *Vanda coerulea* (Aggarwal *et al.,* 2008). Seed germination occurred within 4–5 wks and developed seedlings in subsequent subculture on the same medium (Fig. 1C-D).

Fig. 1. A-D. Seed germination in *D. aphyllum* and *R. retusa*: A, Germination of seeds of *D. aphyllum* on PM medium; B, Germination of seeds of *R. retusa* on MS medium; C, Development of seedlings of *D. aphyllum* and *R. retusa,* respectively.

Table1. Response of different explants of *R. retusa* and *D. aphyllum* to different PGRs and their combinations.

Species	Basal Medium	Explants	IAA (1.5 mgl^{-1})	NAA (1.5 mgl^{-1})	pic (1.5 mgl^{-1})	BAP (1.5 mgl^{-1})	ZN (1.5 mgl^{-1})	KN (1.5 mgl^{-1})	BAP (1.5 mgl^{-1}) + IAA (2.5 mgl^{-1})	IAA (2.5 mgl^{-1}) + ZN (0.5 mgl^{-1})	IAA (1.5 mgl^{-1}) + KN (2.5 mgl^{-1})	NAA (2.5 mgl^{-1}) + BAP (1.5 mgl^{-1})	NAA (2.5 mgl^{-1}) + ZN (1.0 mgl^{-1})	NAA (1.5 mgl^{-1}) + KN (1.5 mgl^{-1})	pic (1.5 mgl^{-1}) + BAP (1.5 mgl^{-1})	pic (2.0 mgl^{-1}) + ZN (1.5 mgl^{-1})	Pic (1.5 mgl^{-1}) + KN (1.5 mgl^{-1})
Rhynchostylis retusa -	MS	LS	C	C	C	-	C	-	-	-	-	-	PLBs	-	-	PLBs
	NS	C	-	C	-	-	-	-	MSB	-	MSB	MSB	-	-	MSB	-
	IS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	PM	LS	C	C	C	-	C	-	-	-	-	-	PLBs	-	-	PLBs	-
		NS	C	-	C	-	-	-	-	MSB	-	MSB	MSB	-		MSB	-
		IS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dendrobium aphyllum -	MS	LS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	NS	-	-	C	C	-	-	MSB	MSB	MSB	MSB	MSB	MSB	MSB	MSB	MSB	MSB
	IS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	PM	LS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		NS	-	-	C	C	-	-	MSB	MSB	MSB	MSB	MSB	MSB	MSB	MSB	MSB
		IS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Results are based on observations recorded from 15 culture vessels; LS , Leaf segment; NS, Nodal segment; IS, Inter-nodal segment; C,Callus; MSB, Multiple shoot buds; PLBs, Protocorm like bodies.

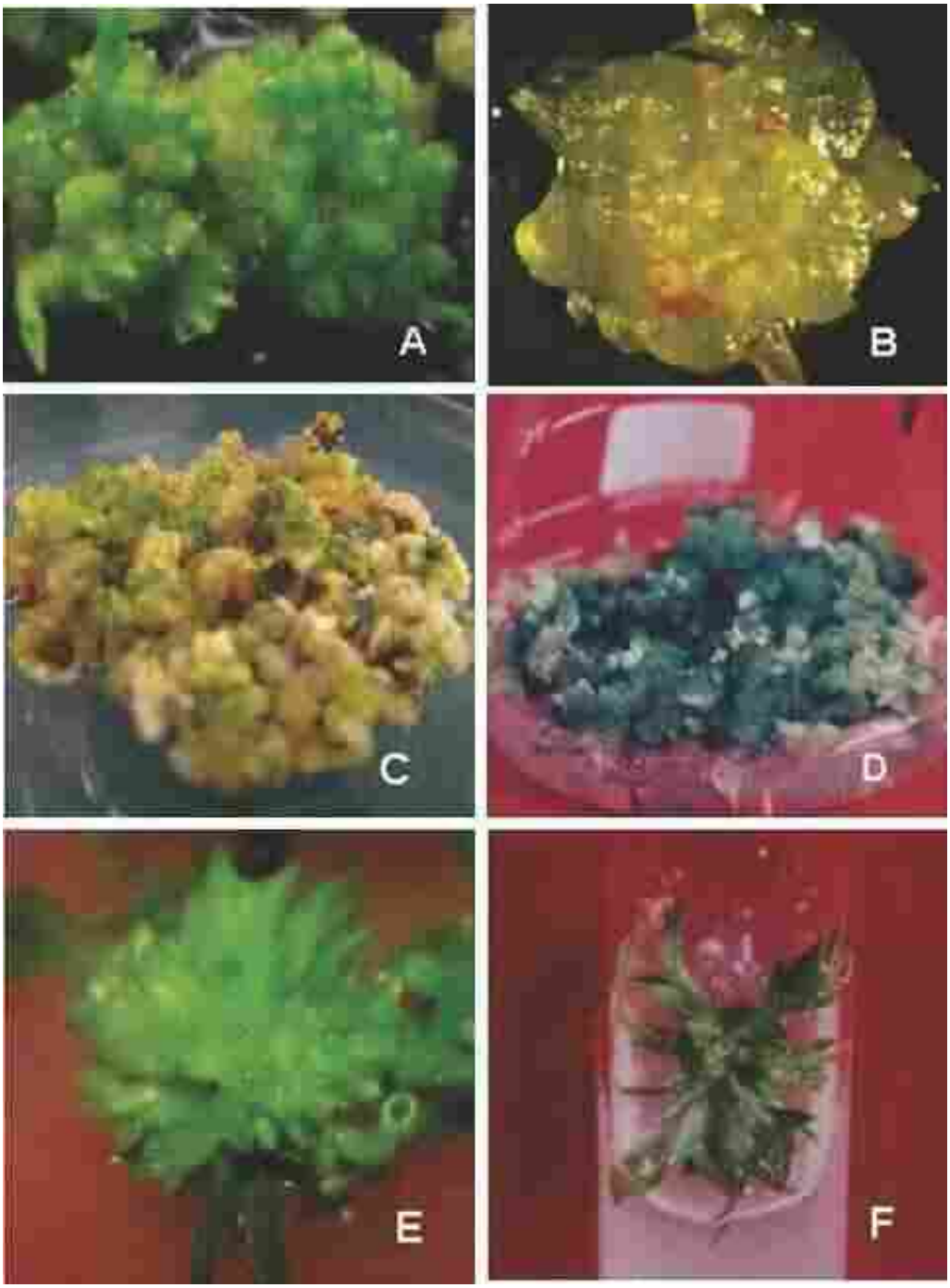

Fig. 2. A-F. *In vitro* propagation of *D. aphyllum* and *R. retusa* using leaf and nodal segments: A, Induction of PLBs in leaf segments of *R. retusa*; B-C, Induction of green, compact callus and loose friable callus in leaf segments of *R. retusa*; D, Green and compact callus differentiated into PLBs; E-F, Induction of multiple shoot buds in nodal segments of *R. retusa,* and *D. aphyllum* respectively.

Effects of PGRs on In Vitro Differentiation

Different explants *i.e*., leaf, nodal and inter-nodal segments of *in vitro* grown seedlings of the two species of different growth types gave differential response to the different PGR concentrations and combinations (Table1). The effects of PGRs on different explants are briefly described below:

Effects of PGRs on Leaf Explants

The response of leaf explants of the two species was quite different in same PGRs supplemented media. In case of *R. retusa,* the leaf segments produced different

Table 2. Effects of subculturing of loose and friable callus tissues on broad spectrum of different PGRs supplemented media.

Basal medium	PGRs	Nature of response		Time (days) required for induction of shoot buds	
		R. retusa	*D. aphyllum*	*R. retusa*	*D. aphyllum*
MS	NAA (2.0 mgl^{-1}) + BAP (2.5 mgl^{-1})	Callus multiplied without differentiation	Shoot bud formation	_	22-25
	IAA(2.5 mgl^{-1}) + BAP (1.5 mgl^{-1})	Shoot bud formation	Shoot bud formation	42-46	32-35
	pic (1.0 mgl^{-1}) + BAP (2.0 mgl^{-1})	Callus multiplied without differentiation	Shoot bud formation	-	2325
	IAA (2.0 mgl^{-1}) + ZN (0.5 mgl^{-1})	Shoot bud formation	Callus multiplied without differentiation	40-42	-
	NAA (2.0 mgl^{-1}) + ZN (1.0 mgl^{-1})	PLB formation	Callus multiplied without differentiation	45-50	-
	pic (2.0 mgl^{-1}) + ZN (1.5 mgl^{-1})	PLB formation	Shoot bud formation	45-48	30-35
PM	NAA (2.0 mgl^{-1}) + BAP (2.5 mgl^{-1})	Callus multiplied without differentiation	Shoot bud formation	_	20-22
	IAA (2.5 mgl^{-1}) + BAP (1.5 mgl^{-1})	Shoot bud formation	Shoot bud formation	42-46	30-32
	pic (1.0 mgl^{-1}) + BAP (2.0 mgl^{-1})	Callus multiplied without differentiation	Shoot bud formation	_	25-27
	IAA (2.0 mgl^{-1}) + (ZN 0.5 mgl^{-1})	Shoot bud formation	Callus multiplied without differentiation	35-38	-
	NAA(2.0 mgl^{-1} + ZN (1.0 mgl^{-1})	PLB formation	Callus multiplied without differentiation	40-42	-
	pic (2.0 mgl^{-1}) + (ZN 1.5 mgl^{-1})	PLB formation	Shoot bud formation	45-48	30-34

types of callus tissue as well as PLBs depending on the PGRs. While the leaf segments of *D. aphyllum* did not give any response to any of the media combinations used. Moreover, it became brown within 3-4 wks of culture and finally died. Induction of PLBs *i.e.*, direct embryogenesis took place in *R. retusa* when the leaf segments were grown on MS or PM medium fortified with i) NAA (2.5 mgl^{-1}) + ZN (1.0 mgl^{-1}) and ii) pic (2.0 mgl^{-1}) + ZN (1.5 mgl^{-1}) (Fig. 2A). Based on the study of the effects of different PGRs *i.e.*, auxins (NAA, IAA, IBA, 2, 4-D) and cytokinins (ZN, KN, BAP, TDZ) *in vitro*, it was documented that PGRs activate the proliferative loci of the leaf segments and regulate subsequent development into plantlets (Arditti, 2008; Vij and Pathak, 1990). Cytokinins have been found to be essential for regeneration from leaf explants in *Acampe praemorsa* (Nayak *et al.*, 1997) and *Aerides maculosum* (Murthy and Pyati, 2001). The embryo formation on leaf explants was retarded by auxins IAA, IBA, NAA, and 2, 4-D but promoted by cytokinins like 2iP, ZN, KN, BAP and TDZ (Chen and Chang, 2001). Chen and Chang (2004) tested the effect of auxins

Table 3. Rooting response in shoot buds of *D. aphyllum and R. retusa.*

Culture medium	Number of roots/shoot buds (mean ± S. E.)		Length of roots (cm) after 30days of culture (mean ± S. E.)	
	D. aphyllum	*R.retusa*	*D. aphyllum*	*R. retusa*
PM	2.60 ± 0.16^{c}	2.70 ± 0.15^{c}	2.52 ± 0.12^{e}	2.50 ± 0.12^{e}
½PM	3.00 ± 0.26^{c}	2.90 ± 0.10^{c}	2.80 ± 0.15^{de}	2.88 ± 0.16^{cde}
½PM + IAA(0.5 mgl^{-1})	5.12 ± 0.29^{a}	5.10 ± 0.25^{a}	4.44 ± 0.16^{a}	4.33 ± 0.10^{a}
½PM + IAA(1.0 mgl^{-1})	4.20 ± 0.25^{b}	4.10 ± 0.31^{b}	4.57 ± 0.26^{a}	4.79 ± 0.23^{a}

Mean values within a column followed by the same letters are not significantly different at $P=0.05$ according to Duncan's multiple range test.

Fig. 3. A-D. *In vitro* propagation of *D. aphyllum* and *R. retusa* using nodal segments: A- B, Induction of green, compact callus in nodal segments of *R. retusa* and *D. aphyllum* respectively; C-D, Lose and friable callus differentiated into PLBs when grown in broad spectrum of PGRs in *R. retusa* and *D. aphyllum.* respectively.

(IAA, 2, 4-D), two auxin transport inhibitors (TIBA and quercetin) and an auxin antagonist (PCIB) on direct somatic embryogenesis from leaf tip region. Except for TIBA, all the other growth regulators retarded embryo formation. Beneficial effect of using combination of auxins and cytokinins has been demonstrated in *Oncidium* (Chen and Chang, 2000), *Renanthera imschootiana* (Seeni and Latha, 1992), *Rhynchostylis retusa* (Vij *et al*., 1984), *Vanda coerulea* (Seeni and Latha, 2000), *Vanda* hybrid (Mathews and Rao, 1985), and *Vanda spathulata* (Decruse *et al*., 2003).

The leaf explants also produced different types of callus tissues depending on the PGRs (Table 1). Green and compact callus were produced in medium containing i) IAA (1.5 mgl^{-1}) and ii) NAA (1.5 mgl^{-1}) (Fig. 2B), while loose and friable callus were produced in medium containing i) pic (1.5 mgl^{-1}) and ii) Zn (1.5 mgl^{-1}) (Fig. 2C). After three subsequent subcultures, the green and compact callus differentiated into PLBs (Fig. 2D). On the other hand, loose and friable callus was, however, failed to undergo either organogenesis or embryogenesis but proliferated without differentiation. Along with the PGRs, orientation of explants on the media, physiological age of leaf and source of leaf are crucial factors for regeneration *in vitro*. The available reports of the physiological age of explants indicated that young leaves respond better than the older ones with respect to the number of regenerants developed upon inoculation in a suitable medium (Chugh *et al*., 2009; Chung *et al*., 2005; Pathak and Vij, 2001; Vij and Pathak, 1990; Vij *et al*., 1986). Available reports affirmed that young leaves show better response in *Vanda* Kasem's Delight Tom Boykin (Vij *et al*., 1994) and *Vanda coerulea* (Vij and

Agarwal, 2003). Tenjensangba and Deb (2005) reported that young leaves (15 weeks old) of *Cleisostoma racimeferum* develop PLBs *in vitro* while older leaves were unable to regenerate.

Effects of PGRs on Nodal Explants

The response of the nodal segments of the two different species to different PGRs and their combinations also differed highly as leaf segments. The nodal segments of *R. retusa* produced multiple shoot buds on i) IAA (2.5 mgl^{-1}) + ZN (0.5 mgl^{-1}), ii) NAA (2.5 mgl^{-1}) + ZN (1.0 mgl^{-1}), iii) NAA (2.5 mgl^{-1}) + BAP (1.5 mgl^{-1}), and iv) pic (2.0 mgl^{-1}) + ZN (1.5 mgl^{-1}). But the average number of multiple shoot buds induced per explant varied in different PGR combinations. The maximum number of multiple shoot buds per explant was recorded on MS + sucrose [2% (w/v)] + IAA (2.5 mgl^{-1}) + ZN (0.5 mgl^{-1}) (Fig. 2E). This finding indicates that high concentration of auxin and low concentration of cytokinin enhanced multiple shoot bud formation in nodal segments of *R. retusa*. On the other hand, the nodal segments of *D. aphyllum* also underwent direct organogenesis producing multiple shoot buds on a number of media compositions used (Table 1) and the maximum number of multiple shoot buds were produced in PM + IAA (1.5 mgl^{-1}) + BAP (2.5 mgl^{-1}) (Fig. 2F). This finding indicates that low concentration of auxin and high concentration of cytokinin enhanced multiple shoot bud formation in nodal segments of *D. aphyllum*. The above findings clearly indicated that the nature and magnitude of the requirement of PGRs is different for monopodial and sympodial orchids. A number of earlier reports demonstrated that, the combinations, concentrations,

Fig.4. A-D. Root induction and seedling development in *D. aphyllum* and *R. retusa*: A-B, Induction of stout root system in the multiple shoot buds as well as PLB derived seedlings of *R. retusa* and *D. aphyllum* respectively (1 = ½PM medium, 2 = ½PM + 0.5 mgl^{-1} IAA and 3 = ½PM + 1.0 mgl^{-1} IAA); C-D, Establishment of *in vitro* grown seedlings of *R. retusa* and *D. aphyllum* in outside pots, respectively.

and the ratio of exogenous PGRs supplements are critically important for morphogenetic response in orchids (Begum *et al*., 1994; Chang and Chang, 1998; Deb and Pongener, 2012; Hossain *et al*., 2013a; Huan *et al*., 2004; Mahendran and Bai, 2012; Malabadi *et al*., 2008; Teixeira da Silva *et al*., 2006, 2007a, b ; Teng *et al*., 1997; Vij *et al.,* 1994)

The nodal segments of both the species also produced different types of callus tissues in some of the PGRs combinations (Table. 2). In case of *R. retusa* green and compact callus were induced in i) IAA (1.5 mgl^{-1}) and ii) Pic (1.5 mgl^{-1}) (Fig. 3A). While, in case of *D. aphyllum* green and compact callus were induced in i) BAP (1.5 mgl^{-1}) and ii) pic (1.5 mgl^{-1}) (Fig. 3B). These findings demonstrated that for induction of green callus in *R. retusa* needs exogenous supply of both auxin / cytokinin while *D. aphyllum* needs only cytokinins. After three subsequent subcultures, the green and compact callus differentiated into PLBs. Thus, indirect embryogenesis was observed. This type of response was not only due to exogenous supply of hormones but also dependent on the endogenous level of hormones. The inter-nodal segments of both the species did not give any response to any one of the media used for leaf and node culture. Thus the overall results indicated that different explants of the same species and the same explants of the two species gave different response depending on various PGRs and their combinations. Appropriate combination of cytokinins with auxins was critically important in induction of somatic embryos or PLBs in orchids as also reported earlier by some workers (Huan *et al*., 2004; Malabadi *et al*., 2008; Roy and Banerjee, 2003; Teixeira da Silva *et al*., 2005, 2006, 2007a, b; Teng *et al*., 1997). PLB production is comparatively more efficient than organogenesis, easy to carry out, and can provide large number of propagules for mass propagation within a short period of time (Hossain *et al*., 2010).

Culture of Loose and Friable Callus

As mentioned earlier, the explants in both the species produced loose and friable callus in some of the PGR combinations and those failed to undergo differentiation, proliferated profusely. For induction of organogenesis or embryogenesis, these callus tissues were further grown in broad spectrum of PGRs supplemented media (Table 2). The callus of *R. retusa* differentiated into PLBs when grown in i) IAA (2.5 mgl^{-1}) + BAP (1.5 mgl^{-1}) and ii) IAA (2.0 mgl^{-1}) + ZN (0.5 mgl^{-1}) iii) NAA (2.0 mgl^{-1}) + ZN (1.0 mgl^{-1}) and iv) pic (2.0 mgl^{-1}) + ZN (1.5 mgl^{-1}) (Fig. 3C). On the other hand, the callus of *D. aphyllum* produced multiple shoot buds in i) NAA (2.0 mgl^{-1}) +BAP (2.5 mgl^{-1}), ii) IAA (2.5 mgl^{-1}) + BAP (1.5 mgl^{-1}), iii) pic (1.0 mgl^{-1}) + BAP (2.0 mgl^{-1}), and iv) pic (2.0 mgl^{-1}) + ZN (1.5 mgl^{-1}) (Fig. 3D). These findings indicated that the concentrations and combinations of PGRs switched the process of differentiation. The comparative results of *in vitro* culture based on the growth pattern of the two species showed remarkable differences. High concentration of auxins and low concentration of cytokinins proved to be effective for differentiation in monopodial orchid, *R. retusa* but such observations, however, could not be made in sympodial orchid *D. aphyllum*.

Rooting and Acclimatization of Plantlets

For induction of stout root system, the multiple shoot buds as well as PLB derived seedlings were grown on different rooting media. Half strength agar solidified PM medium fortified with IAA (0.5-1.0 mgl^{-1}) were used for this purpose. Medium fortified with IAA (0.5 mg l^{-1}) proved to be most effective for induction of well developed root system for both MSBs (>4/MSB) and seedlings (>5/seedling) (Table 3; Fig. 4A, B). The shoot buds or seedlings also produced roots in IAA (1.0 mgl^{-1}) containing combination but those roots were very thin and long, making them fragile and prone to damage during *ex vitro* transfer. PM medium without any PGRs produced a few stunted roots per MSB or seedling. It is pertinent to mention here that roots developed in PLB sourced seedlings were stronger and healthier than those developed in MSBs. Well-rooted plantlets were then transferred to the greenhouse with 90% and 92% survival in *D. aphyllum* and *R. retusa* respectively (Fig. 4C, D). Induction of healthy root system in *in vitro* plantlets is very important for their survival in outside environment. Root development is an innate nature of plants which is controlled by endogenous level of hormones (Jarvis, 1986). Hossain *et al*. (2013 a, b) reported that scarcity of nutrition ions in the culture medium could enhance root induction *in vitro*, most probably to explore nutrient ions and water from the medium. In *in vitro* conditions, addition of exogenous hormone (auxins) to the medium enhances rooting response (Hossain *et al*., 2013 a, b). Stimulatory effects of IAA on rooting were also reported in some orchids (Das *et al*. 2007; Hossain *et al*., 2010). The present study suggested that combined effects of deprived nutrition and additional presence IAA enhanced the development of stout root system in *D. aphyllum*.

Conclusion

The results indicated that the two species of the two different growth groups *i.e.,* monopodial and sympodial, differed highly in terms of their response in tissue

culture. The type of explants and the PGR supplements were found to be equally important for regeneration purpose. Both embryogenesis and organogenesis were induced but the kind of differentiation was species, PGR and explant dependent.

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LESSER KNOWN ORCHIDS OF HIMACHAL PRADESH (NORTHWEST HIMALAYA): II - GENUS *GALEARIS* RAF. AND *PONERORCHIS* RCHB. F.

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Abstract

Genus *Galearis* Raf. is represented by a single [*G. spathulata* (Lindl.) P. F. Hunt] and *Ponerorchis* Rchb. f. by two [*P. chusua* (D. Don) Soó, *P. nana* (King and Pantl.) Soó] species in Himachal Pradesh, NorthWest Himalaya. These species occupy open grasslands at higher altitudes beyond 3000 m amsl in Chamba, Lahaul and Spiti, Shimla, and Sirmaur districts of the state. Present communication provides information on their taxonomy, habitat characteristics, distribution, and flowering and fruiting periods. A brief note is also provided on possible threats and conservation of these orchids.

Introduction

HIMACHAL PRADESH is a mountainous Indian state with vast geographical expense (55672 km^2) and remarkable altitudinal variation (350-7000 m). It is located in NorthWest part (30°22´ to 33°12´N latitude, 75°47´to 79°04´ E longitude) of the Himalayan range. With 85 species, orchids represent an important component of the state Flora (Vij *et al.,* 2013). Many of these are quite popular because of their strikingly beautiful flowers and/ or curative properties. Some of the most fascinating orchid species of Himachal Pradesh include the lady slippers (*Cypripedium* spp.), the fox-tails (*Aerides multiflora, Rhynchostylis retusa*), the jewels (*Goodyera* spp.), marsh orchid (*Dactylorhiza hatagirea*), and species of *Calanthe, Epipactis, Eulophia, Habenaria, Nervilia, Platanthera* and *Vanda.* There are, however, many other species (belonging to genus *Androcorys, Galearis, Pachystoma, Ponerorchis, Zeuxine, etc*.) that are not of much direct importance to man, and are therefore of very little interest for horticulturists and herbalists. Recently, Verma *et al*. (2014) provided details on genus *Zeuxine* Lindl. in Himachal Pradesh, and in present communication notes are provided on taxonomy, habitat characteristics, distribution, and flowering and fruiting periods of two other lesser known orchid genera, *Galearis* Raf. and *Ponerorchis* Rchb. f.

Material and Methods

Present results are based on the orchid collections made in Himachal Pradesh during years 2002-2012. The species were identified following standard Flora (Deva and Naithani, 1986; Duthie, 1906), and information on habitat characteristics, flowering and fruiting periods, and threats was collected during field observations. The reports on occurrence of these species in the state and their general distribution are based on present field trips as well as earlier available records (Aswal and Mehrotra, 1985, 1999; Chowdhery and Wadhwa, 1984; Deva and Naithani 1986; Duthie, 1906; Hooker, 1890; Murti, 2001; Nair, 1977; Subramani and Kapoor, 2011; Vij *et al*., 2013). Plants were described and illustrated from freshly collected materials.

Results

Galearis Raf. is represented by a single [*G. spathulata* (Lindl.) P. F. Hunt] and *Ponerorchis* Rchb. f. by two [*P. chusua* (D. Don) Soó, *P. nana* (King and Pantl.) Soó] species in Himachal Pradesh. All of these species occur in open grasslands at higher altitudes beyond 3000 m amsl. *Ponerorchis nana* is closely allied to, and usually sympatrically distributed with *P. chusua*. In World Checklist of Selected Plant Families (Govaerts *et al.,* 2015), it has been treated as a synonym of *P. chusua*. But such a treatment has not been followed presently because of marked difference in their lip character. *P. nana* has also been treated as an independent species by Jalal *et al*. (2007), Lucksom (2007), and Vij *et al*. (2013).

The first report of occurrence of *Galearis spathulata* in Himachal Pradesh was by Nair (1977); it was based on author's own collection from Chansil pass (*Nair 36118*). The species was later also reported from Rupin valley and Dodra Kanwar. *P. chusua* was first reported from the state by Duthie (1906) based upon its

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collection from Chamba (*Lace, 1992*). Subsequent workers also recorded it from Rohtang pass and Churdhar. Aswal and Mehrotra (1985) first reported *P. nana* from Himachal Pradesh based on its collection from Rohtang slopes (*Aswal 6970*); this also constituted the very first report of this species from NorthWestern Himalayan region. More recently, this species was collected from Churdhar. There is no reference of genus *Ponerorchis* in state Flora compiled by Chowdhery and Wadhwa (1984).

In what follows, taxonomic keys are provided for both genera (*Galearis, Ponerorchis*), and both species of *Ponerorchis*. The genera and their species are described. Species description is followed by notes on their habitat characteristics, distribution, and flowering and fruiting periods.

Key to Genera

1. Plants rhizomatous; leaf solitary, usually spathulate ..... *Galearis*

1′. Plants having tubers; leaves 1-3, linear-oblong or linear-lanceolate ..... *Ponerorchis*

Species Description

1. *Galearis* Raf. Herb. Raf.: 71. 1833. Type: *Galearis spectabilis* (L.) Raf.

Terrestrial herbs. *Stem* arising from long and creeping rhizome, thin. *Roots* many, thin. *Leaf* solitary, arising from the base of the stem. *Inflorescence* raceme or spike, bearing one to four laxly arranged flowers. *Floral bracts* foliaceous, generally exceeding the flowers in length. *Flowers* small, purple or rarely white. *Sepals* and *petals* forming a hood. *Lip* entire or rarely faintly lobed, as long as or longer than sepals, base spurred. *Spur* nearly half of the ovary length, stout or incurved. *Column* short, without foot. *Pollinia* 2, with caudicles and viscid gland, the latter enclosed in a single pouch (bursicula).

The genus comprises of about 10 species distributed from the Himalaya to Russian Far East, and Subarctic America to North Central and Eastern USA. Two species are reported from NorthWest Himalaya (Deva and Naithani, 1986), and only one (*G. spathulata*) occurs in Himachal Pradesh.

***Galearis spathulata* (Lindl.) P. F. Hunt**, Kew Bull. 26: 172. 1971; Vij *et al.*, Orch. Him. Pradesh 73. t. 9. 2013. *Gymnadenia spathulata* Lindl., Gen. Sp. Orchid. Pl. 280. 1835. *Orchis spathulata* (Lindl.) Rchb. f. ex Benth., J. Linn. Soc. Bot. 18: 355. 1880 (*non* L.); Hook. f., Fl. Brit. India 6: 127. 1890; King & Pantl., Ann. Roy. Bot. Gard. (Calcutta) 8: 301. t. 400. 1898; Duthie, Ann. Roy. Bot. Gard. (Calcutta) 9: 174. 1906. *Habenaria spathulata* (Lindl.) Benth., J. Linn. Soc., Bot. 18: 355. 1881. *Galeorchis spathulata* (Lindl.) Soó, Acta Bot. Acad. Sci. Hung. 12: 351. 1966. *Aorchis spathulata* (Lindl.) Verm., Jahresber. Naturwiss. Vereins Wuppertal 25: 33 (1972); Seidenfaden & Arora, Nord. J. Bot. 2: 9. 1982; Deva & Naithani, Orch. Fl. N. W. Himal. 105. t. 47. 1986. **Figs. 1a, 2a-c.**

Terrestrial herbs. *Stem* 4.5-12 cm tall with a thin underground rhizome, 2-2.5 mm thick, erect, base clothed with 1-2 loose tubular sheaths. *Roots ca.* 1 mm thick, present at irregular distances on rhizome. *Leaf* solitary, membranous, ovate to narrow-elliptical or spathulate, petiolate, blade 4-7 × 1.8-2 cm, petiole 1-2.5 cm, sometimes another small leaf present near middle of the scape. *Inflorescence* spike, short, 1-2 flowered. *Floral bracts* leaf-like, lanceolate, subacute, longer than the flower, *ca.* 12 × 5 mm, 5-veined, with highly intricated veinlets. *Flowers* purple or white, 1-1.3 cm across. *Sepals* subequal; the dorsal *ca.* 5 × 2.5 mm, ovate, subacute, connivent with the petals to form a hood; the laterals slightly longer than the dorsal, oblanceolate, subacute, spreading. *Petals* of the size of dorsal sepal, obliquely ovate, subacute. *Lip* almost equaling the sepals, *ca.* 5.5 × 3.5 mm, entire, margins crenulate, broadly elliptic or obovate, spotted near the base, upper surface with many shallow grooves extending from the base nearly to the apex. *Spur* small, nearly straight, about half the length of the ovary. *Column* small, 1-1.5 × 1 mm. *Pollinia* 2, pyriform, caudicles short and tapering.

Etymology

The specific name *spathulata* (Latin: spoon shaped) refers to solitary, spathulate leaf.

Type

India, Kedarkanta, *Royle 55* (holo, K-LINDL).

Habitat Characteristics

Grows in alpine open grasslands (> 3500 m) individually or in groups of 2-3 plants. Grasses, *Fragaria nubicola, Geum elatum, Meconopsis* spp., *Pedicularis* spp., *Potentilla cuneata, Trollius acaulis etc.*, comprise associated vegetation.

Flowers and Fruits

July-October.

Occurrence in Himachal Pradesh

Fig. 1a-c. Genus *Galearis* Raf. and *Ponerorchis* Rchb. f. (Orchidaceae) in Himachal Pradesh: a, *Galearis spathulata* (Lindl.) P. F. Hunt; b, *Ponerorchis chusua* (D. Don) Soó (note the deeply lobed lip); c, *Ponerorchis nana* (King and Pantl.) Soó (note the shallower lip lobes). Scale bars = 1cm.

Shimla (Dodra Kanwar, Larot-Chansil pass, Rupin valley).

Voucher Specimens

Deva 3805 (DD), Nair 36118 (BSD), Vij & Verma 288 (PAN).

Distribution

India (Himachal Pradesh, Uttarakhand), Bhutan, China, Nepal.

2. *Ponerorchis* Rchb. f. Linnaea 25: 227. 1852. Type: *Ponerorchis graminifolia* Rchb. f.

Terrestrial herbs with underground undivided tubers. *Stem* small, erect. Leaves 1-3. Inflorescence many flowered. *Floral bracts* foliaceous, more or less equaling the ovary. *Flowers* small, purple, spurred. *Sepals* and *petals* subequal, dorsal sepal forming hood with petals. *Lip* more or less 3-lobed, usually bent at or below the middle. *Column* short. *Pollinia* 2, each with caudicle and viscidium.

Ponerorchis is a genus of about 20 species distributed mainly in temperate to arctic climates, chiefly in Asia. It is represented by three species in India, all of which are reported from NorthWest Himalaya (Deva and Naithani, 1986). Two species (*P. chusua*, *P. nana*) occur in Himachal Pradesh.

Key to Species

1. Lip deeply 3-lobed, all lobes almost equal, leaves 1-3 ..... *P. chusua*

1/. Lip very shallowly 3-lobed, leaf 1..... ..... *P. nana*

***Ponerorchis chusua* (D. Don) Soó,** Acta Bot. Acad. Sci. Hung. 12: 352. 1966; Seidenfaden & Arora, Nord. J. Bot. 2: 24. 1982; Deva & Naithani, Orch. Fl. N.W. Himal. 195. t. 105. 1986; Aswal & Mehrotra, Fl. Lahaul-Spiti 587. 1999; Vij et al., Orch. Him. Pradesh 135. t. 37. 2013. *Orchis chusua* D. Don, Prodr. Fl. Nepal. 23. 1825; Hook. f., Fl. Brit. India 6: 127. 1890; King & Pantl., Ann. Roy. Bot. Gard. (Calcutta) 8: 303. t. 402. 1898; Duthie, Ann. Roy. Bot. Gard. (Calcutta) 9: 173. 1906. **Figs. 1b, 2d-f.**

Terrestrial. *Tubers* small, elliptic or oblong, 12-15 × 5 mm. *Stem* 13-20 cm long, 2-3 mm thick, with two blunt, tubular sheaths at the base. *Roots* many, 1-2 mm thick. *Leaves* 1-3, spreading, membranous, linear-oblong or linear-lanceolate, acute or acuminate, 5-8

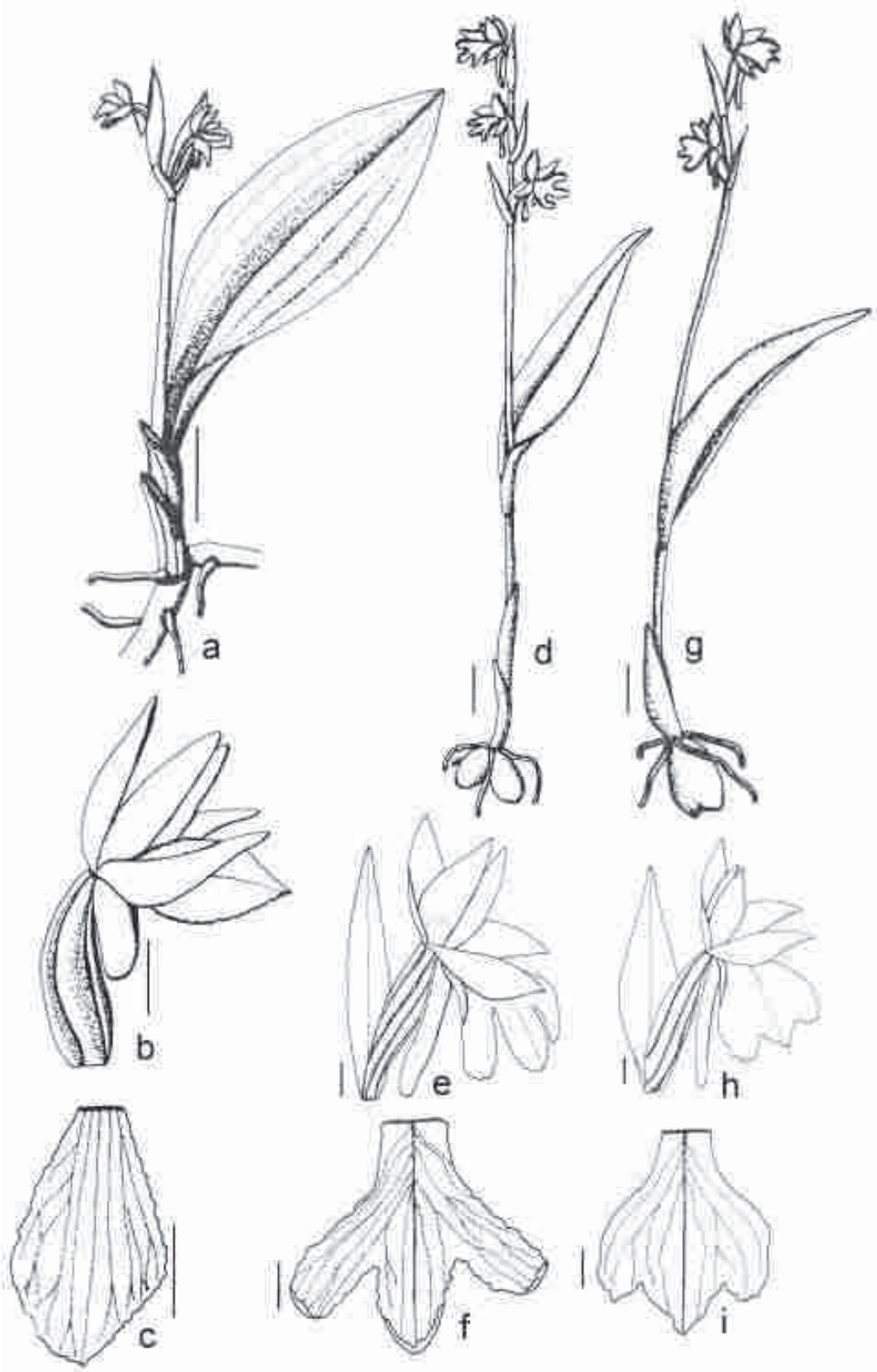

Fig. 2a-i. Genus *Galearis* Raf. and *Ponerorchis* Rchb. f. (Orchidaceae) in Himachal Pradesh. a-c, *Galearis spathulata* (Lindl.) P. F. Hunt: a, plant showing habit; b, flower; c, lip. d-f, *Ponerorchis chusua* (D. Don) Soó: d, plant showing habit; e, flower; f, lip. g-i, *Ponerorchis nana* (King and Pantl.) Soó: g, plant showing habit; h, flower; i, lip. Scale bars: a, d, g = 1cm; b, c, e, f, h, i = 2mm.

× 0.8-1.2 cm. *Inflorescence* spike, erect, 2.5-8 cm long, one or few to many flowered. *Floral bracts* lanceolate, acuminate, 15-16 × 3-4 mm, equaling or slightly longer than ovary. *Flowers* purple, 12-15 mm across. *Sepals* subequal, *ca.* 8 × 3 mm, oblong, obtuse; the dorsal erect; the laterals curved. *Petals* broadly and obliquely ovoid, base truncate, 8-9 × 3 mm. *Lip* longer than the sepals, 11-12 mm long, deeply 3-lobed, the lobes almost equal, oblong, obtuse, the lateral ones diverging. *Spur* stout, cylindric, as long as and lying parallel and compressed to ovary. *Column* short, *ca.* 2 mm long. *Pollinia* 2, ovoid-globose, caudicles long, viscidium small, each lying in a small pouch.

Etymology

The epithet *chusua* (Latin: from Nepalese name Choo Swa) is derived from the Nepalese local name of this species.

Type

China, Yunnan, *Forrest 6464* (holo, K).

Habitat Characteristics

Grows in exposed grasslands individually or in small groups (3400-4500 m). *Gaultheria trichophylla, Thalictrum alpinum,* grasses, junipers, and ferns comprise the associated vegetation.

Flowers and Fruits

July-September.

Occurrence:

Chamba, Lahaul & Spiti (Rohtang pass), Sirmaur (Churdhar, Raicha).

Voucher Specimens

Aswal 10541 (BSD), Lace 1992 (DD), Vij & Verma 311 (PAN).

Distribution

India (Himachal Pradesh to Arunachal Pradesh), Nepal, Bhutan, Tibet, China.

***Ponerorchis nana* (King & Pantl.) Soó,** Acta Bot. Acad. Sci. Hung. 12: 353. 1966; Seidenfaden & Arora, Nord. J. Bot. 2: 24. 1982; Deva & Naithani, Orch. Fl. N.W. Himal. 199. t. 106. 1986; Aswal & Mehrotra, Fl. Lahaul-Spiti 588. 1999; Vij et al., Orch. Him. Pradesh 137. t. 38. 2013. *Orchis chusua* var. *nana* King & Pantl., Ann. Roy. Bot. Gard. (Calcutta) 8: 303. t. 402A. 1898. *O. nana* (King & Pantl.) Schltr., Fedde, Repert. 9: 434. 1911. *Chusua roborowskyi* (Maxim.) Hunt var. *nana* (King & Pantl.) Hunt, Kew Bull. 26: 1976. 1971. *C. nana* (King & Pantl.) Pradhan, Ind. Orch. Guide Ident. & Cult. 2: 678. 1979; Subramani & Kapoor, Int. J. Biol. Tech. 2 (2): 8. 2011. **Fig. 1c, 2g-i.**

Terrestrial. *Tubers* oblong, *ca.* 12 × 5 mm. *Stem* 9-11 cm long, *ca.* 2 mm thick, with one or two, blunt, tubular sheaths at the base, upper portion above the leaf naked. *Roots* many, 1-2 mm thick. Leaf solitary, spreading, membranous, linear-lanceolate, acute or acuminate, 5-7 × 0.7-1.1 cm. *Inflorescence* spike, erect, 2.5-5 cm long, one to three flowered. *Floral bracts* lanceolate, acuminate, 16-18 × 4-5 mm, equaling or slightly longer than ovary. *Flowers* purple, 10-12 mm across. *Sepals* subequal, *ca.* 7 × 3 mm, oblong, subacute; the dorsal erect; the laterals spreading. *Petals* broadly ovoid, *ca.* 8 × 3 mm. *Lip* longer than the sepals, 11-12 mm long, very shallowly 3-lobed, giving an appearance of a broad truncate apex, margins crenate. *Spur* cylindric, equal to but not compressed to ovary. *Column* short, *ca.* 2 mm long. *Pollinia* 2, globose, caudicles long, viscidium small, each lying in a small pouch.

Etymology

The epithet *nana* (Latin: short, small or dwarf) refers to the small sized plants of this species.

Type

India, Sikkim, *Pantling 326* (holo, CAL).

Habitat Characteristics

Grows in subalpine-alpine climates (3000- 4000 m) in exposed situations singly or in small groups. *Gaultheria trichophylla, Polygonum somdevae, Potentilla argyrophylla ,Rhododendron anthopogon, Thalictrum alpinum,* grasses, junipers, and ferns comprise the associated vegetation. It usually grows sympatrically with *Ponerorchis chusua.*

Flowers and Fruits

July-September.

Occurrence

Lahaul & Spiti (Rohtang slopes), Sirmaur (Choordhar).

Voucher Specimens

Aswal 6970 (BSD), Vij & Verma 312 (PAN).

Distribution

India (Himachal Pradesh, Uttarakhand, Sikkim), Nepal.

Threat and Conservation

Anthropogenic activities at high altitude alpine habitats in Himachal Pradesh are very less as compared to subtropical and temperate zones. The only threat to orchids is overgrazing by cattle, sheep and goats. It results in uprooting of herbaceous vegetation, and increases the chances of soil erosion and land slips in affected areas. Grazing should be permitted on rotational basis (same area should not be used for this purpose during every year) so that the affected plant populations may get enough time to get established better.

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