IN VITRO MASS MULTIPLICATION AND CONSERVATION OF TWO RARE AND THREATENED ORCHIDS IN NAGALAND

BY

TEMJENSANGBA

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MOKOKCHUNG 798 601 (UNIVERSITY BRANCH)
NAGALAND, INDIA

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Dedicated

To

My Parents

NAGALAND UNIVERSITY

June 2006

DECLARATION

I, Temjensangba, bearing registration number 164/2004 (1st June 2004), hereby, declare that the subject matter of my thesis entitled "*In vitro* mass multiplication and conservation of two rare and threatened orchids in Nagaland" is the record of work done by me, and that the contents of this thesis did not form the basis for award of any degree to me or to anybody else to the best of my knowledge. The thesis has not been submitted by me for any research degree in any other University/Institute.

This thesis is being submitted to the Nagaland University for the degree of Doctor of Philosophy in Botany.

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Figure 1: Orchid distribution of North-East India including Sikkim

Table 1: Orchid diversity of Nagaland

Sl. No.	Name of the orchid	Flowering season	Status**#
	Epiphytic Orchids		
1	*Acampe multiflora* (Lindl.) Hochr	April-May	-
2	*A. ochraceae* (Lindl.)	Dec-Jan	-
3	*A. papillosa* (Lindl.)	Dec-Jan	-
4	*A. regida* (Buch-Ham.ex. J. E. Sm) Hunt	April-May	-
5	*A. wightiana* Lindl.	Nov-Dec	-
6	*Acanthophippium striatum* Lindl.	July-Aug	-
7	*A. sylhetense* Lindl.	May-June	E,R, T
8	*Aerides crassifolium* Par & Reichb. f.	April-May	-
9	*A.fieldingii* Jenkings	May-June	E,R, T
10	*A. multiflora* Roxb. Corom	June-Aug	-
11	*A odorata* Lour	May-June	-
12	*A. uniflora* (Lindl.) Summer	July-Aug	-
13	*Agrostophyllum brevipes* King & Pantl.	Aug-Sept	-
14	*A. callosum* Reichb	June-July	-
15	*A. khasianum* Griff.	July-Aug	-
16	*Appendicula cornuta* Bl.	Aug	-
17	*Arachnis labrosa* (Lindl. Ex. Paxt) Reichb f.	Aug-Sept	E,R
18	*Ascocentrum ampullaceum* (Roxb) Schltr. f.	March-April	-
19	*A. curvifolium* Schltr	May-June	-
20	*A. micranthum* (Lindl.) Holtt	June-Aug	-
21	*A. miniatum* Schltr	March-May	-
22	*Brachycorytis obcordata* (Buch-Ham. ex. D. Don)	Aug-Sept	-
23	*Bulbophyllum affine* Lindl.	May-June	-
24	*B. acutiflorum* Hook. f.	June-July	-
25	*B. andersonii* (Hook f) J. J. Sm	Sept-Oct	E
26	*B. caryanum* (Hook) Spreng	Oct	-
27	*B. caudatum* Lindl	June-July	E,R
28	*B. cauliflorum* Hook. f.	May-June	-
29	*B. cylindraceum* Lindl	Dec-Jan	-
30	*B. dyeraneum* (King & Pantl) Scidenf	July-Sept	-
31	*B. elatum* (Hook. f.) J. J. Sm.	May-June	E,R
32	*B. eublepharum* Reechb. f.	Aug	E
33	*B. gamblei* Hook. f.	July-Aug	E,R
34	*B. guttulatum* (Hook. f.) Balak	Aug-Sept	-
35	*B. gymnopus* Hook. f.	Oct-Nov	-

Contd...

36	*B. helenae* (Kze) J. J. Sm	May-June	
37	*B. hirtum* Lindl	Nov-Feb	-
38	*B. hymenanthum* Hook. f.	May	-
39	*B. leopardinum* Lindl	Oct-Nov	-
40	*B. leptanthum* Hook. f.	July	E
41	*B. odoratissimum* Lindl	June-Aug	E, R
42	*B. ornatissimmum* (Reichb. F.)J. J. Sm	Oct-Nov	-
43	*B. penecillium* par & Reichb. f.	Aug-Sept	-
44	*B. piluliferum* King & Pantl	May	E
45	*B. polyrhizum* Lindl	March-April	-
46	*B. reptans* (Lindl) Lindl	March	-
47	*B. rigidum* King & Pantl	May-June	E
48	*B. retusiusculum* Reichb f.	July-Aug	-
49	*B. rolfei* (Kutze) Scident	Aug	-
50	*B. rotschildianum* (O' Brien) J. J. Sm	Sept-Oct	E,R,eR,T
51	*B. roxburghii* (Lindl) Reichb	Sept	-
52	*B. secundum* Hook. f	June	-
53	*B. striatum* Reichb. f	Oct	E
54	*B. umbellatum* Lindl	July-Aug	-
55	*B. uniflorum* Griff	July-Aug	-
56	*B. viridiflorum* (Hook. f.) Shltr.	July-Aug	-
57	*B. wallichi* (Lindl) Reichb	Oct-Nov	-
58	*Ceratostylis himalaica* Hook. f.	May-June	E
59	*C. teres* Reichb. f.	June	-
60	*Cleisocentron trichromum* (Reichb) Bruhl	July-Aug	E
61	*Cleisostoma appendiculatum* (Lindl) Benth & Hook	July-Aug	-
62	*C. aspersum* (Reichb. f.) Baray	July	-
63	*C. filiforme* (Lindl) Baray	Aug-Sept	-
64	*C. racimeferum* (Lindl) Baray	July-Aug	eN,T
65	*C. simondii* (Gagnep) Scident	Aug-Sept	-
66	*C. striatum* (Reichb. f.) Baray	July-Aug	-
67	*C. subulatum* Bl. Bijdr	July-Aug	-
68	*C. corymbosa* Lindl	April-May	E
69	*C. cristata* Lindl	March-April	E
70	***C. elata*** Lindl	May-June	E,R
71	*C. fimbriata* Lindl	Sept-Oct	-
72	*C. flaccida* Lindl	March-April	-
73	*C. flavida* Wall ex. Lindl	May-June	-
74	*C. fuscescens* Lindl	Oct-Dec	-

Contd...

75	*C. griffithii* Hook. f.	April-May	E,R
76	*C. hitendrae* Das & Jain	April-May	E,R
77	*C. longipes* Lindl	Feb-March	E,eN,R,T
78	*C. micrantha* Lindl	Feb-March	E
79	*C. nitida* (Wall ex. D. Don) Lindl	May-June	E
80	*C. occultata* Hook. f.	June-July	E,R
81	*C. ovalis* Lindl	Oct-Nov	E
82	*C. prolifera* Lindl	May-June	-
83	*C. punctulata* Lindl	April-May	E,eR
84	*C. raizada* Jain & Das	May-June	E
85	*C. rigida* Par & Reich b. f.	June-July	E,eR
86	*C. schultesii* Jain & Das	May-June	-
87	*C. stricta* (D. Don) Schltr	May-June	-
88	***C. suaveolens*** Hook. f.	May-June	-
89	*C. viscosa* Reichb. f.	Jan-Feb	-
90	*Cryptochilus lutea* Lindl	May-June	-
91	*C. sanguinea* Wall	June-July	E
92	*Cymbidium aloifolium* (Linn) Sw	April-May	E
93	*C. cochleare* Lindl	Oct-Nov	-
94	*C. longifolium* D. Don	Sept-Oct	E,R,T
95	*C. mastersii* Griff ex. Lindl	Oct-Nov	E, R
96	*C. tigrinum* Par ex. Hook	April-May	-
97	*C. tracyanum* Rolfe	Oct-Nov	E,eR,T
98	*Dendrobium acinaforme* Roxb.	June-July	-
99	*D. anceps* Sw	April-May	-
100	*D. aphyllum* (Roxb.) Fischer	April-May	-
101	*D. bensoniae* Reichb. f.	May-June	-
102	*D. bicameratum* Lindl	Aug-Sept	-
103	*D. candidum* Lindl	April-May	-
104	*D. chrysanthum* Wall ex. Lindl	Sept-Oct	E
105	*D. chrysotoxum* Lind	April-May	E,R,T
106	*D. crepidatum* Lindl & Paxt	March-May	E,R,T
107	*D. densiflorum* (Lindl) Wall	April-May	-
108	*D. denudans* D. Don	Sept-Oct	E,eR,T
109	*D. devonianum* Paxt	May-June	-
110	*D. eriaeflorum* Griff	Sept-Oct	E,eR,T
111	*D. falconeri* Hook. f.	April-May	-
112	*D. farmeri* Paxt	April-May	E,R,T
113	*D. fimbriatum* Hook. var. *oculatum*. Hook	April-May	-

Contd...

114	*D. formosum* Roxb	May-June	E,T
115	*D. gibsonii* Lindl	July-Aug	-
116	*D. heterocarpum* Wall ex. Lindl	March-April	E,R,T
117	*D. hookerianum* Lindl	April-May	-
118	*D. infundibulum* Lindl	April-May	E,T
119	*D. jinkinsii* Wall ex. Lindl	April-May	-
120	*D. lindleyi* Steud	Feb-March	-
121	***D. lituiflorum***	April-May	-
122	*D. longicornu* Lindl	Oct-Nov	E,eR,T
123	*D. moschatum* Sw	June-July	E
124	*D. nobile* Lindl	April-May	E,R,T
125	*D. ochreatum* Wall ex. Lindl	April-May	E
126	*D. porphyrochilum* Lindl	Sept-Oct	E,eR
127	*D. primulinum* Lindl	March-April	-
128	*D. pulchellum* Roxb. ex. Lindl	April-May	E,eR
129	*D. stuposum* Lindl	June-July	-
130	*D. terminale* Par & Reichb. f.	Sept-Oct	-
131	*D.thyrsiflorum* Reichb. f.	April-May	E,R
132	*D. transparens* Wall ex. Lindl	April-May	-
133	*D. wardianum* Warner	April-May	E
134	*D. williamsonii* Day & Reichb. f.	April-May	E,eR,T
135	*Diploprora championii* (Lindi) Hook. f.	July-Aug	-
136	*Epigeneium amplum* (Lindl) Summer	Oct-Nov	-
137	*E. fuscescens* (Griff) Summer	Oct-Nov	-
138	*E. navicularis* (Balak & Chowd.) Hynniewta & Wadhwa	Sept	E
139	*E. rotundatum* (Lindl) Summer	April-May	-
140	*Eria acervata* Lindl	June-July	E,R,T
141	*E. alba* Lindl	June-July	-
142	*E. amica* Reichb. f.	April	-
143	*E. bambusifolia* Lindl	Sept-Oct	-
144	*E. biflora* Griff	Aug-Sept	-
145	*E. bractescens* Lindl	April-May	E,R
146	*E. coronaria* (Lindl) Reichb. f.	May-June	-
147	*E. dasyphylla* Par & Relchb. f.	June-July	-
148	*E. excavata* Lindl ex. Hook. f.	June-July	-
149	*E. gramminifolia* Lindl	July-Aug	-
150	*E. muscicola* Lindl	Aug-Sept	-
151	*E. paniculata* Lindl	June-July	-
152	*E. pannea* Lindl	April-May	-

Contd...

153	*E. spicata* (D. Don) Hand & Mazz	July-Aug	-
154	*E. stricta* Lindl	Dec-Feb	E,R,ex?
155	*E. vittata* Lindl	March-April	E,R
156	*Flickingera fimbriata* A.D. Hwakes	May-June	E,R
157	*F. fugax* (Reichb. f.) Scidenf	May	-
158	*F. macrai* (Lindl) Seidenf	July-Oct	-
159	*Gastrochilus acutifolium* (Lindl) Kze	Nov-Dec	-
160	*G. calceolaris* (Buch-Ham ex. Sm) D. Don	March-April	-
161	*G. distichus* (Lindl) Kuntze	Jan-Feb	-
162	*G. inconspicuus* (Wall ex. Hook.f.) Kuntze	June-July	E
163	*Hygrochilus parishii* (Veitch. Reichb.f.)Pfitz	May-June	E
164	*Kingidium deliciosum* (Reichb. f.) Swut	July-Aug	-
165	*K. taenialis* (Lindl) Hunt	May-June	-
166	*Liparis assamica* King & Pantl	Oct-Nov	-
167	*L. bistriata* Par & Reichb. f.	July-Aug	E
168	*L. bootanensis* Griff	Aug-Sept	-
169	*L. caespitosa* (Lam.) Lindl	July-Aug	-
170	*L. delicatula* Hook. f.	Aug-Sept	E
171	*L. distans* Clarke	Oct-Nov	E
172	*L. longipes* Lindl	Oct-Nov	E
173	*L. nervosa* (Thumb) Lindl	July-Aug	-
174	*L. odorata* (Wild) Lindl	July-Aug	-
175	*L. plantaginea* Lindl	June-July	-
176	*L. platyrachis* Hook. f.	Aug-Sept	-
177	*L. pulchella* Hook. f.	Aug-Sept	-
178	*L. resupinata* Ridley	Oct-Nov	-
179	*L. strictlandiana* Reichb. f.	Oct-Nov	-
180	*L. viridiflora* (Bl) Lindl	Oct-Nov	-
181	*Luisia inconspiciua* (Wall ex. Hook. f.) K&P	June-July	-
182	*L. parchystachys* (Lindl) Bl	March-April	-
183	***L. psyche*** Reichb. f.	March-April	-
184	*L. teretifolia* Gand	May-June	-
185	*L. trichorhiza* (Hook) Bl	March-April	-
186	*L. zeylanica* Lindi	March-April	-
187	*Micropera mannii* (Hook. f.) Tang & Wang	June-July	-
188	*M. rostrata* (Roxb) Balak	May-June	E,R
189	*Monomeria barbata* Lindl	Feb	-
190	*Neogyne gardneriana* (Lindi) Reichb. f. ex. Pfitz	Oct-Nov	-
191	*Oberonia acaulis* Griff	Oct-Nov	-

Contd...

192	*O. clarkei* Hook. f.	Oct	-
193	*O. denticulate* Wight	Oct-Nov	E,R
194	*O. emarginata* King & Pantl	Aug-Sept	-
195	*O. ensiformris* (Sm ex. Rees) Lindl	Nov	-
196	*O. griffithiana* Lindl	March-April	-
197	*O. iridifolia* (Roxb) Lindi	Sept-Nov	-
198	*O. longilabris* King & Pantl	July-Sept	-
199	*O. mannii* Hook. f.	June-July	-
200	*O. maxima* Hook. f.	June-Sept	E
201	*O. micrantha* King & Pantl	July-Aug	-
202	*O. obcordata* Laindl	July-Aug	-
203	*O. orbicularis* Hook. f.	Nov-Dec	E
204	*O. pachyrachis* Reichb. f. ex. Hook. f.	Aug-Sept	E
205	*O. pyrulifera* Lindl	Oct-Nov	E,eR
206	*O. recurva* Lindl	July-Aug	-
207	*Ornithochilis dofformis* (Wall ex. Lindl) Scltr	June-July	-
208	*Otochilus alba* Lindl	June	-
209	*O. fusca* Lindl	Nov-Dec	-
210	*O. lancilabius* Seidenf	Sept-Oct	-
211	*O. porrecta* Lindl	Oct-Nov	-
212	*Panisia apiculata* Lindl	Oct-Nov	-
213	*P. tricallosa* Rolfe	Feb-March	-
214	*P. uniflora* (Lindl) Lindl	April-May	-
215	*Papilionanthe longicornu* (Lindl) Garay	Sept	-
216	*P. teres* (Roxb)Shltr	Feb-March	-
217	*Pelatantheria insectifer* (Reichb. f.) Ridl	Aug-Sept	-
218	*Pholidota articulata* Lindl	May-June	-
219	*P. calceata* Reichb. f.	July-Aug	-
220	*P. convallariae* (Relchb. f.) Hook. f.	April-May	E
221	*P. griffithii* Hook. f.	May-June	E
222	*P. imbricata* (Roxb) Lindl. Var. *coriaceae* Hook. f.	June-July	-
223	*P. imbricata* (Roxb) Lindl. Var *imbricata*	June-July	-
224	*P. imbricata* (Roxb) Lindl. Var *sessilis* Hook. f.	May-June	-
225	*P. protracta* Hook. f.	Oct-Nov	-
226	*P. rubra* Lindl	Aug-Sept	-
227	*P. watti* King & Pantl	May	-
228	*Phreatia elegans* Lindl	July-Aug	E,R
229	*Pleione maculata* (Lindl) Lindl	Feb-March	-
230	*Pteroceros suaveolens* (Roxb) Hoitt	March-April	E,eR,ex?

Contd...

231	*Renanthera imschootiana* Rolfe	May-June	-
232	*Rhynchostylis retusa* Bl	May-June	-
233	*Robiquetia succisa* (Lindl) Scedenf & Garay	May-June	E,eN,eR,T
234	*Schoenorchis gemmata* (Lindl) J. J. Sm	May-June	-
235	*Smitinandia micrantha* (Lindl) Holtt	June-July	-
236	*Sunipia bicolor* Lindl	June-July	-
237	*S. candida* (Lindl) Hunt	May-June	-
238	*S. jainii* (Hynniewta et Malhotra)	Nov	E,R
239	*Taeniophyllum khasianum* Joseph & Yog	May-June	E,R
240	*Thelasis longifolia* Hook. f.	June-July	E,R
241	*Trichotosia dasyphylla* (Par & Reichb. f.) Kranzl	May-June	-
242	*Tylostylis discolor* Hook. f.	Feb-March	-
243	*Uncifera acuminata* Lindl	Aug-Sept	-
244	*U. obtusifolia* Lindl	Aug-Sept	-
245	*Vanda alpina* Lindl	June-Aug	-
246	*V. bicolor*	Jan-Feb	-
247	*V. coerulea* Griff. Ex. Lindl	Sept-Oct	-
248	*V. cristata* Wall ex. Lindl	April-May	E,V,eR,ex?
249	*V. pumillia* Hook. f.	May-June	-
250	***V. stangeana*** Reichb. f.	May-June	-
251	*V. tessellata* (Roxb) Hook ex. Garay	May-June	E,eR,ex?
252	*V. testaceae* (Lindl) Reichb. f.	May-June	-
253	*Vandopsis undulata* (Lindl) Sm	April-May	-

Contd...

Terrestrial orchids

Sl. No	Name of the orchid	Flowering Season	Status**#
1.	*Anoectochilus brevilabris* Lindl	Oct-Nov	E,R
2.	*A. crispus* Lindl	June-July	-
3.	*A. elwesii* King & Pantl	June-July	-
4.	*A. griffithii* Hook. f.	Aug-Sept	-
5.	*A. grandiflorus* Lindl	June-July	-
6.	*A. luteus* Lind	July-August	-
7.	*A. roxburghii* (Wall) Lindl	Sept-Oct	-
8.	*A. sikkimensis* King & Pantl	Sept-Oct	-
9.	*Anthogonium gracile* Lindl	Aug-Sept	-
10.	*Arundina graminifolia* (D. Don) Hoehr	June-July	-
11.	*Calanthe alismafolia* Lindl	May-June	-
12.	*C. alpina* Hook. f.	July-Aug	E,R,eN
13.	*C. angusta* Lindl	April-May	E,R
14.	*C. biloba* Lindl	Sept-Oct	E
15.	*C. brevicornu* Lindl	May-June	E
16.	*C. ceciliae* Reichb. f.	Sept	-
17.	*C. chloroleuca* Lindl	Feb-March	E,eN
18.	*C. clavate* Lindl	Feb-March	-
19.	*C. densiflora* Lindl	Oct	-
20.	*C. foerstermannii* Reichb. f.	July	-
21.	*C. gracilis* Lindl	Oct	-
22.	*C. griffithii* Lindl	May	-
23.	*C. herbaceae* Lindl	May-June	E,R
24.	*C. manniii* Lindl	March-April	-
25.	*C. masuca* (D. Don) Lindl	July-Aug	-
26.	*C. plantagina* Lindl	March-April	-
27.	*C. puberula* Lindl	July-Aug	-
28.	*C. tricarinata* Lindl	May-June	-
29.	*C. triplicata* (Willem) Ames	July-Aug	-
30.	*C. vaginata* Lindl	May-Juen	-
31.	*C. vestita* Lindl	Nov-Dec	-
32.	*C. whiteana* King & Pantl	May	E,eN
33.	*Cephalan thera* (Lindl) Fritksch	May-June	-
34.	*Cheirostylis griffithii* Lindl	Oct-Nov	-
35.	*C. pusilla* Lindl	Nov-Dec	-

contd ....

36.	*Corymborkis veratrifolia* Bl	March-April	E
37.	*Cremastra wallichiana* Lindl	April-May	-
38.	*Cymbidium cyperifolium* Lindl	Oct-Nov	-
39.	*C. ensifolium* (L) Sw	Nov-Dec	-
40.	*C. lancifolium* Hook	May-June	E,eN
41.	*Diphylax urceolata* (Clarke) Hook. f.	Aug-Sept	-
42.	*Diplomeris hirsuta* (Lindl) Lindl	June-Aug	-
43.	*D. pulchella* D. Don	Aug-Sept	-
44.	*Eulophia bicallosa* (D. Don) P. F. Heent & Sum	March-April	-
45.	*E. graminea* Lindl	March-April	-
46.	*E. nuda* Lindl	May	-
47.	*Geodorum densiflorum* (Laur) Schltr	June-July	-
48.	*Goodyera foliosa* (Lindl) Benth. ex. Clarke	Sept-Oct	-
49.	*G. fusca* (Lindl) Hook. f.	Aug-Sept	-
50.	*G. hispida* Lindl	Sept	-
51.	*G. procera* (Wall ex. Ker. Gawl) Hook. f.	April-May	-
52.	*G. repens* (Ker-Gawl) Hook. f.	May	-
53.	*G. schlechtendaliana* Reichb. f.	Oct-Nov	-
54.	*G. secundiflora* (Griff) Lindl	Oct-Nov	-
55.	*G. viridiflora* (Bl) Bl	July-Aug	-
56.	*Habernaria acufera* Wall ex. Lindl	June-July	-
57.	*H. arietina* Hook. f.	Sept-Oct	-
58.	*H. dentata* (Sw) Schltr	Aug-Sept	-
59.	*H. furcifera* Lindl	July-Aug	-
60.	*H. intermedia* D. Don	July-Aug	-
61.	*H. malleifera* Hook. f.	Aug-Sept	E
62.	*H. pectinata* var. *arietina* Hook. f.	July-Aug	-
63.	*H. stenopetala* Lindl	Aug-Sept	-
64.	*Herminium lanceum* (Thumb ex. Sw) Vuijk	July-Aug	-
65.	*H. macrophyllum* (D. Don) Dandy	July-Aug	-
66.	*H. monorchis* (L) R. Br	July-Aug	-
67.	*Hetaeria rubens* (Lindl) Benth. ex. Hook. f.	Jan-Feb	-
68.	*Liparis bituberculata* (Hook) Lindl	May-June	-
69.	*L. cordifolia* Hook. f.	Sept-Oct	-
70.	*L. paradoxa* (Lindl) Reichb	July-Aug	-
71.	*L. petiolata* (D. Don) Hunt & Summer	April-May	-
72.	*Malaxis acuminata* D. Don	May-June	-
73.	*M. biaurita* (Lindl) O. Kuntze	July-Aug	-
74.	*M. cylindroatachya* (Lindl) O. Kuntze	July-Aug	-

contd ....

75.	*M. josephiana* (Reichb. f.). Kuntze	May-June	-
76.	*M. khasiana* (Hook. f) Kuntze	June-July	-
77.	*M. latifolia* Smith	June-July	-
78.	*Nephelaphyllum cordifolium* (Lindl) Lindl	June-July	E
79.	*Nervilia aragoana* Gaud	June-July	-
80.	*N. prainiana* (King & Pantl) Scidenf	June-July	-
81.	*Pachystoma senile* (Lindl) Reichb. f.	May-June	-
82.	*Paphiopedilum hirsutissimum* (Lindl) Stein	March-April	E,eR,eN
83.	*P. insigne* (Wall ex. Lindl) Pfitz	April-May	E,eR,eN
84.	*Pecteilis gigantea* (Sm) Rafin	Sept-Oct	-
85.	*P. susannae* (L) Rafin	July-Aug	-
86.	*Peristylus affinis* (D. Don) Waidenf	July-Aug	-
87.	*P. chloranthus* Lindl	July-Aug	-
88.	*P. constrictus* (Lindl) Lindl	July-Aug	-
89.	*P. densus* (Lindl) Sant & Kapad	July-Aug	-
90.	*P. fallax* Lindi	July-Aug	-
91	*P. goodyeroides* D. Don) Lindl	July-Aug	-
92.	*P. lacertif?rus* (Lindl) Sm	June	-
93.	*P. mannii* (Reichb) Mukherjee	July-Aug	-
94.	*P. prainii (*Hook.f) Kranzl	Aug-Sept	-
95.	*Phaius flavus* (Bl) Lindl	March-April	-
96.	*P. longipes* (Hook.f) Hoitt	Nov	E
97.	*P. mishmensis* Reicbh f	Sept-Oct	E,eR
98.	*P. tankervilliae* (Aiton) Bl	April-June	E,eN,R
99.	*Platanthera arcuata* Lindl	June	-
100.	*P. stenantha* (Hook.f) Soo	July-Aug	-
101.	*Satyrium nepalense* D.Don	Sept-Oct	-
102.	*Spathoglothis plicata Bl*	June-July	-
103.	*S. pubescens* Lindl	June-July	-
104.	*Spiranthes sinenses* (Pres) Ames	May-June	-
105.	*Taenia latifolia* Benth.ex.Hook	March-April	-
106.	*T. viridi-fusca* Benth	April-May	-
107.	*Thunia alba* (Lindl) Reicbh.f	June-Aug	-
108.	*T. marshalliana* Reicbh.f	June-Aug	-
109.	*Tropidia curculigoides* Lindl	Sept-Oct	-
110.	*Zeuxine abbreviata* (Lindl) Hook. f.	Aug-Sept	-
111.	*Z. affines* (Lindl) Benth.ex.hook. f	April-May	-
112.	*Z. flava* (Wall.ex.Lindl) Trimen	April-May	-
113.	*Z. goodyeroides* Lindl	Sept-Oct	-

contd ......

114.	*Z. gracilis* (Breda) Bl	Aug-Sept	-
115.	*Z. nervosa* (Wall.ex.Lindl) Benth.ex.Clarke	March-April	-
116.	*Z. strateumatica* (Linn) Schltr	Dec-Jan	-

Saprophytic orchids

1.	*Aphyllorchis monatana* Reichb. f.	July-Aug	-
2.	*A. prainii* Hook. f.	July-Aug	-
3.	*Cymbidium macrorhizon* Lindl.	May-June	E,eR,T
4.	*Galeola falconeri* Hook. f.	July-Aug	E,eR,T
5.	*G. lindleyana* (Reichb. f) Reichb	July-Aug	E,eR,T
6.	*Neottia listeroides* Lindl	July-Sept	-
7.	*Oreorchis foliosa* (Lindl) Lindl	June-July	-
8.	*Yaonia prainii* King & Pantl	May-June	-

Dual habitat

(Epiphyte – Lithophyte)

1.	*Coelogyne barbata* Griff	Oct-Nov	-
2.	*Cymbidium devonianum* Paxt	May-June	E,R,T
3.	*C. elegans* Lindl	Oct-Nov	E,eR,T
4.	*C. irirdioides* D. Don	Oct-Nov	E,eR,T
5.	*C. lowianum* Reichb. f.	April-May	E,R
6.	*Pleione hookeriana* (Lindl) William	May-June	E
7.	*P. humilis* (Sm) D.Don	Feb-March	E
8.	*P. praecox* (Sm) D. Don	Oct-Nov	E,R

Terrestrial – Lithophyte

1.	*Esmeralda carthcartii* (Lindl) Reichhb. f	March-April	R,T

*** Adopted from Deb *et al*, 2003.**

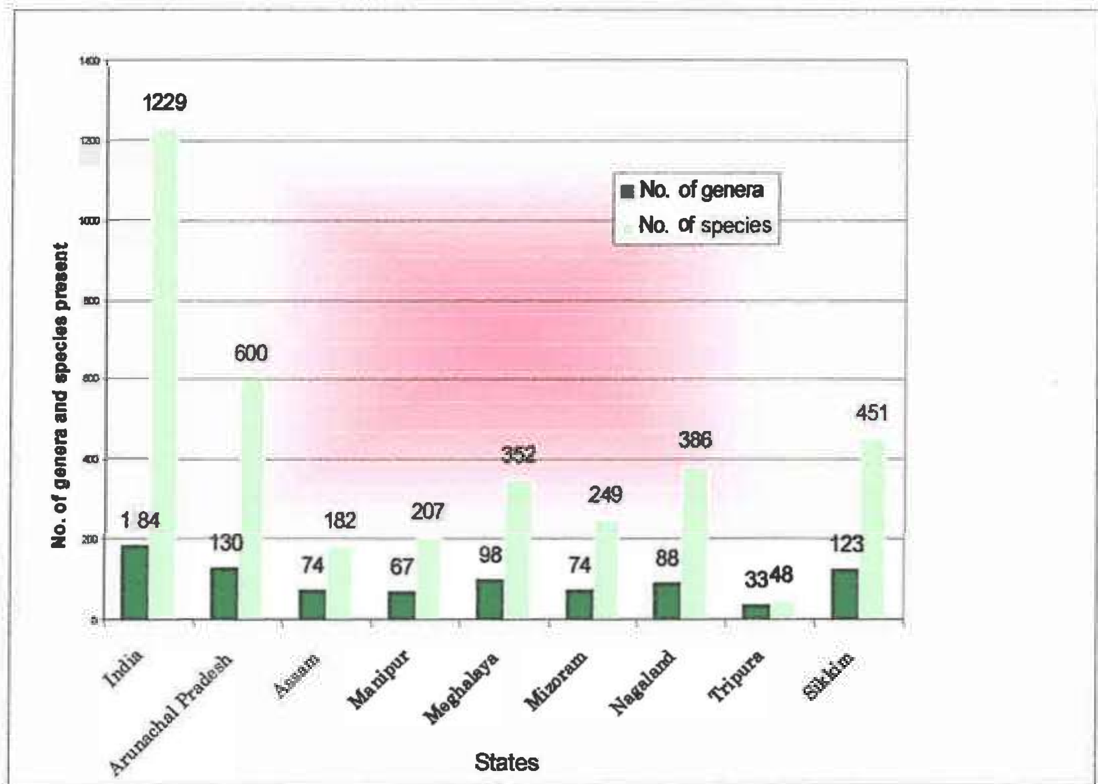

Figure 1: Orchid distribution of North-East India including Sikkim

Chapter–1

Introduction

The word 'Biodiversity' means the multiplicity of life forms including their genetic diversity and the assemblages they form, or in other words, the variety and variability in which they occur. A more comprehensive definition was given by De Long (1996), "*Biodiversity is an attribute of an area and specifically refers to the variety within and among living organisms, assemblages of living organisms, biotic communities and biotic processes, within naturally occurring or modified by humans*". Usually three levels of biodiversity are discussed – genetic, species, and ecosystem diversity. 1. **Genetic diversity** – It consist of all the different genes contained in all individual plants, animals, fungi and other microorganisms. It occurs within a species as well as between species. 2. **Species diversity** – It is all the differences within and between population of species as well as between different species. 3. **Ecosystem diversity** – It is all the different habitats, biological communities, and ecological processes as well as variation within individual ecosystem.

Globally, however, biodiversity is under siege. The 2000 IUCN, `*Red List of Threatened species*` indicates that species extinction is on an increasing spiral. Since

the 1996 assessment of globally threatened species, the number of **`Critically Endangered Primates`** has increased from 13 to 19. Globally 5,611 plants are listed as threatened, but this is based on an assessment of only 4% of the world's total described plants. Therefore, the percentage of threatened plant species is likely to be much higher (IUCN, 2000). According to Myers *et al,* (2000), there are 25 biodiversity hotspots identified globally, they are *Tropical Andes, Meso America, Caribbean, Brazil's Atlantic Forest, ChoeDarien/ Western Ecuador, Brazil's Cerrado, Central Chile, California floristic Province, Madagascar, Eastern Arc and coastal forest of Tanzania/ Kenya, Western African Forests, Cape Floristic Province, Succulent Karoo, Mediterranean Basin, Caucasus, Sundaland, Wallacea, Philippines, Indo-Burma, South Central China, western Ghats/ Sri Lanka, SW Australia, New Caledonia, New Zealand, and Polynesia/ Micronesia,* covering an area of 2,122,891 sq. km with 133,149 endemic plants (44% of total global plants from 300,000 identified and described plants).

Hotspots are geographical areas exhibiting extremely species richness, have high endemism, and are under constant threat. Among 25 hotspots of the world, India is blessed with two of them extending into neighboring countries- the western Ghats/Sri Lanka and the Indo-Burma region (covering the Eastern Himalayas including North-East India) and they figured in top eight hottest hotspots. These areas are particularly rich in floral wealth and endemism, not only in flowering plants but also in reptiles, amphibians, swallow-tailed butterflies, and some mammals. In addition, in India there are 26 endemic centers, which are the home to nearly a third of all the flowering plants identified and described to date. About 7% of the world's total land area is the home to half of the world's species. India contributes

significantly to this latitudinal biodiversity trend. With a mere 2.4% of the world's area, India accounts for 10.78% of the global total flora (Teri, 2000).

The endemism of India is impressively high with about 33% of the country's recorded flora are endemic to the country and are concentrated mainly in the North-East, Western Ghats, North- West Himalaya and the Andaman and Nicobar Islands. Of these endemic species, 3500 are found in the Himalayas and adjoining regions and 1600 species in the Western Ghats alone. India's record in agro-diversity is equally impressive. There are 167 crop species and wild relatives. India is considered to be the centre of origin of 30,000- 50,000 varieties of rice, pigeon-pea, mango, turmeric, ginger, sugarcane, gooseberries etc and ranked seventh in terms of contribution to world agriculture (MoEF, 1999; TERI, 2000). But according to State of Forest report 1999 (FSI, 2000), the forest cover in India is losing at an alarming rate coupled with various factors, which poses a greater threat to the rich biodiversity of the country. The underlying causes of biodiversity loss, however, according to Wood *et al,* (2000) are poverty, macroeconomic policies, international trade factors, policy failures, poor environmental law/weak enforcement, unsustainable development projects and lack oflocal control over resources.

There are 12 biogeographic regions in India representing 3 basic biomes (Udvardy, 1975) with a total land area of about 329 million hectares. Of the 12 biogeographic regions, Northeast India is the most important floristic region owing to its rich biodiversity. The northeastern part of India lies between 22° 9′ - 29° 6′ N latitude and 89° 7' E longitude, is known for its diverse and most extensive lush forest cover is unique structurally and species composition is one of the major regions facing severe deforestation. It is a meeting region of temperate east

Himalayan flora, paleo-artic flora of Tibetan highland and wet evergreen flora of South East Asia and Yunnan forming a bowl of biodiversity. The northeast Indian region host a number of botanical curiosities like *Sapria himalayana, Nepenthes khasiana* and saprophytic orchids like species of *Epipogium and Galeola* and primitive angiospermic plants like *Exbucklandia, Manglietia, Holboellia,* etc. The rich presence of ancient plant like *Magnolia, Michelia, Camellia, Rhododendron,* orchids etc. in the region indicates that evolutionary development in wild and cultivated plants are continuously taking place (Choudhery, 2001). Takhtajan (1969) named the region "*The cradle of flowering plants*". The region is one of the richest and most interesting floristic regions of India, with orchids forming a prominent feature of the vegetation.

According to an official estimate of FSI, Northeastern region has 1, 63,799 sq. km of forest, which is about 25% of the total forest over in the country (Anonymous, 2000). The management of forest has suffered in the recent past due to the pressure on land, decreasing cycle of shifting cultivation, exploitation of forest for timber and lack of scientific management strategy. Further the problem is compounded due to indiscriminate removal of primary forest and trees to satisfy the ever growing need of industries depending on forest products such as paper and pulp, plywood, matchstick etc. Besides these, over exploitation of natural resources, habitat loss and fragmentation are the three major factors that threaten the biodiversity of the region. The nature of forest fragmentation in these landscapes is mainly attributed to shifting cultivation, illicit felling and deforestation for creating agricultural lands (Roy and Joshi, 2002). The impact of shifting cultivation practices was assessed in northeast India under one of the international initiatives TREES-II.

The three sites in Arunachal Pradesh (i.e., Tirap, Patki and Hilla), Nagaland and Barak Valley between 1991 and 1996. The study revealed that area under permanent agriculture has almost been constant; the increase is only in semi-permanent agriculture, witnessing an increase extent of "*Shifting Cultivation*" in the region. In the past with the sufficiently long fallow periods to 20-30 years the system was sustainable but with the reduction in the fallow period of as low as 3 years in recent times the situation has attracted the attention of policy makers and planners. However due to the human pressure and ill management practices not only the primary forests but also the secondary forests are being cleared (Roy *et al*, 2002).

India is considered one of the most diverse bio-geographic regions of the world, has a varied topography from high altitude snow covered Himalayas to plains at sea level, swamps and mangroves forest to tropical evergreen forest, hot desert to cold deserts and climatic conditions ranging from warm tropical to arctic. This great variety of climatic and topographical conditions resulting in varied ecological habitats supports an immensely rich biodiversity with more than 45,000 species of flora, 89,000 species of fauna and an equally rich marine life. A statistical analysis of the angiosperm flora reveals that the family Poaceae with 263 genera and 1291 species is the largest followed by Orchidaceae with 184 genera and 1229 species (Karthikeyan, 2000). In India, orchids are found from sea level to the snow covered alpine regions but their number varies in different regions due to the prevailing climatic conditions, for example, the number of orchid species is very small in the low rainfall, dry, cold north western regions as compared to the high rainfall, warm, humid eastern region (Pradhan, 1976, 1979; Satish Kumar and Manilal, 1994). Orchids are also abundant in peninsular India.

Due to the above factors this North-East region supports almost all types of vegetation from cultivated plains to grasslands, meadows, marshes, swamps, scrub-forest, humid evergreen forest, temperate forests, and alpine vegetation, each with its own characteristic species composition. In addition, the region also has number of 'sacred groves or forest'. According to literature (King and Pantling, 1898; Pradhan, 1979; Kataki, 1986; Satish Kumar and Manilal, 1994; Chowdhery, 1998; Hynniewta *et al*, 2000; Deb *et al*, 2003), it is estimated that out of 1229 species of orchid known from India, about 750 to 800 species are found in N.E. region of the country. A comparative analysis of distribution of orchid species within the region shows (Figure-1) that maximum diversity is found in Arunachal Pradesh followed by Sikkim and the lowest in Tripura. Northeast region has the highest concentration of monotypic orchid genera while large number of saprophytic orchid species belonging to the genera *Aphyllorchis, Cymbidium, Epipogium, Eulophia, Galeola, Gastrodia, Stereosandra*, etc are also found in the region. On the other hand, due to wide altitudinal variation and topographical features supported by favorable climatic conditions endowed the state with a rich floristic biodiversity including huge number of orchid species. The habitats of orchids are classified according to different vegetation and forest types; they are tropical moist evergreen and deciduous forest type (100- 1000 m), subtropical evergreen and semi evergreen forest type (1000- 2000 m), temperate and sub temperate forest type (2000- 3500 m) and alpine zone (3500- 5000 m).

Among the flowering plants, Orchidaceae family is one of the largest with a worldwide distribution comprising about 25,000-35,000 species in some 800 genera (Chowdhery, 2001; Deb *et al*, 2003). More new species are being added every year.

Orchids are found in almost every type of habitat ranging from tropical jungles to the Tundra and to the Arctic Circle. They are found in almost every colour except black. In Australia even two species are found in underground condition e.g. *Rhizanthella gardneri* and *R. slateri* and are extremely rare. Basically, orchid consists of stem or pseudobulbs with leaves (e.g. terrestrial, epiphytic orchids) or without leaves (saprophytes). Roots help them in anchoring with the substratum of their habitat. The majority of orchids are epiphytic, growing on the tree trunks. Some grow as terrestrial on land, as lithophytes on rock/stones and as saprophytes on decaying organic materials. The orchids are distinct in type of vegetative structure and morphology. It exhibits amazing diversity in shape, size and flower color. The smallest orchid measures only 1 (one) mm across (*Bulbophyllum globuliforme*), while *Vanilla* species can climb the tallest tree in the forest and can be up to 20 m long whereas, *Grammatophyllum speciosum* is the biggest orchid in the world. Orchids are differentiated not only by their flowers but also from the leaves and roots. On the basis of vegetative structure and its growth, orchids are of two types: 1. **Monopodial orchids** like *Aerides, Rhynchostylis, and Vanda* etc.– they do not have rhizomes or pseudobulbs but grow from single vegetative apex continuously season after season and 2. **Sympodial orchids** like *Dendrobium, Bulbophyllum* etc. – they have number of vegetative apices situated in the rhizomes. The rhizomes/bulb/pseudobulbs many a times act as reserved organ and help the orchids to combat the extreme drought conditions faced by epiphytic orchids. The orchids are the most advanced and are biologically complex.

Mankind knows the orchids for the last several centuries for their beautiful attractive flowers and as medicinal plants. One may find mentioned in the work

"*Enquiry into Plants*" of Theophrastus, a Greek Philosopher (370-285 BC). The Chinese who have been growing orchids for the last 500 years is the symbol of Scholar–unassuming enduring and ascetic, it also stood as a symbol of loves, beauty, grace nobility and elegance in a women. "*Paint bamboo when you are angry, orchids when you are happy*" is a well-known Chinese saying. Confucius (551-479 BC) praised orchids for their amazing beauty and scent and said: '*acquaintance with good men was like entering a room full of fragrant orchids*'. Orchids have adapted themselves to extremes of the environmental conditions producing thereby great variations in vegetative forms and one may often find it difficult to identify them as orchids if they are not in the flower. Many orchid flowers resemble in shape of a slipper (*Paphiopedilum, Cypripedium*), dancing girl (*Oncidium, Renanthera*), moth (*Phalaenopsis*), spider (*Brassia*), scorpion (*Arachnis*), bee (*Ophrys*), pineapple orchid (*Dendrobium densiflorum*), etc. Orchids are popular worldwide due to their marvellous flower architecture and the spectrum of colours, and comprise one of the most successful group of plants- the orchidaceae. They account for nearly 7% of the total flowering plant species (Pijl and Dodson, 1966) and are still in a state of evolutionary radiation. The orchids stand distinct in having velamenous roots; zygomorphic flowers with well-developed gynostegium, compound pollen, elaborate perianth and resupinate ovaries; and microscopic and non-endospermic seeds with undifferentiated (reduced) embryos. The orchids are adapted to insect pollination; their flowers flaunt a variety of temptations, i.e. bright colours, a safe landing platform in the form of labellum (lip), a nourishing drink, tantalizing odours and even sex to the pollinators (insects).

The state of Nagaland lies between 25°6′ - 27°4′ of latitudes and 93°20′ - 95°15′ E longitudes with geographical area of about 16,57,900 hectares and forest occupy an area of ~8,62,930 hectares. Arunachal Pradesh borders Nagaland to the north, to the south is Manipur, in the east Myanmar and on the west is Assam. Nagaland is a hilly state with few hundreds sq. km stretch of plains along the foothills in the western part of the state. The altitude varies from 300- 3848 m above mean sea level. Naga Hills is topographically divided into three main categories: outer foothills area in the west, medium high hill ranges in the intermediate zone and the high hill ranges in the east. The two highest peaks in the state are Japfu peak with 3814 M in the Barail range and Saramati peak with 3848 M in the Patkai range in the far east of the Indo-Myanmar boundary. Mt. Saramati is the only snowcap mountain other than the Himalaya in the country. The climate over the foothills is warm subtropical and cool temperate over the high hills and moderate sub-montane type in the lower ranges of the western part. The average annual rainfall is 200-250 cm, covering a period of nine months, highest rainfall occurs in the month between July and August. The average relative humidity is 85% but as high as 95% during rainy season. Maximum and minimum temperatures during summer and winter are 32/12°C and 25/5°C respectively. The wide altitudinal variation and topographical features coupled with favorable climatic factors are responsible to rich floristic biodiversity. The different forest vegetation types are Northern Tropical moist/wet evergreen forest covering Namra-Tizit area, Northern Tropical Semi Evergreen forest found in the foothills of Assam-Nagaland border in Mokokchung, Wokha and Kohima districts, Northern Sub-Tropical Broad leaved wet hill forest found in the hill areas below 1000 m and above 500 m in all the districts of Nagaland, Northern

Sub-Tropical Pine forests found in the hills with elevation of 1000 m 1500 m in parts of Phek, Tuensang and Kipheri districts of Nagaland and Northern Mountain wet-Temperate forest found on the higher reaches of the tallest mountains (i.e. 2500 meters) like Saramati and Dzukuo area. Some of the commonly found trees are *Albizzia lebbeck, Artocarpous sp., Bombax ceiba, Castanopsis sp., Dipterocarpus macrocarpus, Gmelina arborea, Melia azederach, Mesua ferra, Michelia champaca, Quercus, Rhododendrons, Shorea sp., Schima wallichi, Terminalis sp., Tetrameles sp*, and different conifer Sp, *etc* and the endemic species of the hills are *Areca nagensis, Begonia wattii, Berberis mucropetala, B. wardii, Capillipedium nagense, C. pteropechys Carex astroi, Chaerophyllum orientale, Clematis meyeniana, C. meyeniana var. insularis, Cocculus prainianus, Coelogyne hitendrae, Corydalis borii, Cotoneaster nagensis, Crotalaria meeboldii, Cyclea wattii, Deyeuxia nagarum, D. borii, Hedychium marginatum, Impatiens cuspedifera, Maesa macrophylla var.magnidentata, Pholidota imbricata, Pimpinella nervosa, Rhynchosia meeboldii, Saussurea nagaensis, Senecio rhapdos, Silene vagans, , Sunepia jainii, Tetragoga nagaensis, Trichosanthes tomentosa,* (Nayar, 1996, Chowdhery and Murti, 2000). Besides these tree species Nagaland is also the native of large numbers of orchids. The orchid flora in Nagaland state is about 368 species of 88 genera (Table 1) including two endemic species, *Coelogyne hitendrae* and *Sunipia jainii* (Deb *et al*, 2003).

The remarkable ease with which orchids form hybrids (interspecific to plurigenic) has proved to be an invaluable asset not only to themselves but also to men. Since production of new species and varieties by hybridization is the speediest form of evolution, the tolerance of even very wide crosses by orchids has enabled

them to go up the ladder of evolution in leaps and bounds. The amenability of these plants to hybridization has successfully exploited by man to raise novel and striking hybrids in his horticultural pursuits (Vij, 2002). This fascinating feature has placed them at the top most position in aesthetic world. Orchids are important in the floriculture industry due to their beautiful foliage, colourful and fragrant flowers of varying shapes, and long vase life of cut flowers. This group of plants is valued highly both in the national as well as in the international markets. However, unlike the south Asian countries, India has not been able to make inroads into this multibillion dollars business despite rich natural wealth of orchid diversity (Satish Kumar and Manilal, 1994).

Although, orchid is one of the largest families among flowering plants, it is also probably among the most seriously threatened group of plants. Their vulnerability stems from two factors: the first being their highly specialized nature of germination and growth in association with a specific fungus and pollinator insects, and second being the attractive and beautiful flower of many species, making them so sought after by man. In recent years though biotechnological means has been adopted for their multiplication in mass scale and a ban has been imposed for their wild collection for trade under *Convention on International Trades in Endangered Species* (CITES) regulations, through wild life conservation laws, still there are events of wild collections. In developing countries like India, shifting cultivation and continual expansion of agricultural land coupled with deforestation for developmental activities have been a major threat to these plants. Each orchid species is adapted to life in a specialized environment. Because of their specialized requirements many orchids are very restricted in distribution and endemism is high

in many cases (Hegde and Sinha, 2002). While commercial orchid growing is yet to be properly organized in the country, the trade, whatsoever, is at present in the hands of hobbyist and nurserymen who collect orchids from naturally grown population, to meet their national and international commitments, and add to conservation related problems (Chadha, 1992). Mass propagation using conventional and tissue culture techniques thus seem to be the only strategy to commercialize orchids and conserve their natural populations from collection pressures (Vij, 2002).

Orchids are very sensitive to ecological disturbances in their habitats. Though orchids enjoy a wide range of habitats relatively little is known about the distribution and ecology of Indian orchids. Some of the more sensitive and specialized orchids are fast disappearing from their known habitats. Habitat destruction and disturbances coupled with lack of ecological awareness of people in general have driven some of the orchids from their natural niches to near extinction. Destruction and fragmentation of forest causes decrease in pollinator population. This results in the low frequency of pollinators visiting the orchid flowers. Nearly 98 per cent of flowering individual fail to set fruits under natural conditions due to lack of pollinators (Calvo, 1993).

The orchid seeds are microscopic and non-endospermous with undifferentiated embryos are produced in large numbers and their germination in nature is dependent upon a suitable association with a mycorrhizal fungus to provide an essential physico-chemical stimulus for initiating germination (Harley, 1959). Ever since Knudson (1922) demonstrated the possibility of bypassing the fungal requirement of orchid seeds during germination *in vitro*, asymbiotic seed germination has been accepted as important tools for propagating orchids (Arditti *et al*, 1982).

The orchids are primarily sexual but they also reproduce and propagate by vegetative means as well through seeds. The rate of vegetative propagation (i.e. keikis, back-bulbs, division of shoots etc.) is very slow in many orchid species and seed germination in nature is also very poor (~0.2-0.3%). The asymbiotic germination potential of fertilized ovules (seeds) has been positively tested in several commercially viable and or threatened Indian taxa (Vij, 2002).

Micropropagation of Orchid and its Role in Conservation: An Overview of Current Status

Orchid seeds due to poor development of endosperm or complete absence require a suitable fungal (mycorrhizal) stimulus for germination in nature. Knudson (1922) for the first time demonstrated the possibility of bypassing the fungal requirements during germination of *Cattleya* seeds (embryo) *in vitro* supply of appropriate organic carbon in the medium, while Tsuchiya (1954) discussed the possibility of germinating orchid seeds from immature pods. The discovery of these two techniques led to the development of 'green pod culture' that enabled to rescue hybrid embryos from desired mating (Sagawa, 1963). However, it calls for devising protocols for rapid cloning for exploitation of elite hybrids. *In vitro* cloning of *Phalaenopsis* using uni-nodal floral stock cuttings was developed by Rotor (1949) whereas; Thomale (1957) successfully cultured the shoot tips of *Orchis maculate,* but the possibility of using aerial roots for micropropagation was first suggested by Beechey(1970). The credit has been attributed to Morel (1960) for mass propagating *Cymbidium* clones by the culture of shoot apical meristem on Knudson 'C' medium for raising virus free clones. Lecoufle (1967) noted that orchid represents the first floricultural crop to be propagated successfully through shoot tip culture. Shoot tips

remain the most commonly used explants for micropropagating *cymbidiums* and other sympodial orchids but their utility is limited in monopodials for the same purpose as its excision endangers the survival of mother stock. Endeavors are therefore made toward exploring an alternative but equally effective technique whose excision will not be detrimental to the survival of the mother plant. Biotechnological tools like plant tissue culture techniques have thus opened new possibilities in conservation. The current status of orchid tissue culture and conservation is given in (Table 2).

Seed/Embryo Culture

Immature orchid seed/immature embryos can also be germinated *in vitro*. The technique is variously referred to as ovule/embryo/green pod/green fruit culture (Sagawa, 1963), which ensures better germination frequency and favours the production of virus free seedlings at a faster rate. Asymbiotic/non-symbiotic seed germination is commonly used in the propagation of tropical orchids, which tend to be easier to grow than their temperate relatives. The media used for asymbiotic germination is more complex than that for symbiotic germination, as all organic and inorganic nutrients and organic carbon source must be in a form readily available to the orchid without the intermediary fungus (Mc Kendrick, 2000). The technique involves an easy procedure for sterilization, ensures better frequency of germination, and reduces the time-lapse between pollination, sowing of seeds and production of virus free seedlings. Since all the seed/embryos are used in a single sowing in this technique, it is important to determine the harvest time of capsule or pod for getting optimal germination. The earliest stage at which the embryos can be cultured successfully varies with the orchid genotype and the local conditions. Very young

Table 2: Micropropagation of orchids: its current status

Taxa	Explant used	Response	Reference
Aerides multiflora Roxb.	(Seeds)	Seedling formation	Vij *et al*, 1981
	-do-	-do-	Katiyar *et al*, 1987
	-do-	Protocorm formation	Sharma & Tandon, 1987
	Protocorm segments	Multiple PLBs formation	Vij *et al*, 1981
	Leaf segments (0.5-1.0 cm)	Direct and callus mediated PLBs; regeneration & plantlet formation	Vij & Pathak, 1990
	root	Callus formation	Vij, 1993
Arachnis labrosa	(seeds)	PLBs formation	Temjensangba & Deb, 2005 b
	leaf	Shoot bud formation	Deb & Temjensangba (In Press)
	root	PLBs formation	Deb & Temjensangba (In press)
Aranda cv. Nancy	leaf tip	PLBs formation	Loh *et al*, 1975
Arundina bambusifolia Lindl.	Shoot tips (0.1-0.2 cm) & stem discs	Leaf development followed by rooting	Mitra, 1971
Bletilla striata	root	Callus formation	Yam & Weatherhead, 1991a
Catasetum fimbriatum	root	PLBs formation	Kerbauy & Colli. 1997, Vaz *et al*, 1998, Peres *et al*, 1999
Catasetum hybrid	root	PLBs formation	Kerbauy, 1984a.
Bulbophyllum comosum Collett& Hemsl	(Seeds)	Protocorm formation	Sharma & Tandon, 1987
Cattleya sp	leaf base	PLBs formation	Champagnat *et al*, 1970.
Cleisostoma Fordii	root	Callus formation	Yam and Weatherhead, 1991a
C. racemiferum	(Seeds)	PLBs formation	Temjensangba & Deb, 2006
	leaf	PLBs, callus and shoot bud formation	Temjensangba & Deb, 2005a
	root	PLBs and shoot bud formation	Deb & Temjensangba, 2005
Clowesia Warscewiczii	root	PLBs formation	Kerbauy & Estelita, 1996.
Coelogyne cristata Lindl	(Seeds)	Protocorm formation	Sharma & Tandon, 1987
C. speciosa	entire leaf	PLBs formation	Abdul & Hairani, 1990
C. porrecta Lindl	seeds	-do-	-do-
C. prolifera Lindl	-do-	-do-	-do-
C. punctulata Lindl	(Seeds)	Seedling formation	Sharma & Tandon, 1986, Katiyar *et al*, 1987.
Cymbidium eburneum Lindl.	Seeds	Seedling formation	Prasad & Mitra, 1975.
C. elegans	(Seeds)	Protocorm formation	Sharma & Tandon, 1987
	seeds	Seedling formation Symbiotic germination)	Raghuvanshi *et al*, 1991
C. giganteum Wall.(= C. *iridioides* D. Don)	Seeds	-do-	-do-
	(seeds)	Seedling transferred to community pots& infected with various mycorrhizal fungi; the infected seedlings grew better	Katiyar *et al*, 1991

Contd...

Taxa	Explant used	Response	Reference
C. longifolium D. Don(= *C. elegans* Lindl)	(Seeds)	Protocorm multiplication & seedling formation	Muralidhar & Metha, 1986
C. macrorhizon Lindl	(Seeds)	Formation of rhizomatous structures;Aerial shoots with scale leaves emergence in a few cultures	Vij & Pathak1988.
C. mastersii Griff.	Seeds	Seedling formation	Prasad & Mitra, 1975
Dendrobium chrysanthum Wall.ex. Lindl	(Seeds)	Seedling formation	Raghuvanshi *et al*, 1986
D. crumenatum	leaf base	callus & PLBs Mediated formation	Manorama *et al*, 1984.
D. farmeri Paxt	(Seeds)	Protocorm formation	Devi et al, 1990
D. fimbriatum Hk.f	Seeds	Protocorm formation via callusing; direct Protocorm differentiation & seedling formation	Mitra *et al*, 1976
D. fimbriatum var. *Oculata* Hk.f. (D. Normale Falc)	(Seeds)	Seedling formation	Devi *et al*, 1990
D. moschatum (Buch-Ham.) Su	(Seeds)	Seedling formation	Devi *et al*, 1990
D. nobile Lindl	(Seeds)	Seedling formation	Raghuvanshi *et al*, 1985, 1986
D. primulinum Lindl	(Seeds)	-do-	Devi *et al*, 1990.
Doritis pulcherrima	entire leaf	PLBs formation	Tanaka, 1987.
Eulophia hormusjii Duth.	Rhizome segments (0.3-0.4 cm) with or without differentiated nodes	Direct shoot bud& PLBs regeneration; plantlet formation	Vij *et al*, 1989.
Luisia teretifolia Gaud.	leaf segment (0.5-1.0 cm)	Direct or callus mediated PLBs regeneration & plantlet formation	Vij & Pathak, 1990
L. trichorhiza	entire leaf	callus & PLBs	Vij & Pathak, 1990
Mormodes histrio	root	PLBs formation	Arditti & Ernst, 1993
Neottia nidu-avis	root	PLBs & Shoot bud formation	Champagnat *et al*, 1971
Oncidium varicosum	root	PLBs & shoot bud formation	Kerbauy, 1984b.
Paphiopedilum	leaf tip	Callus mediated	Allenberg, 1976
Phalaenopsis Intermedia	entire leaf	PLBs formation	Tanaka, 1987.
P. pulchra	entire leaf	PLBs formation	-do-
P. sanderiana	-do-	-do-	-do-
P. stuartiana	-do-	-do- -do-	
P. amabilis	root tip	PLBs formation	Tanaka *et al*, 1976.
P. amabilis hybrid	entire leaf	-do-	Tanaka & Sakanishi, 1977, 1985
Pholidota cantonesis	root	shoot bud formation	Yam & Weatherhead, 1991a

Contd...

Taxa	Explant used	Response	Reference
P. chenensis	root	Shoot bud formation	Yam & Weatherhead, 1991a
Rhynchostylis retusa (Linn) Bl.	(Seeds)	Direct and callus mediated protocorms; Seedling. formation	Vij *et al*, 1981.
	-do-	seedlings transferred to pots.	Nath *et al*,1991.
	Root segments (0.2-0.8 cm)	Direct PLBs regeneration, multiplication& plantlet formation	Chaturvedi & Sharma, 1986.
	Root segments (0.5-1.0 cm)	Direct shoot bud& PLBs regeneration & plantlet formation.	Sood & Vij, 1986
	-do-	Direct PLBs regeneration & plantlet formation	Vij *et al*, 1987.
R. retusa	Leaf segment	No response From *in vivo* grown plants	Vij *et al*, 1984.
	Leaf segments (0.5-1.0)	Direct PLBs regeneration & plantlet formation	-do-
	Leaf segments	Direct PLBs regeneration, multiplication & plantlet formation	Chaturvedi & Sharma, 1986.
	Leaf segments (0.5-1.0)	Direct PLBs regeneration & plantlet formation	Vij & Pathak, 1990
Saccolabium Calceolare Lindl.	(Seeds)	Direct & callus mediated protocorms; Seedling formation	Vij *et al*, 1981.
Sarcanthus pallidus Lindl	(Seeds)	Protocorm formation	Sharma & Tandon1987.
	-do-	Seedling formation	Raghuvandshi *et al,* 1985, 1986.
Satyrium nepalense D. Don.	Leaf segments (0.5-1.0)	Callusing & direct PLBs regeneration& plantlet formation	Vij & Pathak, 1990
Thunia alba (Lindl) Reichb.	Leaf segments	Protocorm formation	Sharma & Tandon, 1987.
	Seeds	seedling formation (Symbiotic germination)	Raghuvanshi *et al*, 1991.
Vanda cristata Lindl.	leaf segments (0.5-1.0)	Direct or callus mediated PLBs, Regeneration & Plantlet formation	Vij & Pathak, 1990
V. testaceae Lindl). Reichb.f.	(seeds)	Direct or callus mediated protocorms; Seedling formation	Vij *et al*, 1981
	Leaf segments	Direct PLBs regeneration & plantlet formation	Vij *et al*, 1986
	Leaf segments (0.5-1.0 cm)	Callusing: Direct regeneration & plantlet formation	Vij & Pathak, 1990
Vanda Kasem's Delight 'Tom Boykin'	root	PLBs formation	Vij & Sharma,1997

*Abbreviation used- PLBs: Protocorm like bodies

** Different plant parts used were taken from *in vitro* raised seedlings (unless mentioned otherwise); (seeds): from undehisced capsule; seeds: from dehisced capsule.

ovules do not form suitable explants in orchids because the embryo sac development is a post pollination phenomenon and fertilization a prerequisite for obtaining seedlings. However as the ovules can be used for raising cultures immediately after fertilization, the importance of information on time interval between pollination and fertilization has often been stressed (Valmayor and Sagawa, 1967). *Doritis* ovules from pollinated ovaries germinated readily after getting fertilized *in vitro* (Yasugi, 1984) suggesting that fertilization is a pre-requisite for germination. Yam & Weatherhead (1988) also noted that immature embryo germinates better than the mature ones due to their distended testa cells and metabolically awakened embryos; they also lack dormancy or inhibitory factors. *Eulophia hormusjii* embryos, procured between 8 and 16 weeks after pollination (WAP), germinate readily but their germination frequency declines sharply with further passage of time; when obtained later than 16 WAP. Likewise, *Satyrium nepalense, Nephalaphyllum cordifolium, Phaius tankervilliae* and *cymbidiums* germination frequency shows sharp decline when the embryos are collected 3-4 weeks prior to fruit dehiscence. Mitra (1986) also stressed the importance of information on histochemical and biochemical features for selecting the fruits for the right type of immature ovules (seeds). Supporting this view, Vij (1995) reported that the fruit that develop prominent ridges along the valves and cease to grow in diameter responds better, a marker useful in selecting the right stage of fruits for embryo culture.

Meristem Culture

Resident meristem: A great deal of heterozygosity is produced in their progeny in orchids owing to its out breeding characteristic because of this embryo culture appears to be a disadvantageous proposition in cut-flower industry where pure lines

of desired genotypes are preferred. It was Morel (1960) who demonstrated the possibility of using excised shoot-meristem for regenerating complete plant from *cymbidiums in vitro*, and Wimber (1963) formulated, described and published a procedure for the purpose. This technique of using resident meristem (shoot-tips, axillary bud) has opened new vistas in orchid micropropagation (Arditti and Ernst, 1993). Through this technique, from a single resident meristem upto 200,000 plants can be regenerated within a year, however, it has limited utility in monopodial taxa as it involves the sacrifice of the growing tip thereby, endangers the survival of the mother plant.

Adventive meristems: The regenerative competence or the proliferative potential of adventitious meristem, whose excision is not detrimental to the survival of the mother plant, has been positively tested in many orchid taxa, viz: leaf explants (Vij *et al*, 1984; Mathews and Rao, 1985; Chaturvedi and Sharma, 1986; Seeni, 1988; Vij and Pathak, 1988, 1990; Seeni and Latha, 1992; Temjensangba and Deb, 2005a), root (Chaturvedi and Sharma, 1986; Sood and Vij, 1986; Vij and Pathak, 1988, Vij, 1993; Deb and Temjensangba, 2005), flower stalks (Singh and Prakash, 1984; Kaur and Vij, 1995; Vij *et al*, 1997). The source and genetic constitution and physiological age of the explants are however, some of the important factor for regeneration. The juvenile tissues from greenhouse grown plants respond better than the mature ones grown outdoors. Generally, the proliferative loci get activated in the dermal cells and soon develop into somatic embryos and or protocorm-like-bodies (PLBs). Somatic embryogenesis is either direct or callus mediated development, multiplication and differentiation of the PLBs is influence by the chemical stimulus present in the nutrient pool (Vij and Pathak, 1990; Seeni and Latha, 1992).

The advantages of leaf and root segment culture are apparent for more than one reason: they are easy to obtain, easier to disinfect, and their excision does not endanger the mother plant. Furthermore, as the regeneration occurs in the dermal cells, which is cytologically more stable, mass production of genetically uniform plant from this is within the realm of reality (Vij, 2002).

Different species of orchids exhibits specific needs in respect to nutritional requirement and treatment with plant growth regulators (PGRs) for their growth and development. Most commonly employed basal medium for orchid tissue culture are Knudson 'C' (1946), Mitra *et al*, (1976), Murashige and Skoog (MS) (1962), Nitsch and Nitsch (1969), Vacin and Went (1949). The use of α-Naphthalene acetic acid (NAA) and one of the cytokinins like Benzyladenine (BA) and kinetin (Kn) yields a rich crop of PLBs in *Luisia trichorhiza, Satyrium nepalense, Vanda cristata* and *Vanda testaceae* leaf segment culture (Vij, 1995). Similarly, in *Rhynchostylis retusa*, a synergistic action of Kn and indole 3-acetic acid (IAA) or NAA in peptone enriched medium favours enhanced production of PLBs while yeast extract (YE) is obligatory for regeneration in *Aerides multiflorum, Papilionanthe teres* and *Satyrium nepalense* foliar cultures and peptone in those of Vanda (Vij, 2002)

The phenolic exudation by the explants into the medium is a serious problem in orchid tissue culture as it impairs the growth of plant /regenerates considerably. But this can be offset by the use of activated charcoal (AC) in the medium. Activated charcoal favors better health of the cultures because of its ability to absorb exudates/growth inhibitors, enhance medium aeration, and absorb light and provide enhanced quantum of energy per unit plant material (Yam *et al*, 1989).

The biotechnological tools like tissue culture technique offer an exciting opportunity for germplasm storage and conservation as, the *in vitro* storage of tissues/germplasm requires less space under different physico-chemical conditions, such as, light, reduced temperature or altered media. Seed storage is the most commonly used method for storage of plant genetic resources (Englemann, 1997). However, seed storage is prone to destruction by pathogen/pests, loss of viability and while, cryopreservation of germplasm/somatic embryos/propagules is an expensive approach. On the other hand, the technique of *in vitro* germplasm conservation or storage through slow-growth of culture is cost effective and does not interfere with the viability of regenerates, thus provides an efficient system, which has been applied to several plants including orchids (Aggrawal *et al*, 1992; Ichihashi and Hiraiwa, 1996; Chang and Chang, 1998 and Vij *etal*, 2000a&b).

The minimal or slow growth technique is one of the techniques usually employed and provides a convenient option for *in vitro* germplasm conservation of short to medium term storage. A reduction in growth can be achieved in several ways: incubation of plants at low temperature, growth maintained at reduced light/temperature, lowering of the available oxygen levels, reduced nutritional status particularly carbon levels and by incorporation of osmoticum like mannitol, sub lethal level of growth inhibitor or retardants in the medium (Engelmann, 1997). The addition of activated charcoal (AC) to the culture medium is also been reported to be beneficial in minimal growth condition (Paul, 1999).

The minimal growth method is advantageous because the cultures can be maintained and subculture at an intervals ranging from 6 months or less to 2 years, and can be readily revert back to normal growth condition for resumption of growth

to produce plants. However, it is not suited for long-term programmes, because of risks of selection due to stresses imposed on the cultures during storage (Withers, 1991). There is also continued concern about the level of somaclonal variation under slow growth conditions (Jaret and Gawel, 1991). However, cassava could be stored for 10 years under slow growth condition and remained genetically stable (Angel *et al*, 1996).

With a view to mass propagate and conserve some rare/endemic/ threatened/ endangered orchids of North-East India in general and Nagaland in particular, I selected two rare and threatened orchids (*Arachnis labrosa* and *Cleisostoma racemiferum*) of Nagaland for my Ph.D degree with the following objectives:

I) Initiation of cultures from different explants like seeds of various developmental stages, leaves and roots from *in vitro* raised plants etc.

II) Regeneration of plants and mass multiplication of the selected orchids.

III) Short to medium-term *in vitro* storage of the selected plants using slow-growth technique

IV) Reintroduction of *in vitro* raised plants in their natural habitats.

General Characteristic of the Selected Orchids

Arachnis labrosa (Lindi. Ex Paxt.) Reichb. f.

Carl Ludwig Blume established the genus in 1825 A.D. The generic name has been derived from the Greek word '*arahne*' (spider) due to resemblance of the flower to a spider. The genus *Arachnis* is represented by about 6 species in South-East Asia and 2 species are found in NE India (India). *Arachnis labrosa* is a monopodial epiphytic orchid of Vandaceous orchid. The flower is pale yellow with irregular dark brown marking (Figure 2 a and b). This species flowers in the month

Figure 2: Photographs of selected orchid. a. Plant of *Arachnis labrosa* (vegetative), b. *A. labrosa* flower, c. Plant of *Cleisostoma racemiferum* (vegetative) and d. Flowers of *C. racemiferum*.

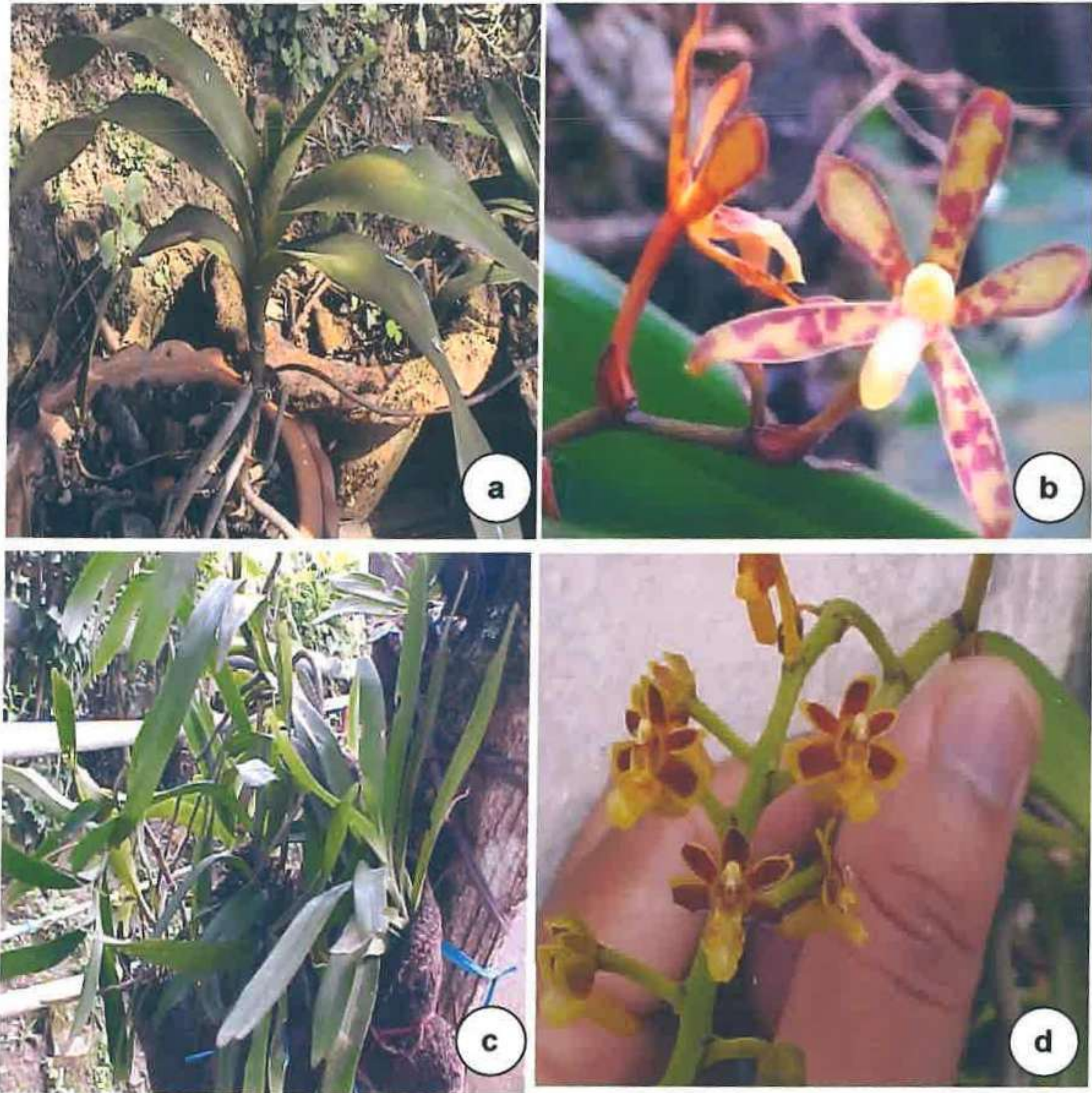

Figure-2

of August and September. They are sparingly distributed in North East India. This species is under threat in natural habitat due to habitat destruction.

***Cleisostoma racemiferum* (Lindl.) Garay**

The generic name is derived from the Greek words '*kleistos*' (closed) and '*stoma*' (mouth), referring to the inflated lip and calli, which are almost closing the mouth of the spur. The genus is represented by about 100 species of which 19 and 8 species are reported from India and Nagaland respectively. *C. racemiferum* is a monopodial epiphytic orchid. Many flowered racemes, small and are dull brown and edged with yellow (Figure 2 c and d). It flowers during the month of July-September. This species is listed endangered and under threat due to habitat destruction.

Chapter-2

Materials and Methodology

The conventional breeding of orchids by seed is a long term process and it has a low survival rate due to lack of endosperm or poorly developed at maturity while their vegetative propagation through *keikis*, back-bulbs, division of shoots etc is rather slow and the production is extremely meagre. Therefore, it is necessary to look for alternative technique for rapid mass multiplication and conservation of the germplasm. The *in vitro* technique like micropropagation has opened a new route in conservation and commercialisation of orchids. In fact, orchid is the first floricultural crop to propagate successfully through this technique. Following this technique, round the year propagation of genetically uniform, disease free, fast maturing and high yielding plants are made possible; and enabled to exploit the regenerative competence more effectively than the conventional method. Moreover, the micropropagated plants grow more vigorously due to their better health status. Ever since, Knudson (1946) demonstrated the ability to by-passed the fungal requirements for germination of *Cattleya* seeds/embryos *in vitro* by using appropriate carbohydrates in the culture medium, and remarkable progress has been made in micropropagation of orchids (Arditti *et al,* 1982; Mitra, 1986; Yam and

Weatherhead, 1988; Vij and Pathak, 1990; Seeni and Latha, 1992; Arditti and Ernst, 1993; Bejoy *et al*; 2004; Chen *et al*, 2004; Deb and Temjensangba, 2005; Temjensangba and Deb, 2005a&b). The immature embryos and the shoot meristem are the most commonly used explants for *in vitro* propagation of orchids. However, success to a great extent depends on the selection of right explants, physiological age, media composition, exogenous growth regulators and culture conditions.

2.1: Plant Materials

Seeds: Immature seeds/green pods of different developmental ages [8- 20 weeks after pollination (WAP)] were collected at 2 wk intervals for both *Arachnis labrosa* and *Cleisostoma racemiferum* from outdoor grown plants. The green pods collected were stored in a sealed polythene bags at 4°C until used.

Leaf: Leaf explants (size: 1.5-2.0 cm) were collected from outdoor grown plants of 15-20 wk after emergence. The leaves were also collected from 15-20 wk old from axenic culture of both the selected orchids. Immediately after harvesting the leaves from both the sources were soaked in sterilized distilled water for 15-20 min.

Roots: Roots from plants grown out door were collected at 2-14 wk after emergence while the roots of 15 – 20 wk were harvested from axenic cultures of both species.

2.2: Sterilization of Plant Materials

Seeds: The green pods/capsule of various developmental stages were harvested and scrubbed cleaned with Extran (commercial lab. detergent, make: Merck) and rinsed them under running tap water. Pods were surface sterilized by 0.5 % aqueous solution of Mercuric Chloride ($HgCl_2$) (w/v), rinsed 4-5 times with sterile doubled distilled water. Thereafter, the pods were dipped in 70% ethanol (v/v) for 30 sec and

rinsed them 3 - 4 times with sterilized doubled distilled water. Prior to dissection of embryos the pods was dipped in alcohol and flamed.

Leaf: Leaf explants of both the species (*A. labrosa* and *C. racemiferum)* collected from plants grown out door were surface cleaned by laboratory detergent (1: 100) (Extran) and rinsed them under running tap water for 10 min. Explants were sterilized with 0.25% (w/v) aqueous solution of $HgCl_2$ for 5 min and washed 4-5 times with sterilized double distilled water. Explants were further sterilized in 70% ethanol for 30 sec and rinsed 3-4 times with sterile double distilled water. The explants collected from both the sources were soaked in sterile distilled water for 15-20 min before inoculation. The leaves were cut into segments of 0.5 cm and cultured on different initiation media.

Roots: The root explants of both species (*A. labrosa* and *C. racemiferum*) collected from plants grown out door were brush - scrubbed cleaned with 'Extran' and rinsed them under running tap water. Thereafter the roots were sterilized with 0.25% (w/v) aqueous solution of $HgCl_2$ for 5 min and washed 4-5 times with sterilized distilled water. The roots were further sterilized by dipping in 70% ethanol for 30 sec and rinsed with sterilized distilled water. While root explants from axenic culture were washed with sterile distilled water to remove the agar. Prior to culture, explants were soaked in sterilized water for 15-25 minutes.

2.3: Tissue Culture

Media: For initiation of embryo cultures/seeds, various media like Knudson C (Knudson, 1946), Mitra *et al*, (Mitra *et al*, 1976) and Murashige and Skoog (MS) (Murashige and Skoog, 1962) media were used. For immature seed culture of both

the species, the basal media were fortified with coconut milk (CW) (0-20%) (v/v), Casein hydrolysate (CH) (0-500mg$^{-1)}$ (w/v) and sucrose 0-3.0% (w/v) as carbon source. For root and leaf culture initiation of both species all the three basal media were fortified with sucrose (0-3%) (w/v), Casein hydrolysate (CH) (0-500mg$^{-1)}$ (w/v), CW (0-20%) (v/v) and citric acid 100 mgl^{-1} (as an antioxidant).

For initiation of culture from immature embryo/seeds from both the species the basal media were further supplemented with different levels of NAA and BA (0-32 µM) singly or in combination. In case of leaf and root culture of *Arachnis labrosa* all the basal media were conjunct with IAA, NAA, BA and Kn (0-6 µM) singly or in combination. While, for leaf and root culture of *C. racemiferum* all the media were enriched with IAA, NAA, BA and Kn (0-2 µM) singly or in combination.

Difco-bacto agar (0.8%) (w/v) was incorporated as gelling agent after adjusting the *p*H of the medium to 4.8 to 6.2 using 0.1 N NaOH and 0.1 N HCl with an increment of 0.10. About 15 ml medium was dispensed into each test tube (size 150 mm x 25 mm) and 30 ml into 500 ml culture bottle (diameter: 70 mm). The media were autoclaved at 1.05 Kg cm^{-2} pressures and at 121°C for 20 minutes. The test tubes were slanted at 45°C and solidified.

2.3.A: Micropropagation

2.3.A.1: Initiation of Cultures

Immature embryos/green pods: The immature embryos (seeds) of different developmental stages from both the species were dissected out from the sterilized green pods with the help of scalpel blade under a laminar flow chamber and cultured on different basal media. For each treatment 20 numbers of culture vials were used and all the experiment was repeated thrice.

Leaf: The sterilized leaves from both *in vivo* and *in vitro* sources of the selected species were cut into 0.5 cm size and cultured on different initiation media. In each culture vial four numbers of leaf segments were cultured. To determine the effect of orientation, explants were placed in a slanted (~45°) and horizontal position. For each treatment 40 explants were used and all the experiment was repeated thrice.

Root segment: The aerial roots from both *in vivo* and *in vitro* sources were sterilized and cut into 0.5 cm size and cultured on different initiation media. Explants were placed both horizontally and slanted (~45°) to study the effect of orientation of explants. In each test tube two numbers of root segments were cultured and about 50 numbers of root segments were used for each treatment. All the experiment was repeated thrice.

The cultured embryos were incubated under different light conditions viz. dark, diffused (20 µmol m^{-2} s^{-1}) and full light (40 µmol m^{-2} s^{-1}) at 12/12 hr light/dark photo cycle. But the inoculated leaf and roots were incubated in the dark for first 48 hr followed by full light conditions. All the cultures were maintained at 24±1°C and sub-cultured at 4-5 wk interval until mentioned otherwise.

The Protocorm like bodies (PLBs) developed from the germinated seeds, leaf and root segments were maintained on the same initiation medium for 2-3 passages for further differentiation.

2.3.A.2: Regeneration of Plantlets and Mass Multiplication

The advanced stage PLBs (with first set of leaflets) formed from the cultured immature embryos, leaf and root segments were maintained further for 2 passages on the optimum initiation conditions for formation of tiny plantlets. The tiny plantlets so formed were separated from the clumps and transferred on two different basal media

(MS and Mitra *et al*) containing sucrose (3%), CW (15%), citric acid (100 mgl^{-1}) different levels of plant growth regulators (PGRs) like IAA, NA, BA and Kn (0- 32 µM) singly or in combination for regeneration of plantlets and mass multiplication. In every sub-culture the shoot buds formed were separated and transferred on fresh regeneration medium. In another set of regeneration medium AC (0-0.3%) (w/v) was incorporated to study its effect on morphogenetic response and mass multiplication. The plantlets were maintained for 4-5 passages on regeneration medium.

2.3.A.3: Hardening of Regenerated Plants

The regenerated plantlets with 2-3 roots (~ 3-4 cm long) of both the species were hardened for considerable period prior to transferring in the potting mix. Two different techniques were followed for *in vitro* hardening the regenerates. In conventional technique the tiny well rooted plantlets from all the explants sources of both the species were taken out and were transferred to highly reduced strength of MS liquid medium ($1/10^{th}$ strength) supplemented by sucrose (2%) (w/v). Alternatively in another set of culture the agar was washed out carefully using a soft brush and cultured in culture vials containing $1/10^{th}$ MS salt solution devoid of sucrose but containing small chips of charcoal, brick pieces and mosses in different combinations. Before using the MS salt solution and substrate (charcoal, bricks and mosses) were put into a conical flask, cotton plugged and autoclaved at 121°C of 1.05 kg cm^2 pressures for 30 min. Solution of basal medium was dispensed to the test tube (size 150 mm x 25 mm) of about 10 ml, to this added the chips of charcoal or bricks or mosses. This operation was done under aseptic condition. The exhausted medium was replaced at 6 wk interval by fresh medium. The plantlets were cultured

and maintained under the full light (40 µmol m^{-2} s^{-1}, provided by fluorescent tube) condition for about three to four months.

2.3.A.4: Potting Mix and Transplantation of Regenerates

For transplanting the hardened plantlets of both the orchid species (*A. labrosa and C. racemiferum*), the community potting mix were prepared by mixing different substrates like decayed wood, charcoal pieces, brick chips and coconut husk in different combinations in the ratio of 1:1:1:1 with a layer of moss. The hardened plants were washed carefully to remove the traces of agar and transferred to community potting mix. The potted plantlets were kept in the polyhouse for acclimatization for about 2-3 months before transferring to the wild. The newly potted plants in the community potting mix were then fed with MS liquid salt solution ($1/10^{th}$ strength) at one-week interval for about two months.

2.4: Short to Medium-Term *In Vitro* Storage

For *in vitro* short to medium-term storage of germplasm by slow growth of culture was attempted. The advanced stage PLBs and tiny plantlets were subjected to *in vitro* conservation using slow growth method. The different types of propagules were cultured on different levels of basal media (0, $1/4^{th}$, 1/2, $3/4^{th}$ and full strength) conjugated with varying concentrations of organic carbon sources like sucrose, mannitol, fructose, glucose and dextrose (0, 1, 2 and 3%) (w/v). The cultures were maintained at different temperature (10, 20 and 25°C) and different light conditions [dark, diffused (20 µmol m^{-2} s^{-1}) and full light (40 µmol $m^{-2}s^{-1}$)]. The cultures are sub-cultured at 15-16 wk interval.

Chapter-3

Results

3.1: Initiation of Cultures

3.1.A: Immature Embryos/Seed

The immature seeds/embryos were cultured on different basal medium containing different supplements. In *Arachnis labrosa* the first sign of germination was observed as nodular swelling and or greening of embryos after 25 days of culture while, in case of *Cleisostoma racemiferum* similar response was registered only after 52 days of culture (Figure 3 a & 4 a). In *A. labrosa* better germination of immature embryos were obtained on Mitra *et al* medium where ~81% embryos germinated successfully followed by MS medium and Knudson 'C' respectively. But in case of *C. racemiferum* the optimum germinating response was achieved on MS medium (90%) followed by Mitra *et al* and Knudson 'C' respectively (Table 3). Green pod/capsule from both the selected species representing different physiological age was used for initiation of non-symbiotic seed germination.

The developmental stages of the immature embryos showed a profound effect on successful non-symbiotic germination. Seed pod age of 18 WAP was found to be optimum for *A. labrosa* where ~81% germination was registered while in the case of *C. racemiferum*, seed pod age of 16 WAP registered ~90% germination (Table 3). In

Table 3: Effect of seed pod age on non-symbiotic germination of *Arachnis labrosa* and *Cleisostoma racemiferum*

Basal media	Age of capsule (WAP)	*Arachnis labrosa*			*Cleisostoma racemiferum*		
		response (%) (± SE)	greening (D)*	Protocorm formation (D)*	response (%) (± SE)	greening (D)*	protocorm formation (D)*
	8	-	-	-	-	-	-
	10	-	-	-	-	-	-
	12	-	-	-	-	-	-
Knudson 'C'	14	-	-	-	50 (± 2.0)	84	60
	16	25 (± 2.0)	32	40	60 (± 1.5)	68	56
	18	44 (± 1.5)	28	28	50 (± 1.5)	75	70
	20	-	-	-	-	-	-
	8	-	-	-	-	-	-
	10	-	-	-	-	-	-
	12	-	-	-	28 (± 2.0)	80	-
Mitra *et al,*	14	30 (± 2.0)	51	26	50 (± 2.5)	63	42
	16	60 (± 1.5)	35	27	60 (± 1.5)	50	53
	18	**81 (± 1.2)**	**25**	**27**	50 (± 1.0)	58	60
	20	60 (± 2.0)	40	32	40 (± 2.0)	60	–
	8	-	-	-	-	-	-
	10	-	-	-	-	-	-
	12	-	-	-	30 (± 2.0)	120	68
Murashige and Skoog	14	30 (± 2.0)	40	27	60 (± 1.5)	70	47
	16	55 (± 1.5)	40	27	**90 (± 1.0)**	**52**	**40**
	18	67 (± 1.5)	20	25	70 (± 0.5)	55	40
	20	60 (± 1.2)	36	28	50 (± 2.0)	70	60

Data represents the mean of three replicates, * No. of days taken, ± SE – standard error, medium containing sucrose (3%), coconut water (15%), and NAA (20 µM) and BA (16 µM) in combination for *A. labrosa* while NAA (10 µM) and (16 µM) in combination for *C. racemiferum*.

Figure 3: Different stages of plant regeneration and mass multiplication of *A. labrosa* from immature embryos/seeds. a. Germinating seeds showing nodular swelling on germination medium and formation of PLBs, b. Advanced stage PLBs and young plantlets formed from PLBs and c. Multiple shoots and rooted plants developed on regeneration medium.

Figure-3

Table 4: Effect of growth adjuvant (sucrose and coconut water) on non-symbiotic seed germination of *A. labrosa* and *C. racemiferum*

Basal medium	Growth adjunct(%)		% germination (±SE)*	
	Sucrose	coconut water	*A. labrosa*	*C. racemiferum*
Knudson 'C'	0	0	-	-
	1	5	-	-
	1	10	-	-
	1	15	-	40 (± 2.0)
	1	20	-	-
	2	5	-	-
	2	10	55 (± 1.5)	40 (± 2.5)
	2	15	-	40 (± 2.5)
	2	20	25 (± 2.0)	30 (± 2.0)
	3	5	40 (± 2.5)	30 (± 2.0)
	3	10	44 (±2.0)	30 (± 2.0)
	3	15	44 (±2.0)	60 (± 1.5)
	3	20	30 (±2.0)	40 (± 2.5)
Mitra *et al*	1	5	-	-
	1	10	-	-
	1	15	-	45 (± 2.0)
	1	20	-	40 (± 2.0)
	2	5	-	20 (± 2.5)
	2	10	-	57 (± 2.0)
	2	15	67 (± 2.0)	80 (± 1.5)
	2	20	60 (± 1.5)	60 (± 1.0)
	3	5	55 (± 2.0)	45 (± 2.0)
	3	10	70 (± 1.0)	50 (± 2.0)
	3	**15**	**81 (± 1.0)**	50 (± 2.0)
	3	20	64 (± 2.0)	45 (± 1.5)
MS	1	5	-	34 (± 1.5)
	1	10	-	43 (± 1.0)
	1	15	50 (± 1.0)	50 (± 1.5)
	1	20	-	55 (± 2.0)
	2	5	-	30 (± 2.0)
	2	10	-	56 (± 2.5)
	2	15	60 (± 1.5)	60 (± 2.0)
	2	20	60 (± 1.5)	58 (± 2.0)
	3	5	55 (± 2.0)	50 (± 2.5)
	3	10	67 (± 2.5)	55 (± 2.0)
	3	**15**	67 (± 2.5)	**90 (± 1.5)**
	3	20	-	70 (± 1.5)

Data represents the mean of three replicates,*± SE: Standard error,
Medium containing NAA (20 µM) and BA (16 µM) in combination for *A.labrosa* while NAA (10 µM) and (16 µM) in combination for *C. racemiferum*.

Figure 4: Different stages of plant regeneration and mass multiplication of *C. racemiferum* from immature embryos/seeds. a. germinating seeds showing nodular swelling on germination medium and formation of PLBs, b. Advanced stage PLBs and young plantlets formed from PLBs and c. Multiple shoots and rooted plants developed on regeneration medium

Figure–4

both species the young embryos either failed to germinate or required longer germination period. In case of *A. labrosa* seeds <14 WAP did not germinate on any of the medium tested while, seeds of <12 WAP from *C. racimeferum* exhibited similar response. The seeds from the older pods exhibited poor response accompanied by fungal contamination.

The *p*H of the medium showed a pronounced effect on successful culture initiation. The *p*H range of 4.8 to 5.4 did not support culture initiation, as the medium was softer. The healthy culture could be initiated with medium adjusted to *p*H 5.6 to 5.7 while, a *p*H range >5.7 resulted harder medium and culture degenerated after 1-2 passages (Figure 5).

Different concentrations of sucrose and CW were incorporated in the initiation media and both the adjuvant showed varied effect on seed germination in the two orchid species under study. In *A. labrosa*, sucrose (3%) and CW (15%) favoured ~81.0% germination (Table 4) in Mitra *et al* medium followed by 67% germination in MS basal medium containing sucrose (3%) and CW (10%), where as in *C. racemiferum*, the optimum germination was ~90% on MS medium containing sucrose (3%) and CW (15%) followed by 80% germination on Mitra *et al* medium containing sucrose (2%) and CW (15%) (Table 4). However, the incorporation of CH in the germination medium did not show any marked effect in both the species (Table 5).

Amongst the different levels PGRs used for non-symbiotic seed germination *A. labrosa* the Mitra *et al* medium containing NAA + BA (20.0 + 16.0 µM respectively) in combination supported optimum germination (~81%) and healthy PLBs formation. The singly treatment of NAA formed fewer PLBs while, BA

Figure 5: The effect of *p*H of the basal medium on non-symbiotic seed germination of *A. labrosa* and *C. racemiferum*

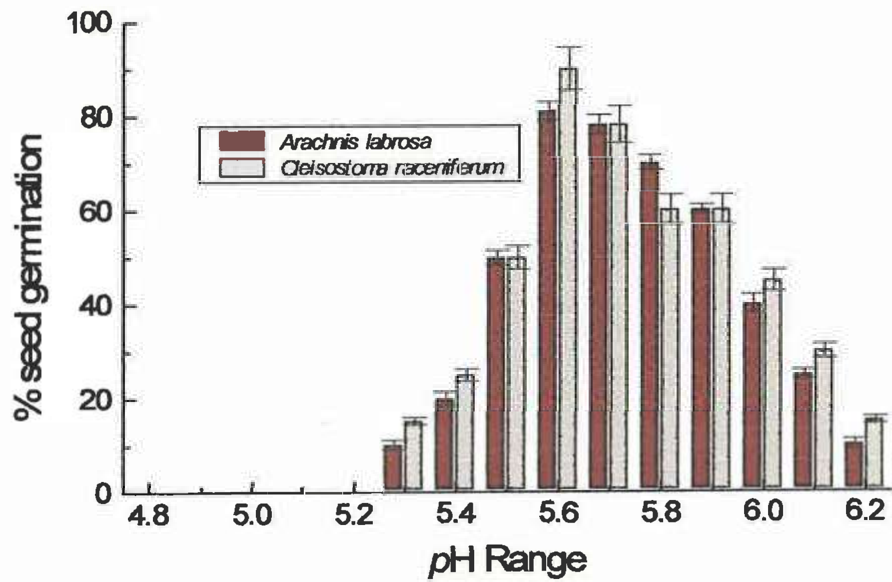

Figure 5: The effect of *p*H of the basal medium on non-symbiotic seed germination of *A. labrosa* and *C. racemiferum.*

Table 5: Effect of casein hydrolysate on *in vitro* regeneration from different explants sources like seed, leaf and roots of *A. labrosa* and *C. racemiferum*.

Casein hydrolysate (mgl^{-1})	% response (±SE)[@]					
	Seed		**Leaf**		**Root**	
	Al*	**Cr****	**Al**	**Cr**	**Al**	**Cr**
0	81 (± 1.0)	90 (± 1.5)	45 (± 1.0)	51 (± 1.5)	55 (± 2.0)	50 (± 1.0)
100	72 (± 1.0)	84 (± 1.0)	-	**82 (± 1.34)**	-	68 (± 1.06)
200	72 (± 1.0)	75 (± 2.0)	53 (± 1.39)	78 (± 0.81)	64 (± 1.36)	**80 (± 1.98)**
300	60 (± 2.0)	60 (± 2.5)	50 (± 0.77)	61 (± 1.14)	61 (± 2.64)	70 (± 1.49)
400	58 (± 1.5)	50 (± 1.0)	70 (± 0.97)	75 (± 0.97)	71 (± 1.41)	50 (± 1.13)
500	40 (± 2.5)	44 (± 1.0)	**84 (± 0.89)**	71 (± 1.15)	**75 (± 0.95)**	55 (± 0.95)
600	40 (± 1.0)	44 (± 1.0)	64 (± 0.97)	71 (± 1.79)	-	61 (± 3.23)

Data represents the mean of three replicates, @: Standard error, * Al- *A. labrosa* , ** Cr- *C. racemiferum*, Medium containing sucrose (3%), NAA (20 µM) and BA (16 µM) in combination for *A. labrosa* while NAA (10 µM) and (16 µM) in combination for *C. racemiferum.*

enriched media did not support healthy growth of the culture and degenerated subsequently. Only the combined treatment of NAA and BA supported healthy culture growth (Table 6). But MS medium containing NAA + BA (10.0 + 16.0 µM respectively) in combination registered optimum germination for *C. racemiferum* while, media enriched with either NAA or BA singly supported fewer PLBs formation (Table 7).

Three different light conditions were tested for *in vitro* seed germination for both the species. Of the three light conditions, diffused light (20 µmol m^{-2} s^{-1}) supported better germination followed by full light conditions (40 µ mol m^{-2} s^{-1}) in both the species. The cultures maintained in the dark either failed to germinate or callused and degenerated subsequently (Table 8).

The germinated seeds/embryos of both the species showed initial response of germination as nodular swelling and or greening of seeds. These nodular structures during subsequent sub-culture converted into PLBs (Figure 3 b & 4 b). The PLBs so formed on initiation media were maintained on the optimum initiation medium for 2 more passages and during this period the PLBs flushed the first set of leaflets (advanced PLBs).

3.1.B: Leaf

The cultures were also initiated from the foliar explants of *A. labrosa* and *C. racemiferum*. However, the foliar explants from *in vivo* source remained recalcitrant to *in vitro* morphogenetic response but in some cases slight swelling was observed at the basal part of the explants.

***Arachnis labrosa*:** In *A. labrosa* direct shoot buds/young plantlets were induced in the basal part of the *in vitro* raised foliar explants. The better morphogenetic

Table 6: Effect of PGRs on non-symbiotic seed germination of *A. labrosa*

Growth regulator (µM))		Days taken to germinate	Type of response	Remarks*
NAA	BA			
0	0	-	-	-
10.0	0	23	GreenPLBs	++
20.0	0	23	GreenPLBs	++
30.0	0	25	No germination	-
0	08.0	25	Degenerated in subsequent subculture	-
0	16.0	25	As above	-
0	24.0	23	In few case formed green PLBs	+
10.0	08.0	23	Green PLBs formed	++
10.0	16.0	27	Few green PLBs formed	+
10.0	24.0	23	Few green PLBs formed	+
20.0	08.0	23	Few green PLBs formed	+
20.0	**16.0**	**25**	**Healthy green PLBs formed**	**+++**
20.0	24.0	24	Green PLBs formed	++
30.0	08.0	24	Green PLBs formed	++
30.0	16.0	25	Few green PLBs formed	+
30.0	24.0	26	Green PLBs formed	++

Data represents the mean of three replicates,* - No response,+ Poor; ++ Average; +++ Optimum, on Mitra *et al,* medium containing sucrose (3%) and CW (15%).

Table 7: Effects of PGRs on non-symbiotic seed germination of *C. racemiferum*

Growth regulators (µM)		Days taken to germinate	Type of response	Remark*
NAA	BA			
0	0	-	-	-
10.0	-	49	GreenPLBs	++
20.0	-	45	Few PLBs formed	+
30.0	-	45	PLBs developed	++
0	8.0	43	As above	++
0	16.0	43	As above	++
0	24.0	45	Few PLBs formed	+
10.0	8.0	40	PLBs developed/formed	++
10.0	**16.0**	**40**	**Healthy green PLBs formed**	**+++**
10.0	24.0	47	Few green PLBs formed	+
20.0	8.0	45	Only few green PLBs formed	-
20.0	16.0	47	PLBs turned necrotic	-
20.0	24.0	47	PLBs degenerated	-
30.0	8.0	47	As above	-
30.0	16.0	47	Only few green PLBs formed	-
30.0	24.0	43	Moderate green PLBs formed	++

Data represents the mean of three replicates, * - no response, + Poor, ++ Average, +++ optimum response, on MS medium containing sucrose (3%) and CW (15%)

Table 8: Effect of light on asymbiotic seed germination

Species	Light Condition	Days taken	Response (%)	Remarks
	Dark	45 and above	35	Callus formed but degenerated subsequently
Arachnis labrosa	Diffused (20 µmol cm^{-2} s^{-1})	28-30	80	Healthy PLBs formation
	Full (40 µmol cm-2 s-1)	34-38	66-70	Moderate PLBs formation
	Dark	60 and above	40-45	Callus formation and subsequent degeneration
Cleisostoma racemiferum	Diffused	30-35	80-90	Healthy seed germination and PLBs development
	Full	38-40	65-75	Few green PLBs formed

Data represents the mean of three replicates

response was registered with the foliar explants of ~15 wk old after emergence. The explants placed slanted (45°C) exhibited better morphogenetic response. Initiation of meristematic activity was observed initially at the basal/cut ends of the explants after 7-8 wk of culture. Mostly the meristematic activity was restricted towards the lower basal parts and or cut ends (Figure 6 a). Of the different media used the optimum morphogenetic response was recorded with MS medium containing sucrose (3%), CH (500 mgl^{-1}).

Amongst the various plant growth regulators (PGRs) tested for the initiation of culture, the singly treatment of NAA and IAA did not support optimum initiation and growth of culture whereas a singly treatment of BA (4 μM) was found most suitable followed by singly treatment of Kn (2 μM). As many as 21 shoot buds and PLBs per explants were developed on BA enriched medium after 7-8 wk of culture where ~85% of the explants responded. (Table 9). The combined treatment of different PGRs exhibited differential response. In general the combined treatment of NAA and BA supported better PLBs formation initially followed by differentiated into young plantlets. The optimum response in this combination was registered with 2.0 μM each where ~60% explants responded. In case of NAA-Kn combined treatment though there was response initially, differentiation impaired and culture degenerated subsequently. A similar response was registered with the combined treatment of IAA-BA. While, with a combined treatment of IAA-Kn optimum response (~66%) was recorded with medium containing 2 μM each where it supported direct shoot buds formation. The PLBs and shoot buds formed on initiation medium were maintained for another 2 passages on MS medium containing BA (4 μM) along with other adjuvant for further differentiation.

Figure 6: Different stages of in *vitro* regeneration of plantlets from foliar explants of *A. labrosa*, a. Leaf explant swelling at the basal part and formed shoot buds, b. PLBs/shoot buds/young plantlets formed from the cultured leaf and c. Multiple shoot bud formed on regeneration medium.

Figure- 6

Table 9: Effect of different plant growth regulators on morphogenetic responses of foliar explants of *Arachnis labrosa* from *in vitro* sources

PGRConc. (µM)@	% response (±SE)*	No. of regenerates /explants	Type of response**
0	-	-	No regeneration
NAA_2	30 (±1.0)	2	PLBs formation
NAA_4	-	-	No response
NAA_6	-	-	No response
IAA_2	-	-	No response
IAA_4	30 (± 2.0)	3	Regeneration impaired
IAA_6	-	-	No response
BA_2	40 (± 2.0)	5	PLBs mediated regeneration
BA_4	**85 (±3.0)**	**21**	**Simultaneous PLBs and shoot bud formation**
BA_6	40 (± 3.0)	8	PLBs mediated regeneration
Kn_2	61 (± 3.0)	12	Multiple PLBs and shoot bud formation
Kn_4	45 (± 2.0)	4	PLBs mediated regeneration
Kn_6	40 (± 3.0)	3	PLBs mediated regeneration
$NAA_2 + Kn_2$	30 (± 2.0)	5	Direct shoot bud formation
$NAA_4 + Kn_4$	30 (± 1.50)	3	Regeneration impaired
$NAA_6 + Kn_6$	20 (± 1.0)	1	Regeneration impaired
$NAA_2 + BA_2$	60 (± 2.0)	10	PLBs mediated regeneration
$NAA_4 + BA_4$	60 (± 3.0)	8	PLBs mediated regeneration
$NAA_6 + BA_6$	45 (± 3.0)	6	PLBs mediated regeneration
$IAA_2 + BA_2$	55 (± 3.0)	7	Direct shoot bud formation
$IAA_4 + BA_4$	45 (± 2.0)	4	PLBs mediated and direct regeneration
$IAA_6 + BA_6$	-	-	No response
$IAA_2 + Kn_2$	66 (± 2.0)	8	Multiple shoot bud formation
$IAA_4 + Kn_4$	55 (± 2.0)	6	Shoot bud regeneration
$IAA_6 + Kn_6$	40 (± 2.0)	2	Regeneration impaired

Data represents the mean of three replicates,* ±SE- standard error,** On MS medium containing Sucrose (3%), CH (500 mgl^{-1}) and 100 mgl^{-1} citric acid and * PLBs-Protocorm like bodies. @ value in subscript indicates µM conc. of the growth regulators

Cleisostoma racemiferum: There was no distinct response from the explants collected from *in vivo* source. Though there was swelling initially at the basal part of the explants but failed to differentiate into either shoot buds or PLBs. The *in vitro* source leaf explants of ~15 wk old tested positively for regeneration competence. Amongst the different media tested MS medium supported optimum response (~80% response) when supplemented with sucrose (3%), CH (100 mgl^{-1}). The first sign of regeneration competence was initiated at the basal part and or cut ends of the foliar explants after ~40 days of culture initiation which subsequently spread the entire surface of the explants (Figure 7 a). The orientation of explants showed a marked effect on morphogenetic response. The explants cultured normal slanted condition (~45° and up side up condition) produced better response. While, the explants cultured horizontally and vertically did not support healthy culture initiation.

The responding foliar explants formed PLBs, shoot buds and callus on initiation medium containing different PGRs. The different levels of PGRs exhibited varied effect to stimulate meristematic activity from the explants. In medium containing singly treatment of IAA and NAA registered poor response and impaired regeneration. The simultaneous formation of PLBs and shoot buds was observed on medium containing BA (2 μM) singly treatment with ~80% frequency after 40 days of culture followed by BA (2 μM) singly (65% response). In BA (2 μM) containing medium there was as many as 18 PLBs/shoot buds formation per explants (Table 10). In general the medium containing IAA-BA combination supported very poor culture initiation and growth and in most of the cases callus mediated shoot bud formation was registered. While, the medium containing NAA-BA, mostly the explants either failed to respond/impaired regeneration or in some cases callus

Figure 7: Different stages of *in vitro* regeneration of plantlets from foliar explants of *C. racemiferum*, a. Leaf explants swelling at the basal part, b. PLBs/shoot buds formed from the cultured leaf and c. Multiple shoot bud formed on regeneration medium

Figure- 7

Table 10: Effect of plant growth regulators on morphogenetic response* of foliar explants (15-20 week old) of *Cleisostoma racemiferum*

PGRs Conc. (μM)		(%) response (± SE))[@]	No. of regenerates per explants	Type of response**
0		-	-	No response
IAA				
0.5		-	-	No response
1.0		10 (± 2.0)	2	Callus mediated shoot bud formation Impaired subsequently
2.0		15 (± 2.0)	2	As above
NAA				
0.5		30 (± 2.5)	-	Callus formation without regeneration
1.0		35 (± 3.0)	-	As above
2.0		50 (± 2.0)	-	As above
BA				
0.5		20 (± 2.0)	5	PLBs formation
1.0		65 (± 2.5)	10	PLBs mediated regeneration
2.0		**80 (± 1.5)**	**18**	**Multiple PLBs formation**
IAA	**BA**			
0.5	0.5	-	-	-
1.0	0.5	-	-	-
2.0	0.5	20 (± 2.0)	3	Callus mediated shoot bud formation
0.5	1.0	25 (± 2.0)	3	PLBs formation
1.0	1.0	50 (± 1.5)	4	Callus mediated shoot bud formation
2.0	1.0	50 (± 1.5)	-	Impaired regeneration
0.5	2.0	40 (± 2.0)	4	PLBs formation
1.0	2.0	55 (± 1.5)	6	PLBs mediated regeneration
2.0	2.0	50 (± 1.5)	7	As above
NAA	**BA**			
0.5	0.5	40 (± 2.0)	4	PLBs formation
1.0	0.5	40 (± 2.0)	4	As above
2.0	0.5	40 (± 2.0)	-	Impaired regeneration
0.5	1.0	10 (± 3.0)	2	PLBs formation
1.0	1.0	30 (± 2.5)	-	Impaired regeneration
2.0	1.0	60 (± 2.0)	-	As above
0.5	2.0	55 (± 1.5)	7	PLBs mediated regeneration
1.0	2.0	65 (± 2.0)	7	As above
2.0	2.0	68 (± 1.0)	-	Impaired regeneration

Data represents the mean of three replicates,* On MS medium containing sucrose (3%), CH 100 mgl^{-1}, citric acid 100 mgl^{-1}, **PLBs- protocorm like bodies; @ Standard error.

mediated regeneration registered but the cultures degenerated subsequently. A similar response was observed in combination of IAA-Kn (Table 9). The propagules so formed on initiation medium were maintained for another 2 passages on MS medium containing optimum growth conditions (BA 2 μM) for further differentiation. The PLBs/shoot buds were differentiated and converted to rooted plantlets in the initiating medium after ~30 days of culture.

3.1.C: Root

For both the selected species besides immature seeds/embryos and foliar explants, cultures were also initiated from the aerial roots of both *in vivo* and *in vitro* source. The aerial root explants were collected from *in vivo* as well as *in vitro* source of 15-20 wk old after emergence. Among the different media studied for culture initiation from aerial roots of both the species it was found that MS medium supported better culture initiation and growth.

***Arachnis labrosa*:** In *A. labrosa* the aerial root explants from *in vivo* source failed to elicit morphogenetic response under *in vitro* condition. Initially some swelling was observed in few explants but degenerated subsequently. However, healthy PLBs and shoot buds were formed from the culture of *in vitro* sourced aerial roots of 15-20 wk old. After ~5 wk of culture the first sign of morphogenetic response was recorded as swelling at the basal part/cut ends followed by PLBs and or shoot buds formation (Figure 8 a). In most of the explants morphogenetic response was restricted to the basal cut end and no response was recorded from the root cap region. In root culture also the orientation of explants on the medium exhibited a marked effect on culture initiation. Better morphogenetic response was recorded from explants cultured in

Figure 8: Stages involved in plantlet regeneration of *A. labrosa* from aerial roots.

a. Initiation of culture from aerial root segments showing swelling and PLBs formation,

b. Shoot buds and plantlets formed from the responding root segment and

c. Multiple shoots and rooted plantlets produced on regeneration medium.

Figure- 8

Table 11: Effect of PGRs in initiation medium on morphogenetic response[*] of *Arachnis labrosa* aerial roots from *in vitro* source

PGRs Conc. (μM)[@]	% response (±SE)[**]	No. of regenerates/ explants	Type of response***
0	-	-	-
IAA_2	45 (± 1.5)	4	Regeneration impaired
IAA_4	-	-	No response
IAA_6	-	-	No response
NAA_2	48 (± 2.0)	4	Regenerates growth slightly impaired
NAA_4	48 (± 2.0)	2	Regeneration impaired
NAA_6	-	-	No response
BA_2	52 (± 1.5)	3	Multiple PLBs formed
BA_4	45 (± 2.5)	3	As above
BA_6	43 (± 2.0)	2	As above
Kn_2	45 (± 1.5)	3	Shoot formation
Kn_4	50 (± 2.5)	5	As above
Kn_6	30 (± 2.0)	3	Poor regeneration
NAA_2 +BA_2	65 (± 2.0)	5	Shoot bud formation
NAA_4+BA_4	68 (± 1.5)	5	As above
NAA_6+BA_6	55 (± 2.0)	4	Poor regeneration
NAA_2 + Kn_2	25 (± 2.0)	1	Shoot bud formation
NAA_4+Kn_4	20 (± 1.5)	-	Impaired regeneration
NAA_6+Kn_6	25 (± 2.0)	1	Poor regeneration
IAA_2+BA_2	63 (± 2.5)	2	Regeneration impaired
IAA_4 + BA_4	60 (± 2.0)	3	Multiple shoot bud formation
IAA_6+BA_6	55 (± 2.0)	2	Regeneration impaired
IAA_2 + Kn_2	56 (± 2.0)	3	Shoot bud formation
IAA_2 + Kn_4	70 (± 1.5)	4	As above
IAA_2 + Kn_6	55 (± 2.0)	3	As above
IAA_4 + Kn_2	57 (± 2.5)	4	Multiple shoot formation
IAA_4 + Kn_4	70 (± 2.0)	7	As above
IAA_4+Kn_6	**80 (±2.5)**	**10**	**Multiple shoot/buds formation**
IAA_6+Kn_2	55 (± 2.0)	3	Multiple PLBs formation
IAA_6+Kn_4	55 (± 2.0)	3	Multiple PLBs formation
IAA_6+Kn_6	60 (± 2.0)	7	Multiple shoot/buds formation

Data represents the mean of three replicates, * On MS medium containing sucrose (3%) (w/v), CH (500 mgl^{-1}), ** ±SE: Standard error, *** PLBs- Protocorm-like bodies. @ value in subscript indicates the μM conc. of the growth regulators

slanted conditions (~45°). There was no response with the explants cultured horizontally.

About 80% of the explants responded positively on MS medium containing sucrose (3%), CH (500 mgl^{-1}) and IAA (4 µM) and Kn (6 µM) in combination after 5 wk of culture where as many as 10 PLBs/shoot buds formation per explants was registered. Both the auxin sources used (IAA and NAA) singly impaired regeneration but enhanced considerably when used in conjunction with either of cytokinins (BA and Kn) (Table 11). The different levels PGRs combination showed differential effect on the initiation of aerial root culture. In medium containing IAA-BA moderate PLBs formation was recorded. While in NAA-BA (2 + 4 µM respectively) combination formation of PLBs registered in ~75% of cultured explants. The morphogenetic response in medium containing NAA-Kn was insignificant, impaired regeneration and degenerated on prolonged culture.

***Cleisostoma racemiferum*:** The culture initiation on different media using the aerial root explants of *C. racemiferum* from *in vivo* source was poor and only ~20% of the explants responded positively to *in vitro* regeneration. The morphogenetic response of aerial root was recorded optimum on MS basal medium containing sucrose (3%), CH (200 mgl^{-1}) and citric acid (100 mgl^{-1}). The optimum response was recorded only after 75 days of culture on MS medium containing IAA-Kn (2 µM each) combination. While, about 100% morphogenetic response was registered from *in vitro* grown roots on MS medium enriched with IAA (1 µM) and kinetin (1 µM) in combination only after 25 days of culture initiation (Table 12). The initial response was observed at the basal cut ends and subsequently spread along the entire length of the root segments (Figure 9 a). The explants orientation exhibited a similar response

Figure 9: stages involved in plantlet regeneration of *C. racemiferum* from aerial roots

a. Initiation of culture from aerial root segments showing swelling and PLBs formation,

b.Shoot buds and PLBs formed from the responding root segment and

c. Multiple shoots and rooted plantlets produced on regeneration medium.

Figure- 9

Table 12: Effects of PGRs in the initiation medium* on morphogenetic response of *in vitro* grown aerial roots of *Cleisostoma racemiferum*

PGRs Conc. (µM)@	% response	No. of regeneratcs/ explants (±SE)**	Type of response***
0	0	-	No response
$IAA_{0.5}$	45 (± 2.0)	2	Callus mediated shoot bud formation
IAA_{1}	48 (± 1.0)	3	Callus mediated PLBs formation
IAA_{2}	45 (± 2.0)	-	Impaired regeneration
$BA_{0.5}$	50 (± 1.5)	5	PLBs formation
BA_{1}	49 (± 1.0)	6	Shoot bud formation
BA_{2}	40 (± 1.5)	5	As above
$Kn_{0.5}$	50 (± 2.0)	4	Callus mediated PLBs formation
Kn_{1}	55 (± 1.0)	4	As above
Kn_{2}	55 (± 2.0)	-	Impaired regeneration
$IAA_{0.5}$ + $BA_{0.5}$	50 (± 2.5)	5	PLBs formation
$IAA_{0.5}$ + BA_{1}	50 (± 2.5)	7	PLBs and shoot bud formation
$IAA_{0.5}$ +BA_{2}	60 (± 1.5)	7	As above
$IAA_{0.5}$ + $Kn_{0.5}$	40 (± 1.5)	3	PLBs formation
$IAA_{0.5}$ + Kn_{1}	45 (± 2.0)	8	PLBs and shoot bud formation
$IAA_{0.5}$+ Kn_{2}	55 (± 2.0)	7	Callus mediated PLBs formation
IAA_{1} + $Kn_{0.5}$	70 (± 2.5)	10	PLBs formation
IAA_{1} + Kn_{1}	**100 (± 1.0)**	**15**	**Multiple PLBs formation**
IAA_{1} + Kn_{2}	80 (± 1.0)	9	Callus mediated PLBs formation
IAA_{2} + $Kn_{0.5}$	55 (± 2.0)	8	As above
IAA_{2} + Kn_{1}	60 (± 1.0)	7	As above
IAA_{2} + Kn_{2}	60 (± 1.0)	6	Callus mediated PLBs formation

Data represents the mean of three replicates.

* On Ms medium containing sucrose (3%), CH (200 mgl^{-1}), ** Standard error,

*** PLBs- Protocorm like bodies, @ value in subscript indicates the µM conc. of growth regulators

in *C. racemiferum* at par with the leaf and root culture of *A. labrosa,* where the explants cultured in slanted position (~45°) showed better morphogenetic response over other condition i.e. horizontal and vertical condition. Both IAA and kinetin singly stimulated mostly callusing of the explants and restricted the further differentiation. After 40 days of culture initiation ~75% of explants responded positively and formed PLBs on medium enriched with IAA-BA (1 + 2 μM respectively) in combination. A synergistic action of auxins and cytokinins was observed in the medium containing NAA-BA (1 + 2 μM respectively) in combination, which resulted to multiple PLBs formed on medium. But the medium enriched with NAA (2 μM) alone supported callus mediated PLBs formation.

Though the incorporation of CW in the initiation media for immature seed culture did not show any significant effect but helped in early differentiation of PLBs into plantlets in subsequent subcultures. The effect of CH on initiation of cultures of leaf and root explants was tested in both the selected orchid species. Different levels of CH exhibited a varied effect on culture initiation of explants. In *A. labrosa,* CH (500 mgl^{-1}) showed optimum response for both leaf and root explants while in *C. racemiferum* optimum response was obtained with CH (100 mgl^{-1}) for leaf explants and CH (200 mg^{-1}) in case of root explants (Table 5).

The PLBs and shoot buds formed from the cultured aerial root segments of both the species were maintained for another 2 passages for further differentiation on optimum growth conditions. The advanced stage PLBs, shoot buds developed from the germinated seeds, foliar explants and cultured aerial roots were subjected to regeneration of plantlets and mass multiplication.

Table 13: Effect of different levels of PGRs for regeneration and mass multiplication of *A. labrosa on MS medium****

PGRs Conc. (µM)		No. of shoot bud/explants	Type of response	Remarks***
0		-	No shoot bud formation	-
NAA				
10		-	Leaves small & no regeneration	+
20		-	As above	+
30		-	Plantlets elongated but no regeneration	-
IAA				
10		2	Multiple PLBs formation	++
20		1	Few PLBs and shoot bud formation	+
30		1	Direct shoot bud formation	+
BA				
8		-	No regeneration	-
16		2	Multiple PLBs formation	++
24		2	Multiple PLBs formation	++
Kn				
8		-	No regeneration	-
16		-	As above	-
24		-	As above	-
NAA	**BA**			
10	**8**	**17**	**Multiple PLBs and shoot bud formation**	**+++**
10	16	1	Single shoot bud formation	+
10	24	1	Shoot bud formation	+
20	8	1	Only PLBs formation	+
20	16	1	As above	+
20	24	1	Single shoot bud formation	+
30	8	4	Callus mediated PLBs formation	++
30	16	1	Single PLBs formation	+
30	24	1	Only PLBs formation	+
NAA	**Kn**			
10	8	-	No regeneration	-
10	16	1	Single PLBs formation	+
10	24	3	PLBs mediated regeneration but plantlets were etiolated	++
20	8	1	Single PLBs formation	+
20	16	-	No shoot bud formation	-
20	24	-	As above	-
30	8	-	As above	-
30	16	-	As above	-
30	24	1	Only PLBs formation	+
IAA	**BA**			
10	8	-	No regeneration	-
10	16	8	Few repetitive PLBs formation	+
10	24	-	Subsequently degenerated	-
20	8	-	No shoot bud formation	-
20	16	-	Callus degenerated without differentiation	-

Contd ....

PGRs (μM)	Conc.	No. of shoot bud/explants	Type of response	Remarks***
20	24	-	No regeneration	-
30	8	-	Callus degenerated without differentiation	-
30	16	-	As above	-
30	24	-	No regeneration	-
IAA	Kn			
10	8	-	No shoot bud formation	-
10	16	-	As above	-
10	24	2	Direct plantlet/shoot bud formation	+
20	8	-	No shoot bud formation	-
20	16	4	PLBs formation	++
20	24	-	No shoot bud formation	-
30	8	-	No shoot bud formation	-

Data represent the mean of three replicates

* Propagules raised from all the three explants sources,** containing sucrose (3%), CW (15%),

*** -No response, + Poor, ++ Average, +++ Optimum response

Table 14: Effect of different levels of PGRs for regeneration and mass multiplication of *A. labrosa on Mitra *et al,* medium****

PGRs Conc. (µM)		No. of shoot buds/explants	Type of response	Remarks***
0		-	No response	-
NAA				
10		-	No response	-
20		-	No regeneration though plantlets were healthy	-
30		-	As above	-
IAA				
10		-	No regeneration	-
20		-	As above	-
30		-	As above	-
BA				
8		3	PLBs mediated regeneration	+
16		4	As above	+
24		4	PLBs formation	+
Kn				
8		-	No regeneration though plants were healthy	-
16		-	As above	-
24		-	No regeneration	-
NAA	**BA**			
10	**8**	**14**	**Multiple PLBs & shoot bud formation**	+++
10	16	3	PLBs mediated regeneration	++
10	24	3	As above	++
20	8	1	Only PLBs formation	+
20	16	2	PLBs mediated regeneration	+
20	24	-	No regeneration	-
30	8	-	No response	-
30	16	-	As above	-
30	24	-	As above	-
NAA	**Kn**			
10	8	1	Single PLBs formation	+
10	16	3	PLBs mediated regeneration	++
10	24	-	No regeneration	-
20	8	-	No response	-
20	16	2	Direct shoot bud formation	+
20	24	1	Single PLBs formation	+
30	8	-	No regeneration	-
30	16	-	As above	-
30	24	-	As above	-

Contd ....

PGRS conc. (μM)		No. of shoot buds/explants	Type of response	Remarks***
IAA	**BA**			
10	8	-	No regeneration though plantlets were healthy	-
10	16	7	Repetitive PLBs formation	++
10	24	-	No regeneration	-
20	8	-	No regeneration	-
20	16	-	No regeneration	-
20	24	-	No regeneration	-
30	8	-	No regeneration	-
30	16	-	As above	-
30	24	-	No regeneration	-
IAA	**Kn**			
10	8	1	Single PLBs formation	+
10	16	3	PLBs mediated regeneration	++
10	24	-	No regeneration	-
20	8	2	Direct shoot bud regeneration	+
20	16	3	As above	+
20	24	-	No regeneration	-
30	8	-	No regeneration	-
30	16	-	As above	-
30	24	-	As above	-

Data represents the mean of three replicates

* Propagules raised from all the three explants sources viz: seed, leaf and root explants,

** Medium containing sucrose (3%), CW 15%.

*** - no response,+ Poor,++ Average,+++ Optimum response

3.2: Regeneration and Maintenance of Cultures

The advanced stage PLBs developed from the cultured immature embryos/seeds, leaf and root explants of *A. labrosa* and *C. racemiferum* was maintained further for 2 passages on the optimum initiation conditions for further differentiation. The differentiated PLBs and young plantlets (~1.5 cm) with 1-3 root produced from different propagules were then separated from the clumps and maintained on Mitra *et al* and MS medium. Both the media were conjunct with sucrose (3%), CW (15%) and various levels of PGRs [IAA, NAA, BA and Kn (0-32 μM) singly or in combination]. Amongst the two media tested MS medium fared better over Mitra *et al* for regeneration of plantlets and mass multiplication from all the explants of both the species. The growth of the PLBs was healthy and luxuriant on MS medium complemented with greater proliferation and regeneration of new PLBs (Table 13 & 15). On the other hand Mitra *et al* medium did not support healthy culture growth and proliferation of culture (Table 14 & 16). Alternatively, in one set of experiments for both the species, AC was also incorporated to the regeneration medium to study its effect on morphogenetic response, regeneration and mass multiplication. All the cultures were maintained for 4-5 passages on regeneration media for plantlet formation and mass multiplication.

3.2.A: Effect of PGRs on Regeneration and Mass Multiplication

The differentiated PLBs and or the young plantlets obtained from germinated immature embryos, leaf and root explants of both the species were subjected to regeneration and mass multiplication. The different plant growth regulators exhibited differential effect on regeneration and mass multiplication.

Table 15: Effect of different levels of PGRs for regeneration and mass multiplication of *C. racemiferum* from immature seed on MS medium*

PGRs. Conc (μM)		No. of shoot bud/explant	Type of response	Remarks**
0		-	No regeneration	-
NAA				
10		-	Plantlet short stout with broad leaf without regeneration	-
20		1	Plantlets slightly elongated, leaf long and linear	+
30		1	As above	-
IAA				
10		4	Healthy growth with few PLBs	++
20		3	As above	++
30		3	Few PLBs formation	+
BA				
8		8	Regeneration through PLBs	++
16		9	As above	++
24		5	Few PLBs formation	++
Kn				
8		5	Healthy growth few PLBs	++
16		8	As above	++
24		1	Slightly stunted growth with a single PLBs	+
NAA	BA			
10	**8**	**21**	**Healthy plantlets with repetitive/ multiple PLBs proliferation**	+++
10	16	9	Few PLBs formation	++
10	24	3	Slightly etiolated with few PLBs	+
20	8	2	Plant growth normal, leaf thin and fewer shoot bud formation	+
20	16	2	As above	+
20	24	-	Normal growth with no PLBs formation	-
30	8	1	Slightly etiolated plant, leaves slender with single PLBs	+
30	16	1	As above	+
30	24	-	No regeneration	-
NAA	Kn			
10	8	1	Growth stunted, slightly etiolated with single PLBs formation	+
10	16	4	Moderately healthy with few PLBs formation	+
10	24	1	Elongated plantlets with single PLBs formation	+
20	8	-	Well developed PLBs with no PLBs formation	-
20	16	-	Well developed plantlets but slightly retarded with no PLBs formation	-
20	24	-	As above	-

Contd...

PGRs. Conc (µM)		No. of shoot bud/explant	Type of response	Remarks*
30	8	1	Well developed plantlets and single PLBs formation	+
30	24	-	Well differentiated but growth stunted with no PLBs formation	-
IAA	**BA**			
10	8	6	Healthy plantlets with few PLBs formation	++
10	16	17	Repetitive PLBs formation and healthy plantlets	+++
10	24	6	Few PLB formation, plantlet healthy, leaf broad and long	++
20	8	1	Slightly stunted growth with short and slender leaf and a PLBs	+
20	16	1	Growth slightly retarded and etiolated with a PLBs formation	+
20	24	4	Differentiated plantlets but short and stout with few PLBs	++
30	8	2	Plantlets elongated and slender but two PLBs formation	+
30	16	1	Elongated plantlets with single PLBs formation	+
30	24	1	As above	+
IAA	**Kn**			
10	8	8	Healthy growth with few repetitive PLBs formation	+++
10	16	3	Growth stunted, leaf linear but few PLBs formation	+
10	24	1	Slightly stunted growth with single PLBs formation	+
20	8	-	Growth stunted, leaves small and linear with no PLBs formation	-
20	16	6	Healthy growth with few PLBs formation	++
20	24	3	Healthy plantlets but leaves small and few PLBs formation	++
30	8	-	Subsequently degenerated without regeneration	-
30	16	-	As above	-
30	24	-	As above	-

Data represents the mean of three replicates
*on medium containing sucrose (3%), CW (15%)
** - No response,+ Poor,++ Average,+++ Optimum response

Table 16: Effect of different levels of PGRs for regeneration and mass multiplication of *C. racemiferum* for immature seed on Mitra *et al* medium*

PGRs Conc. (μM)		No. of shoot buds/explants	Type of response	Remarks**
NAA				
10		-	Plantlet degenerated subsequently without regeneration	-
20		-	As above	-
30		1	Plantlets small and etiolated but with a PLBs formation	+
IAA				
10		4	Moderately healthy growth with few PLBs	++
20		1	Small plantlets with a PLBs formation	+
30		-	No regeneration	-
BA				
8		8	Regeneration through PLBs but plantlets short & stout	+++
16		1	Slightly stunted but with a PLBs	+
24		1	As above	+
Kn				
8		1	Healthy growth though slightly stunted and single shoot bud	+
16		1	Slightly stunted growth with a PLBs	+
24		1	Slightly stunted growth with a single PLBs	+
NAA	**BA**			
10	8	4	Plantlets short thin and etiolated but with few PLBs	+
10	16	-	Plantlets slender, thin and no PLBs formation	-
10	24	3	Plant growth normal with few	+
20	8	2	Plant growth slightly retarded, but leaf broad and two PLBs	+
20	16	2	Moderately healthy with few PLBs formation	+
20	24	-	No PLBs formation	-
30	8	1	Small plantlets with a single PLBs formation	+
30	16	1	As above	+
30	24	1	As above	+
NAA	**Kn**			
10	8	1	Growth stunted, slightly etiolated with single PLBs formation	+
10	16	1	As above	+
10	24	1	As above	+
20	8	1	As above	+
20	16	1	As above	+
20	24	-	Plantlets etiolated and degenerated without regeneration	-
30	8	1	Well developed plantlets and single PLBs formation	+
30	16	1	Though growth slightly stunted a PLBs was formed	+
30	24	1	As above	+
IAA	**BA**			
10	8	2	Slightly stunted growth but with PLBs was formed	+
10	16	1	Stunted growth with few PLBs formation	+
10	24	1	As above	+
20	8	2	Slightly stunted growth and etiolated but with PLBs	+
20	16	2	As above	+

Contd...

PGRS Conc. (μM)*		No. of shoot buds/ Explants	Type of response	Remarks**
20	24	2	As above	+
30	8	2	Plantlets slightly elongated but with PLBs formation	+
30	16	1	Elongated plantlets with single PLBs formation	+
30	24	1	Moderate plant growth with a PLBs formation	+
IAA	**Kn**			
10	8	4	Moderately healthy growth with few PLBs	++
10	16	1	Growth slightly stunted, leaf linear but with a PLBs	+
10	24	1	Slightly stunted growth with single PLBs formation	+
20	8	3	growth healthy with few single PLBs formation	++
20	16	4	As above	++
20	24	3	Healthy plantlets but leaves small and few PLBs	+
30	8	-	Slender plants with no PLBs formation	-
30	16	-	As above	-
30	24	-	As above	-

Data represents the mean of three replicates

* On medium containing sucrose (3%), CW (15%)

**- no response,+ Poor,++ Average, +++ Optimum response

Arachnis labrosa: In *A. labrosa* among the different quality and quantity of PGRs used a combined treatment of **NAA-BA** (10 + 8 μM respectively) produced multiple shoots/buds (Figure 3 c, 6 b & 8 b). As many as 17 shoots/buds developed after ~20 days of culture (Table 13). The singly treatment of NAA and IAA supported the stunted growth of the PLBs but there was no regeneration. The regeneration medium fortified with singly treatment of Kn did not support the growth of culture and degenerated while, the propagules cultured on media enriched with BA singly supported the multiple PLBs/shoot buds formation but regeneration impaired. Only the combined treatment of **NAA-BA** (10 + 8 μM) supported regeneration of plantlets and multiple shoot buds formation but at higher concentrations resulted callus mediated PLBs formation and impaired regeneration.

In general the regeneration media supplemented with **NAA-Kn** stimulated the conversion of plantlets from the PLBs, which were slender, slightly etiolated, and leaves were light green in colour. The optimum response in this combination was recorded on medium containing NAA + Kn (10 + 16 μM respectively). The proliferation rate was very poor and fewer repetitive PLBs and shoots developed. But the regeneration and multiplication on Mitra *et al* medium was very poor and did not support healthy conversion. While, in case of **IAA-BA** combination the optimum response was registered with 10 + 16 μM respectively. The plantlets on this medium exhibited slightly retarded growth, slender with small leaves and light green in colour but root growth was very healthy. But **IAA-Kn** combination did not support healthy culture growth and formed long and thin plantlets accompanied by poor rooting.

In general the cultures maintained on Mitra *et al* medium conjunct with different combinations of PGRs were also exhibited a similar response (Table 14). In

A. labrosa the regeneration of plantlets and mass multiplication was achieved on the same regeneration medium.

***Cleisostoma racemiferum*:** The advanced stage PLBs from cultured seeds started converting into young rooted plantlets and repetitive PLBs within 3-4 wk on regeneration media. Amongst the two media tested the optimum growth and differentiation into rooted plantlets was supported by MS medium (Table 15 and 16). It was observed that the singly treatment with different PGRs at lower concentrations resulted stunted growth of the regenerates while, at higher concentrations the regenerates showed etiolated growth. None of the concentrations could support the formation of multiple shoots and only the combined treatments of different PGRs produced multiple shoots/propagules. The optimum regeneration and multiplication of cultures was accomplished on MS medium containing sucrose (3%), CW (15%). Among the different PGRs combinations a combined treatment of NAA-BA (10 + 8 μM respectively) resulted optimum regeneration and multiplication where as many as 10-12 plantlets /shoot buds formed after 3-4 wk on regeneration medium (Figure 4 c). The performance of cultures on regeneration medium containing different PGRs combinations are given below:

The number of multiple shoot buds formation was significantly higher on a MS basal medium containing NAA (10 μM) and BA (8 μM) in combination but in some cultures leaves were thin and slightly etiolated (Table 15). But at higher concentrations the regenerates etiolated. While, the culture on Mitra *et al* medium the plantlets were small, short and stunted growth and most of the cases very few propagules formed (Table 16).

In general, the regenerates on **NAA-Kn** combination containing medium appeared stunted and did not support multiple shoot buds formation. While, the cultures on Mitra *et al* medium showed similar response but in some cases cultures degenerated. The **IAA-BA** combination of PGRs) resulted in multiple shoot buds with stunted growth and poorer root formation. But in IAA (10 µM) and BA (16 µM) in combination, the plantlets were well rooted, healthy, leaf long and broad with PLBs development. But on Mitra *et al* medium the plantlet growth was stunted and etiolated and no repetitive PLBs formation was achieved. In **IAA-Kn,** the optimum regeneration and multiplication of culture accompanied with well root formation registered on MS medium containing IAA (10 µM) and Kn (8 µM) in combination. This treatment supported moderate regeneration and shoot buds/ repetitive PLBs formation. While on Mitra *et al* medium with the same combination the plantlets showed a stunted growth and formed etiolated shoots.

The PLBs, callus and shoot buds produced from the cultured foliar explants of *C. racemiferum* converted into multiple shoots on regeneration medium after 8-10 wk of culture (Figure 7 b) (Table 17). The better response was recorded on MS medium containing IAA and Kn (2 µM each in combination). As many as, 10-12 shoot buds were formed on IAA-Kn enriched medium. While, media enriched with BA along with other PGRs produced multiple shoot buds (up to 15 to 16 per explants).

The propagules developed from the cultured roots of *C. racemiferum* started responding to the stimulus of PGRs after ~30 days of transfer on regeneration and multiplication medium and the PLBs differentiated into shoots/buds. Multiple shoot/buds development (Figure 9 b) was highest at IAA and Kn (4 µM each in

Table 17: Effect of different levels of PGRs for regeneration* from the PLBs and shoot buds obtained from leaf culture of *C. racemiferum*

PGRs conc. (µM) IAA	Kn	No. of regenerates/ Explants	Type of response
0	0	-	No regeneration
2	0	-	Plantlets differentiated without regeneration
4	0	1	Plantlets healthy with single shoot bud
6	0	-	No regeneration though well developed plantlets
0	2	-	Well differentiated plantlets without regeneration
0	4	2	Poor shoot bud formation
0	6	5	Healthy plantlets with direct shoot bud formation
2	2	**12**	**Repetitive PLBs/shoot bud formation from the healthy plantlets**
2	4	9	Multiple shoot buds formation
2	6	6	Well differentiated plantlets produces few directly induced shoot buds
4	2	4	Few PLBs were formed from the well developed plantlets
4	4	7	Simultaneous PLBs and shoot buds formation
4	6	2	Poor PLBs formation
6	2	1	Single shoot bud formation
6	4	1	As above
6	6	-	Healthy and well differentiated plantlets but no regenerates were formed

Data represent mean of three replicates
*MS medium containing sucrose (3%) and CW (15%)

combination) followed by IAA and Kn (2 and 6 µM, respectively in combination) (Table 18). While, IAA singly (4 µM) induced PLBs formation and Kn (6µM) induced direct shoot buds.

3.2.B: Effect of Activated Charcoal on Regeneration and Mass Multiplication

In one set of experiment AC (0-0.3%) was incorporated in the regeneration medium to study its effect on morphogenetic response and mass multiplication. The propagules like PLBs, shoot buds raised from different explant sources of both the species were transferred on AC rich medium and maintained for 2-3 passages. In general the AC rich medium promoted the healthy culture growth and induction of repetitive PLBs.

***Arachnis labrosa*:** Healthy and elongated plantlets with multiple PLBs formation were recorded on regeneration medium containing AC 0.15% (Table 19) (Figure 10 a). In AC 0.01% rich medium did not support multiple shoot buds formation while with increase in AC concentration the repetitive PLBs formation was also increased considerable. But the repetitive PLBs formation declined on medium containing AC (> 0.2%) (w/v) and at higher concentration of AC (0.3%) (w/v) plants and PLBs turned necrotic and degenerated subsequently.

***Cleisostoma racemiferum*:** The incorporation of AC in the regeneration medium had a significant and pronounced effect on the growth and development of the plantlets. Within 2-3 wk on AC rich medium the culture started proliferating and formed reparative PLBs. Healthy and elongated growth with broad leaves was recorded in a medium containing AC 0.1% (w/v) followed by AC 0.01% where multiple PLBs formation was recorded (Table 19) (Figure 10 b). At higher concentration the effect negated and most of the cases culture degenerated.

Table 18: Effects of different levels of PGRs in the regeneration medium* on plantlet regeneration of *C. racemiferum* from PLBs and shoot buds obtained from aerial root culture

PGRs Conc. (µM) IAA	Kn	No. of regenerates/ explant	Type of response
0	0	-	-
2	0	2	Poor shoot bud formation
4	0	4	Mostly callused and few shoot buds formed
6	0	4	Mostly callused
0	2	5	Repetitive PLBs and shoot bud formed
0	4	6	Shoot buds and PLBs formed
0	6	8	Direct plantlet regeneration and few shoot buds formed
2	2	10	Shoot buds formed
2	4	10	As above
2	**6**	**16**	**Multiple shoots developed**
4	2	7	Shoot buds and PLBs formed
4	4	10	Multiple shoot buds formation
4	6	5	Multiple shoot buds formation
6	2	5	Shoot buds with callusing at the base
6	4	5	As above
6	6	-	No regeneration

Data represents mean of three replicates

* MS medium containing sucrose (3%) and CW (15%)

Figure 10: Effect of activated charcoal in the regeneration medium. a. Repetitive PLBs and shoot buds formation of *A. labrosa* on AC enriched regeneration medium. b. *C. racemiferum* culture produces repetitive PLBs on AC enriched medium.

Figure - 10

Table 19: Effect of activated charcoal on morphogenetic response and regeneration* of *A. labrosa* and *C. racemiferum*

Specimen	AC Conc.	Type of response	Performance**
	0.00	No effect on growth of the plantlets	-
	0.01	As above	-
	0.05	Moderate plant growth with shoot bud formation	++
A. labrosa	0.1	Elongated plants and shoot bud formation	++
	0.15	Healthy plantlets and multiple PLBs formation	+++
	0.2	Poor plant growth of plantlets	+
	0.25	As above	+
	0.3	Plants degenerated subsequently	-
	0.01	Multiple PLBs formation	+++
	0.05	Healthy growth and but fewer shoot formation	++
	0.1	Healthy and luxuriant growth	+++
C. racemiferum	0.15	Moderately healthy plants and slightly etiolated	+
	0.2	Moderate plant growth and elongated leaves	+
	0.25	Plants turned necrotic and degenerated	-
	0.3	As above	-

* MS medium containing sucrose (3%)
** - no response.+ Poor,++ Average, +++ Optimum response

The well rooted (with 2-3 roots) plantlets of both the species obtained from different explants sources were maintained for 2-3 passages on regeneration medium. The plantlets of ~3-4 cm size were taken out from the regeneration medium and were subjected to *in vitro* hardening before transplanting into potting mix and in the wild.

3.3: Hardening and Field Trial of the Regenerates

In the current investigation, the regenerates were hardened following the two different approaches. Besides the conventional approach part of the regenerates was also maintained following a new approach. In conventional approach the well rooted plantlets of both the species (*A. labrosa* and *C. racemiferum*) from the regeneration medium were taken out and separated the individual plantlets. Thereafter these plantlets were cultured on highly reduced MS medium (1/10th strength) containing sucrose (2%) devoid of any PGRs (Figure 11 a & b). The cultures were maintained for 10-12 wk under normal culture conditions and 12/12 hr photoperiod. In another set of experiment the tiny rooted plantlets were taken out from the regeneration medium and washed out the agar adhering to the roots and transferred in 1/10th strength of MS salt solution devoid of sucrose and PGRs. In each culture tube small chips of charcoal, brick pieces and mosses were incorporated separately before the plantlets are transferred (Figure 11 c & d). Culture vials were maintained for three to four months. Incorporation of charcoal pieces, brick pieces and mosses helped in two ways: 1. these materials acts as supporting materials and prevented the tiny plantlets from submerging in the liquid medium, 2. the roots of the plantlets get attached to the materials in prolonged cultures. It was observed that the plantlets used the charcoal pieces and mosses as better substratum/supporting material.

Figure 11: Hardening of regenerates and transplantation of hardened plants in community potting mix. a. *A. labrosa* plantlets on hardening medium (conventional method), b. *C. racemiferum* regenerates on hardening medium (conventional method), c. *A. labrosa* plantlets under hardening (new approach), d. *C. racemiferum* plantlets under hardening (new approach), e. The hardened plants of *A. labrosa* transferred to community potting mix and f. The hardened regenerates of *C. racemiferum* after transferring to community potting mix.

Figure–11

The plantlets from the hardening conditions were taken out and the roots were washed using a soft brush to remove the agar and transferred to community potting mix (Figure 11 e & f). About 400 plants of each species were potted. The potted plants were initially covered with perforated transparent plastic for about one wk to retain the moisture. The potted plants were then transferred in polyhouse and maintained for 2-3 months. The plants were fed with $1/10^{th}$ MS salt solution at one wk interval. The acclimatized plants were transferred to Departmental Botanical Garden and the performances of these transplanted plants were monitored at regular interval.

In this new hardening technique the regenerates were very healthy and performed better when potted in potting mix than the regenerates hardened following conventional technique. This new technique of *in vitro* hardening of regenerates developed during the current investigation. It was observed that following this technique survival frequency of potted plants was enhanced significantly to about 95% in both the species compare to 40% in *A. labrosa* and 45% in *C. racemiferum* following conventional technique/control after two months of transfer (Figure 12). It was also observed that the transplanted regenerates were dark green and healthy and new roots started emerging after one month of transfer in the potting mix.

3.4: Short to Medium Term *In Vitro* Storage

In the present investigation, attempt was made to conserve the germplasm under *in vitro* condition. The different types of propagules like advanced stage PLBs and young plantlets of both the species raised in culture were used for short to medium-term *in vitro* conservation of germplasm. The propagules were cultured under different physico-chemical conditions. It was observed that among the

Figure 12: Comparative survival frequency of transplanted regenerates of *A. labrosa* and *C. racemiferum* following two different hardening techniques.

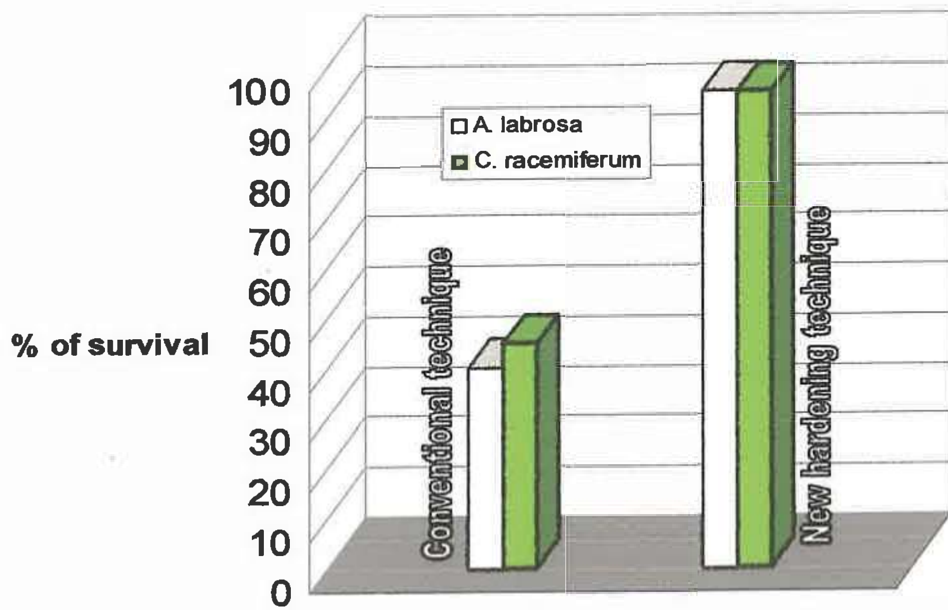

Figure 12: Comparative survival frequency of transplanted regenerates of A. labrosa and C. racemiferum folowing two different hardening techniques

Table 20: *In vitro* conservation of *A. labrosa* through slow growth method*

Organic carbon source(%)	Conc. (%)	Strength of MS medium	Type of response	Performance**
	0	0	All the explants degenerated	-
Sucrose	1	1/4th	Plant growth highly stunted degenerated	-
		2/4th	Plant growth retarded and degenerated	-
		3/4th	Plant slightly stunted and degenerated	-
		Full	Plant growth retarded and stunted	++
	2	1/4th	Plant growth stunted and slowed	++
		2/4th	Plant growth retarded and healthy	+++
		3/4th	Plant growth slightly retarded	++
		Full	Plant growth slightly etiolated and retarded	+
	3	1/4th	Plant growth slightly retarded but remains healthy	+++
		2/4th	Plant growth less retarded and etiolated	+
		3/4th	Plant growth slightly retarded	+
		full	No sign of slow growth and stunting	-
Mannitol	1	1/4th	Plant growth highly retarded but degenerated subsequently	-
		2/4th	Plant growth highly retarded but dies subsequently	-
		3/4th	Plant growth retarded but dies later	-
		full	Plant growth retarded, stunted and etiolated	+
	2	1/4th	Plant growth retarded, stunted and elongated leave	++
		2/4th	Plant slightly stunted and growth retarded	++
		3/4th	Plant slightly stunted and growth slowed	++
		full	Plant growth retarded with elongated leave	++
	3	1/4th	Plant growth stunted with elongated leave	+
		2/4th	Plant growth slightly retarded and stunted	+
		3/4th	Healthy & normal plant growth	-
		full	Plant growth not effected but healthy	-

Contd ...

Organic carbon source(%)	Conc. (%)	Strength* of MS medium	Type of response	Performance**
Fructose	1	1/4th	Plant growth highly stunted & degenerated	-
		2/4th	Plant growth highly stunted & degenerated	-
		3/4th	Plant growth highly retarded and stunted but degenerated subsequently	-
		Full	Plant growth highly retarded, stunted with elongated leaves but degenerated later	-
	2	1/4th	Plant growth retarded, stunted with elongated leaves	++
		2/4th	As above	++
		3/4th	Plant growth slightly stunted with etiolated leaves	+
		Full	As above	+
	3	1/4th	Plant slightly stunted but healthy	++
		2/4th	Plant growth slightly retarded	++
		3/4th	Plant growth less retarded	+
		Full	Plant growth not effected and healthy	-
Glucose	1	1/4th	Plant highly retarded, stunted but degenerated subsequently	-
		2/4th	Plant highly stunted but degenerated later	-
		3/4th	Plant retarded, stunted but degenerated later	-
		Full	Plant highly stunted but degenerated later	-
	2	1/4th	Plant growth retarded and healthy	++
		2/4th	Plant growth retarded with elongated leaves	++
		3/4th	Plant growth slowed with elongated leaves	+
		Full	Plant growth slightly retarded and healthy	++
	3	1/4th	Plant slightly retarded with elongated leaves	+
		2/4th	Plant growth retarded and stunted	+
		Full	No sign of Slowed growth but luxuriant healthy growth	-
Dextrose	1	1/4th	Plant growth highly retarded , stunted and dies later	-
		2/4th	Plant growth retarded, stunted, dies later	-
		3/4th	Plant growth retarded but dies later	-
		Full	Plant growth retarded, stunted with elongated leaves	++
	2	1/4th	Plant growth retarded and stunted , yet healthy	+++
		2/4th	Plant growth slightly retarded and stunted	++
		3/4th	Plant growth slightly stunted with elongated leave	+

Contd...

Organic carbon source(%)	Conc. (%)	Strength* of MS medium	Type of response	Performance**
		Full	Plant growth slightly stunted with Elongated leaves	++
	3	1/4th	Plant growth slightly retarded with elongated leaves	++
		2/4th	Plant growth slightly retarded with elongated leaves	++
		3/4th	Plant growth less retarded and stunted	+
		Full	Plant healthy and luxuriant with no sign of slowed growth	-

* On MS medium, ** -no response. + Poor, ++ Average, +++ Optimum response

different culture conditions studied, a diffused light (20 μmol m^{-2} s^{-1}) condition at 20°C was found to be suitable for both *A. labrosa* and *C. racemiferum*. The Cultures maintained in the dark mostly callused and in some cases cultures degenerated. The callused cultures failed to grow upon returning to normal light conditions and degenerated. While, the cultures maintained in normal light condition and 25°C temperature maintained a healthy culture growth and demanded rapid sub-culture.

Arachnis labrosa*:** In *A. labrosa* among the different levels of MS medium used strength of half strength MS regeneration medium found most suitable for the purpose. Amongst the different organic carbon sources used for ***in vitro conservation an optimum response was achieved on ½MS medium containing sucrose (2%) over other organic carbon sources (Table 20). In this concentration plants exhibited slow and stunted growth without any gross abnormality of plant morphology (Figure 13 a) and regained normal growth when transferred on normal regeneration medium. In lower level of MS medium (1/4th strength) the cultures degenerated on prolonged culture.

The culture growth retarded on medium containing lower levels of mannitol (1 & 2%) and degenerated subsequently while, at higher level (3%) there was no adverse effect on culture growth and maintained normal growth. In general the medium containing fructose supported stunted growth but most of the culture degenerated in subsequent sub-cultures. But the cultures maintained on glucose rich medium supported retarded growth and could be retrieved the normal growth on normal regeneration. The optimum response was registered on medium containing 2% glucose. While, with dextrose a similar performance was recorded on 1/4thMS medium containing dextrose (2%).

Figure 13: *In vitro* storage of germplasm through slow growth method:

a. *A. labrosa* cultures on slow growth conditions showing retarded growth

b. *C. racemiferum* cultures on slow growth conditions showing retarded growth.

c & d. Resumption of normal growth after reverting to normal regeneration medium of *A. labrosa* and *C. racemiferum* respectively.

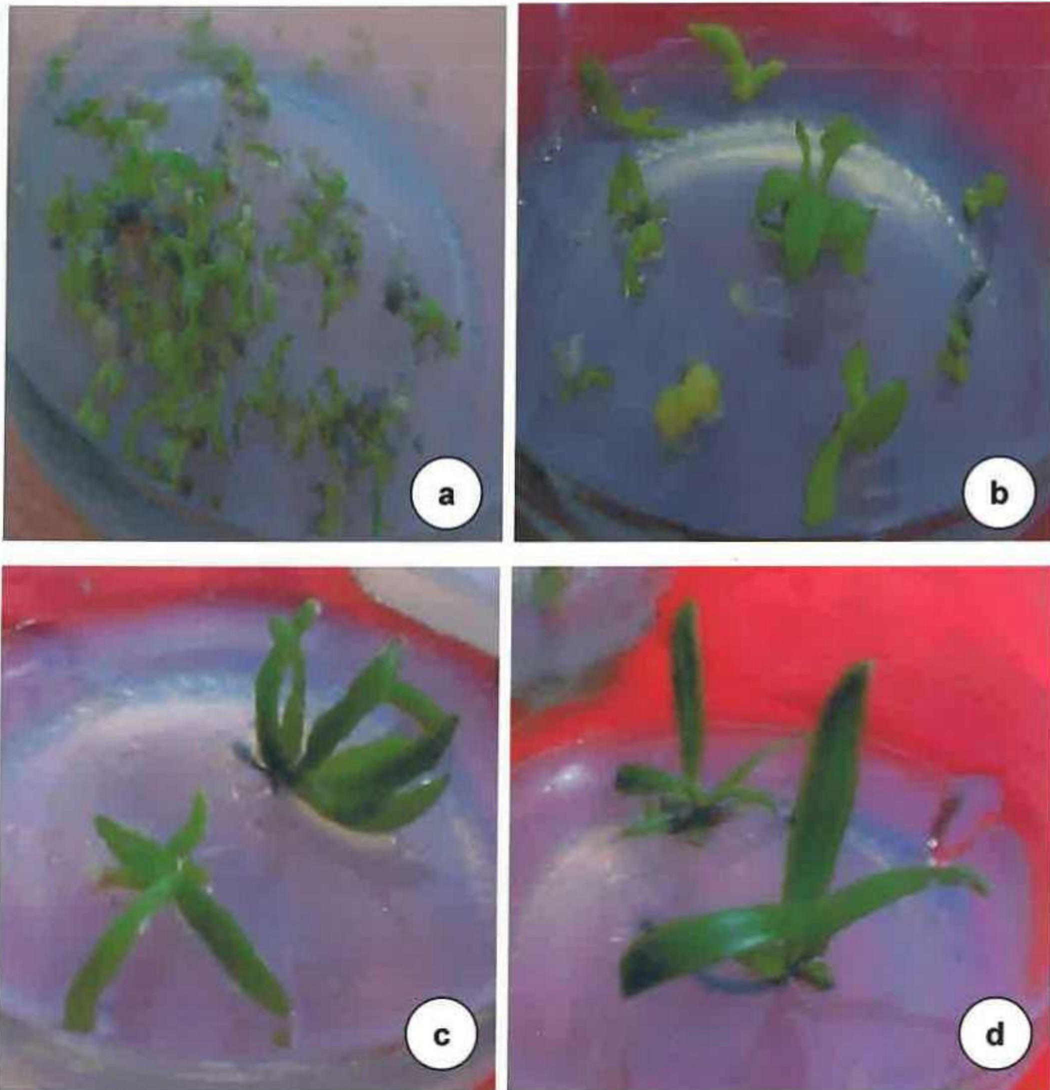

Figure-13

Table 21: *In vitro* conservation of *C. racemiferum* through slow growth method*

Organic carbon source(%)	Conc. (%)	Basal medium strength	Type of response	Performance**
	0	0	All the plantlet degenerated subsequently	-
Sucrose	1	1/4th	Plantlets etiolated and degenerated	-
		2/4th	As above	-
		3/4th	As above	-
		Full	Plantlets moderately healthy but stunted and elongated	++
	2	1/4th	Plantlets degenerated subsequently	+
		2/4th	Growth are highly retarded and stunted but healthy	+++
		3/4th	Growth slightly retarded and stunted	++
		Full	Slightly better growth and elongated	+
	3	1/4th	Growth slightly better and elongated	+
		2/4th	Plant growth healthy	-
		3/4th	Healthy plantlet growth	-
		Full	As above	-
Mannitol	1	1/4th	Plant degenerated after sometime	-
		2/4th	Highly retarded growth & degenerated subsequently	-
		3/4th	Retarded growth with etiolated leaves	+
		Full	Slightly retarded growth with etiolated leave	+
	2	1/4th	Plant growth retarded and stunted	++
		2/4th	As above	++
		3/4th	Plant growth stunted and leave slender	+
		Full	Plantlet healthy and luxurious	-
	3	1/4th	Plantlet healthy and less stunted	+
		2/4th	Growth less retarded and stunted	+
		3/4th	Plantlet growth highly retarded but healthy	++
		Full	Plantlet healthy, no slowed growth	-

Contd....

Organic carbon source(%)	Conc. (%)	Basal* medium strength	Type of response	Performance**
Fructose	1	1/4th	Slow growth and plant degenerated after sometime	-
		2/4th	Unhealthy plantlets and degenerated	-
		3/4th	Growth slightly better and stunted growth seen	++
		Full	Growth stunted and leaves etiolated	+
	2	1/4th	Plant growth retarded and stunted with leaves etiolated	+
		2/4th	As above	+
		3/4th	Plant growth slightly better and healthy	+
		Full	Healthy plantlet, less growth retarded and stunted	+
	3	1/4th	Healthy plantlet, less stunted and etiolated	+
		2/4th	Plant growth retarded and stunted	+
		3/4th	Healthy plantlet with greater retardation of growth	++
		Full	Healthy plantlet, no signs of retarded growth and stunting	-
Glucose	1	1/4th	Stunted growth and degenerated	-
		2/4th	As above	-
		3/4th	Retarded plant growth but degenerated subsequently	-
		Full	Stunted growth but degenerated later	-
	2	1/4th	Growth highly retarded and degenerated after sometime	-
		2/4th	Retarded plant growth and leaves etiolated	+
		3/4th	Stunted growth and etiolated	+
		Full	Plant growth less stunted but healthy	+
	3	1/4th	Plantlet highly stunted and degenerated subsequently	-
		2/4th	Plantlet growth slightly better and healthy	+

Contd ...

Organic carbon source (%)	Conc. (%)	Basal* medium strength	Type of response	Performance**
		3/4th	Plantlet growth not effected	-
		Full	Healthy and luxuriant plant growth	-
Dextrose	1	1/4th	Plantlet growth highly retarded degenerated subsequently	-
		2/4th	Plantlet growth highly retarded, stunted and degenerated subsequently	-
		3/4th	As above	-
		Full	Plantlet growth retarded, but degenerated	-
	2	1/4th	Plantlet growth highly stunted, degenerated subsequently	-
		2/4th	Plantlet growth highly retarded and stunted growth seen	++
		3/4th	Plant less stunted and etiolated	+
		Full	Moderately healthy plantlet	+
	3	1/4th	Plantlet growth less stunted and retarded	+
		2/4th	Plantlet growth slightly better and healthy	+
		3/4th	Plantlet not effected, no sign of slowed growth	-
		Full	Plantlet growth healthy and luxurious	-

* On MS medium, ** - no response+ Poor,++ Average, +++ Optimum response

***Cleisostoma racimeferum*:** In general the ½MS medium performed optimally over other strength of MS medium. The different combinations of MS medium and organic carbon sources performed differently. A similar optimum response were registered with ½MS + sucrose (2%), 1/4th MS medium+ mannitol (2%), 1/4th MS medium+ glucose (3%) and 3/4thMS medium+ fructose (1%) combinations (Table 21). In these concentrations the culture growth arrested without causing any damage to the plants (Figure 13 b). At lower concentrations of most of the carbon sources the culture degenerated subsequently.

The cultures from both the species could be sub-cultured successfully at 15-16 wk interval. The cultures are being maintained from last three years. The cultures resumed their normal growth on transferring on normal regeneration medium (Figure 13 c & d). The complete protocol for culture initiations from different explants, regeneration and mass multiplication of both the species is computed in Table 22.

Table 22: Complete media formulation for initiation of asymbiotic seed germination, regeneration from leaf and root explants and mass multiplication of *A. labrosa* and *C. racemiferum*

Components	Initiation of cultures						Mass multiplication and maintenance of coltures					
	Al^s	Cr^s	Al^l	Cr^l	Al^r	Cr^r	Al^s	Cr^s	Al^l	Cr^l	Al^r	Cr^r
Basal media*	MR	MS	MS		MS		MS		Ms		Ms	

Supplements (g/l)

Components	Al^s	Cr^s	Al^l	Cr^l	Al^r	Cr^r	Al^s	Cr^s	Al^l	Cr^l	Al^r	Cr^r
Sucrose	30	30	30	30	30	30	30	30	30	30	30	30
Coconut water	150	150	150	150	150	150	150	150	150	150	150	150
Meso-inositol	-	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Casein hydrolysate	-	-	0.5	0.1	0.5	0.2	-	-	-	-	-	-
Activated charcoal	-	-	--	-	-	-	1.5	1.0	1.5	1.0	1.5	1.0
Citric acid	-	-	0.1	0.1	0.1	0.1	-	-	-	-	-	-

Growth regulators (µM)

Components	Al^s	Cr^s	Al^l	Cr^l	Al^r	Cr^r	Al^s	Cr^s	Al^l	Cr^l	Al^r	Cr^r
NAA	20	10	-	-	-	-	10	10	10	-	10	-
IAA	-	-	-	-	4.0	1.0	-	10	-	10	-	10
BA	16	16	4.0	2.0	-	-	8	16	8	16	8	16
Kn	-	-	-	-	6.0	1.0	-	9.3	-		-	

*** MR- Mitra *et al*, MS- Murashige and Skoog, Al- *A. labrosa*, Cr - *C. racemiferum*, s- Seed germination, l- leaf culture, r- root culture**

Chapter-4

Discussions

The development of seeds in orchid is very poor even at maturity. They are microscopic, lack endosperm and require a suitable fungal stimulus for germination in nature. The mycorrizal fungus is believed to provide an essential physico-chemical stimulus for initiating germination (Harley, 1959). *In vitro* germination of orchid seed is an important part of the propagation and conservation programme, as the '*dust seeds*' are tiny and contain limited food reserves. Knudson (1922) demonstrated the possibility of by passing the fungal requirement of orchid seeds during *in vitro* germination and since then asymbiotic/non-symbiotic seed germination has emerged as an important tool for propagating a large number of orchid species and hybrids (Arditti *et al*, 1982).

The orchids are propagated by vegetative means (like keikis and or shoot buds) as well as through seeds. The rate of vegetative propagation is very slow and seed germination in nature is also very poor i.e., 0.2-0.3% (Vij, 2002). The fertilized ovules/immature embryos are used successfully for Micropropagation and rapid mass multiplication of several commercially viable and or threatened orchids (Sharma and

Tendon, 1990; Devi *et al*, 1998; Pathak *et al*, 2001; Temjensangba and Deb, 2005b&c). The successful non-symbiotic seed germination and or immature embryo culture of orchids are greatly influenced by several factors like seed pod age, different nutrient media, different media supplements, plant growth regulators and other culture conditions (Sharma and Tandon, 1990; Jamir *et al*, 2002). However, the media used for asymbiotic germination is more complex than that for symbiotic germination, as all organic and inorganic nutrients and sugars must be in a form readily available to the cultured immature embryos/seeds without the intermediary fungus (Mc Kendrick, 2000). But none of these basal nutrient media with different adjuvant fulfil the requirements of the entire orchidaceous group.

In the present study with *Arachnis labrosa* and *Cleisostoma racemiferum the* asymbiotic seed/immature embryo germination was initiated on three different basal media viz: Knudson 'C', Mitra *et al* and MS medium. Cultures were initiated from immature embryos/seeds representing different developmental age (8-20 WAP). The first sign of seed germination in both the species was characterized by the nodular swelling of the immature embryos/seeds with a visible change in colour (circular yellowish embryos) due to physiological changes of cultured embryos followed by greening of the germinating embryos. The formations of nodular embryos were observed in both the selected species and these nodular embryos with the passages of time differentiated into protocorm like bodies (PLBs). The relative time taken for the germination varied with the species. In *A. labrosa* the first sign of germination was observed after 25 days of culture while, a similar response in *C. racemiferum* was registered only after 52 days of culture initiation.

In the present study the physiological age of the green capsule was the key factor for successful non-symbiotic seed germination in both the species. Different species of orchids exhibits a particular threshold, a factor genetically structured in the organism. The influence of physiological age varies with the genus, species within the genus. For every species of orchid there is a window period of seed development, which supports optimum *in vitro* germination. The earliest stage at which the embryos can be cultured successfully varies within the orchid genotype and local conditions (Sauleda, 1976). Therefore it is desirable to determine the right stage to harvest the green capsule/pods to achieve an optimal germination. The culture of immature green pod/embryos ensures sterility but may require prolong period for germination as the seeds are immature. While, the culture of comparatively mature capsules/embryos before dehiscing may support better germination but the chances of contamination increases due to establishment of mycorrizal association (Mc Kendrick, 2000). In the present investigation, the green pod age of 18 WAP supported optimum germination (81%) in *Arachnis labrosa,* while in *Cleisostoma racemiferum* the optimum germination was achieved from green pod of 16 WAP where about ~90% germination recorded. The green pod age <14 WAP in *A. labrosa* and <12 WAP in *C. racemiferum* either failed to germinate or germinated after prolonged period of culture. In *Malaxis khasiana* seeds of 8 WAP supported 75% germination (Temjensangba and Deb, 2005c) while, in *Dacylorrhiza hatagirea* seeds of 16 WAP exhibited better germination (Vij *et al,* 1995) but 12 WAP and 120 days old seeds were the ideal planting materials in *Cymbidium macrorhizon* and *C. iridioides* respectively (Vij and Pathak, 1988, Jamir *et al,* 2002). The ability of immature embryos to germinate better than the mature ones is due to their distended testa cells

and metabolically awakened embryos and lack of dormancy and inhibitory factors (Yam and Weatherhead, 1988).

In the present investigation, three different basal media were used for culture initiation from immature seeds of both the species. Among the different media tested in *A. labrosa* optimum germination was achieved on Mitra *et al* medium followed by MS basal medium and Knudson 'C' medium and registered ~81%, 66% and 55% respectively. While, in case of *C. racemiferum* ~90% germination was recorded in MS medium followed by Mitra *et al*, (80%) and Knudson 'C' medium (60%). The orchids of different species exhibit a preferential requirement to specific nutrient media for seed germination; as such no standard media can be prescribed for all the orchid taxa. Temjensangba and Deb (2005c) reported better seed germination of *Malaxis khasiana* on MS medium while, in *Cymbidium macrorhizon* on Mitra *et al* medium (Vij and Pathak, 1988), *Dactylorhiza hatagirea* in Knudson 'C' medium (Vij *et al*, 1995), *Dendrobium chrysotoxum* in Vacin and Went (Rao *et al*, 1998), *Vanda coerulea* in Ichihashi & Yamashita (Rao *et al*, 1998) and VW medium (Devi *et al*, 1998), *Aerides rosea* in Knudson 'C', VW and MS media (Sinha *et al*, 1998), *Cymbidium iridioides* in Nitsch medium (Jamir *et al*, 2002). In the present study with the two species Knudson 'C' medium found to be least suitable for non-symbiotic seed germination. A similar response was also reported with other orchid species like *Renanthera imschootiana* (Laishram and Devi, 1999), *Geodorum densiflorum* (Sheelavanthmath *et al*, 2000) and *Cymbidiums* (Nagaraju and Upadhyaya, 2001).

The *p*H of the medium showed a marked effect on successful seed germination. In the present study, *p*H levels of 5.5 to 5.7 was found suitable, however, 5.6 were recorded to be optimum for all the culture. Most orchids germinate on media

of *p*H 5.5, but Andean species may favour slightly higher *p*H (5.6 to 5.9) (Mc Kendrick, 2000), however Sinha *et al,* (2002) reported that a *p*H of 5.6 supported the optimum seed germination of *Renades* Arunodaya.

Besides basal medium the sucrose concentration in the initiation media showed a marked effect on seeds germination. Presence of sucrose in the initiation medium was pre-requisite and there was no germination in the absence of sucrose on any of the media tested. The different levels of sucrose showed differential effect on seed germination. The requirements of the quantity and quality of exogenous supply of the organic carbon sources and or sucrose varies with the species, the media compositions used, the endogenous level of organic carbon and the developmental stage of the cultured embryos (Temjensangba and Deb, 2005c). Sharma and Tandon (1990) reported that 2-3% sucrose, D-Fructose and D-Glucose were the suitable organic carbon source for *in vitro* seed germination of *Cymbidium elegans* and *Coelogyne punctulata*. In the present investigation it was observed that the optimum seed germination of *A. labrosa* and *C. racemiferum* were obtained on basal media containing 3% sucrose and CW (15%). But in *C. racemiferum* on Mitra *et al* medium optimum germination was recorded with sucrose (2%).

The incorporation of CW (15%) in the germination media of both the species did not show any significant effect on germination but showed a marked effect and accelerated the early differentiation of PLBs. The stimulatory effect of CW was reported in *Dendrobium aphyllum* (Talukdar, 2001), in *Vanda coerulea* (Devi *et al,* 1998). Better seedling differentiation was achieved in *Peristeria elata* when medium supplemented with CW (10%) and proved to be advantageous over peptone (Bejoy *et al,* 2004) while, Sinha *et al* (2002) and Vij and Aggarwal (2003) reported the

enhanced repetitive PLbs formation in Renades and *Vanda coerulea* respectively when media were supplemented with CW (15%). However, Sheela *et al* (2004) reported a rapid multiplication of PLBs as well as the development of shoots in *Dendrobium* CV Sonia when media is supplemented with 7.5% CW in the presence of BA (1.5 mgl^{-1}). Leetham (1974) reported that a plant growth regulator like substance (cytokinins) is present in coconut water whereas Lee *et al* (1995) attributed its effect to its sugar and cytokinin content. But according to Jamir *et al* (2002) the use of CW failed to elicit any effect on its growth in *Cymbidium iridioides* and *C. lowianum*.

In the present study with the two selected orchids a diffused light condition of 20 μmol m^{-2} s^{-1} was found suitable for initiation of asymbiotic germination followed by full light (40 μmol m^{-2} s^{-1}) conditions at 12/12 hr photoperiod while culture maintained in the dark degenerated mostly. Earlier Temjensangba and Deb (2005c) reported the effect of light on immature seed germination and observed that diffused light condition supported higher rate of germination in *Malaxis khasiana*. It is believed that this particular light condition activate and sensitise the photoreceptor of the germinating embryos and break down the endogenous growth inhibitors responsible for dormancy in seeds resulting in the optimal germination. The quality and quantity of light effect the germination, growth and differentiation (Economou and Read, 1987; Ichihashi, 1990). Islam *et al,* (2003) reported that the *Phalaenopsis* callus growth and development of PLBs was better in illumination than in dark. The effect of light for embryogenic culture initiation in conifers is well documented (Von Arnold, 1987; Gupta and Grob, 1995, Deb and Tandon, 2004). In *Pinus kesiya*, light was reported inhibitory for initiation of embryogenic cultures and produced more

non-embryogenic cultures. Dark was optimum while diffused light formed moderate embryogenic cultures (Deb and Tandon, 2004).

In the present investigation even in the absence of PGRs nodular swelling of the immature embryos were possible but failed to differentiate further. The PGRs in the germinating medium showed a marked effect on the growth, differentiation and development of PLBs. In the present study seed germination of *A. labrosa* was recorded as high as ~81% on Mitra *et al* medium containing NAA-BA (20 + 16 µM respectively) in combination but in *C. racemiferum* higher seed germination was recorded optimum on MS medium containing NAA-BA (10 + 16 µM respectively) in combination. The spherules/nodular swelled embryos developed and differentiated into PLBs and subsequently to seedlings/plantlets on initiating medium after 25 days and 40 days respectively. A synergistic action of auxin and cytokinins was observed in asymbiotic seed germination in the present investigation. The present observation is in agreement with Sharma and Tandon (1986) where they reported the stimulatory effect of NAA (0.1 mgl^{-1}) in conjunction with cytokinins (Kinetin 1 mgl^{-1}) in *Coelogyne punctulata* for both seed germination and seedling growth. But Nagaraju *et al* (2004) reported that in *Dendrobium* hybrid the single leaflet in the protocorm developed when basal medium was supplemented by BAP alone while, BA singly supported better germination in *Malaxis khasiana* (Temjensangba and Deb, 2005c).

In the current investigation cultures were also initiated from the foliar explants of both the species. Wimber (1965) successfully developed PLBs from the leaves of *Cymbidiums*, thus opened up an effective alternative to apical shoot meristem culture. Since then the regenerative competence of foliar explants were positively tested for more than 60 orchid species (Vij *et al*, 2000a). However, the success is restricted

mostly with epiphytic orchids and only three species from terrestrial orchids suggesting thereby the ground orchids are less amenable to *in vitro* regeneration. In the present investigation, foliar explants of both the species from *in vivo* source however mostly remain recalcitrant though there were initial swellings at the basal part of the explants. The previous works suggest that the green house sourced explants are less responsive to *in vitro* regeneration due to profuse phenolic exudation from mature tissues, lose of chlorophylls, failure to developed meristematic activity probably due to tissue damage during surface sterilization (Vij *et al*, 2000a) and present investigation is in agreement with this opinion. However, direct shoot buds/PLBs/young plantlets were induced from the basal part of the *in vitro* raised ~15 wk old foliar explants in *A. labrosa*. A similar response was recorded from the foliar explants of *C. racemiferum*. In both the species it was observed that the young leaf explants (~1 cm size) of 15 wk old were more suitable than the mature ones (~20 wk old) for regeneration. The better morphogenetic potential of the young tissue was attributed to the physiologically active state of the constituent cells owing to their less rigid cell walls (Misra and Bhatnagar, 1995). Vij *et al* (1984) reported that the juvenile leaves proliferate along the surface where as the response is restricted to the basal region in relatively older explants. The importance of explants age is well documented in other orchid species, viz: in *Vanda coerulea* (Vij and Aggarwal, 2003), in*Aerides multiflora* (Vij *et al*, 2004a).

In *A. labrosa* the meristematic activity was restricted to the basal cut end of the foliar explants. However, in *C. racemiferum* the meristematic activity was subsequently developed and spread along the entire surface of foliar explants. The morphogenetic potential of leaf base has been reported in *Coelogyne*, *Dendrobium*,

Oncidium and *Phalaenopsis* (Abdul Karim and Hairani, 1990); *Acampe praemorsa* (Nayak *et al*, 1997), in *V. coerulea* (Vij and Aggarwal, 2003). Mathews and Rao (1985) considered the leaf base to be the decisive factor for culture initiation from foliar explants. Sinha and Hegde (1999) reported the development of meristematic activity along the entire in Renades Arunoday Hybrid. In the present study the orientation of explants was observed to be another crucial factor for morphogenetic response in both the species. Slanted orientation (~45° angle) of the explants showed better response than horizontally placed. Earlier, Nayak *et al* (1997) in *Acampe praemorsa* reported the influence of explants orientation of shoot development.

The incorporation of PGRs to the basal medium was obligatory for the initiation of culture. The explants of both the orchid species failed to respond, when cultured on PGRs free medium. The role of growth hormones in stimulating meristematic activity and promoting proliferation in leaf explants is well documented in orchids (Abdul Karim, 1990; Vij and Pathak, 1990; Arditti and Ernst, 1993; Vij *et al*, 1994; Nayak *et al*, 1997; Yam and weatherhead, 1991b). Murashige (1974) opined that *in vitro* plant regeneration occurs frequently through adventitious shoot formation and rarely through somatic embryogenesis. In the present study, BA (4 μM) singly supplemented to basal medium successfully initiated meristematic activity in ~85% of cultures in *A. labrosa* and as many as 21 shoot buds were formed from a single explants after 7-8 wk of culture. Kinetin registered a modest response but regeneration was much lower than the BA. This result is in line with the earlier report that BAP was better used at 2 mgl^{-1}, which favoured development of multiple shoot buds (Vij *et al*, 2000b). While in *Dendrobium* 'Sonia' Saiprasad *et al* (2003) reported that BA enriched cultures produced more PLBs than Kn enriched culture. A similar

response was also registered with *C. racemiferum* during present study. Healthy and better PLBs developed over the entire leaf surface and ~80.0% explants responded positively on MS medium containing BA (2µM) singly. However, combine treatment of BA and NAA (2µM) each resulted callus mediated regeneration. While, IAA-Kn (2µM each) in combination stimulated shoot buds mediated regeneration in ~66% culture. Nagaraju *et al*, (2003) reported that supplementation of media with low concentration of BAP (0.5 mgl^{-1}) produced the maximum numbers of protocorm and shoots in *Cymbidium* and *Cattleya* culture. Cytokinins were successfully used to enhance the regeneration frequency and to multiply the cultures of several orchids (Vij and Pathak, 1990; Kaur, 1996; Temjensangba and Deb, 2005a). Earlier, Sinha and Hegde (1999) reported a 90% response in leaf explants containing 2 mgl^{-1} BA in a medium.

In the present study, the root explants of both the selected orchid species were cultured on MS basal medium and the explants failed to regenerate in absence of PGRs. In *A. labrosa* the aerial roots from *in vivo* source remained recalcitrant to morphogenetic response whereas a poor morphogenetic response (~20%) was recorded in *C. racemiferum* on medium containing IAA and Kn (2µM each in combination). From most of the previous reports with root culture reported that the roots from out door source remained recalcitrant to regeneration but selective proliferation from those with ill-defined root caps depending upon their nutrient regimes and the species (Vij and Aggarwal, 2003). Hence, Vij (1993) considered the importance of tissue juvenility and the presence of stimulus in nutrient pool for successful regeneration from aerial roots. About 15-20 wk old aerial roots of *A. labrosa* from axenic culture responded positively on MS medium enriched with

sucrose (3%), CH (500 mgl^{-1}) and in the presence of IAA-Kn (4 µM + 6 µM respectively) in combination. About 80% cultures responded positively and as many as 10 PLBs or shoot buds developed per explants within five wk of culture initiation. While in *C. racemiferum,* the juvenile root (~15-20 wk old) from axenic culture exhibited 100% meristematic activity on basal medium containing sucrose (3%), CH (200 mgl^{-1}) and IAA-Kn (1 µM each) in combination enriched by sucrose (3%) and CH (200 mgl^{-1}) after 25 d of culture. In both the species, the regeneration response was restricted to the basal cut end and no response was recorded from the root cap region, but at later stage, meristematic activity spread to the surface of root explants in case of *C. racemiferum.* In both the species auxins (IAA and NAA) singly impaired regeneration but enhanced considerably when used in conjunction with either of cytokinins (BA and Kn). However, Vij *et al* (2004b) reported that prolonged cultures in the medium containing NAA proved useful for PLBs mediated culture multiplication in *Cymbidium* root and combined treatment with NAA and BAP induced direct callus mediated development and enhanced response frequency in root explants.

In *A. labrosa,* the propagules developed on initiation media started differentiating into rooted plantlets after 7-8 wk on regeneration medium. The optimum response was registered on MS medium containing NAA-BA (10 + 8 µM respectively) in combination whereas, as many as 10-12 shoot buds/PLBs developed. But in *C. racemiferum* the optimum shoot buds/PLBs formation was recorded from cultured aerial roots on MS medium containing IAA-Kn (4 µMeach) in combination followed by IAA-Kn (2 + 6 µM). Philip and Nainer (1988) observed that the requirement of exogenous supply of PGRs is species specific and it varies during

initiation, multiplication and differentiation of cultures. The transformation of root meristem to shoot meristem is positively influenced by the endo- and or exogenous level of auxin. In another report by Peres *et al,* (1999) observed that the exogenous PGRs markedly influenced the morphogenesis by alternation in the endogenous IAA/cytokinin balance. Present investigation is in agreement with the other reports where a synergistic action of auxin and cytokinin has been reported with several other orchids (Vij and Pathak, 1990; Seeni and Latha, 1992). The incorporation of CH in the initiation medium for leaf and root culture of both the species showed a marked effect on PLBs formation. In *A. labrosa,* 500 mgl^{-1} CH proved to optimum for both leaf and root culture while, in *C. racemiferum* 100 mgl^{-1} and 200 mgl^{-1} were the optimum concentrations for leaf and root culture respectively. The incorporation of CH to the basal medium was reported to enhance cell proliferation and rich crop of PLBs within 4 wk in pseudobulb segment culture of *Malaxis acuminata* (Vij and Kaur, 1998). Similarly, Puruhit *et al* (2002) reported to increase somatic embryo formation of *Quercus floribunda* in the CH enriched medium.

The soaking of excised root explants in sterile water prior to culture in basal medium promoted better regeneration response in both the species. This observation is in agreement with the earlier report of Sanchez (1988), where a similar finding was reported in *Cyrtopodium punctatum.* It is believed that this type of treatment accelerate the differentiation process either by diluting the inhibitory substances present in the root-tip or favouring the formation ofpromotory substance(s) under low oxygen condition of water. The orientation of the explants on initiation medium showed a marked effect on successful initiation of culture. The explants cultured in slanted position (~45°) performed better over the explants horizontally placed. In both

the species a similar response was registered. Earlier Nayak *et al* (1997) also reported a similar response in *Acampe praemorsa.*

The PLBs and tiny plantlets/seedlings obtained from cultured immature embryos of *A. labrosa* and *C. racemiferum* were maintained on two different basal media (Mitra *et al* and MS medium) in conjunction with different adjuvant for regeneration of plantlets and mass multiplication. The seedlings with tiny leaf sprouting were differentiated into plantlets after 4 wk on transferring to a regeneration medium. Amongst the two media tested, MS basal medium supported an optimum growth and differentiation into rooted plantlets in both the species under study. While Mitra *et al* medium supported poor culture differentiation and growth. This is perhaps could be due to difference of chemical constituents with MS medium or deficient as to the requirement of the developing seedlings. George and Sherrington (1984) and Chen *et al* (2004) stated that the change in culture conditions and media could alter the pattern of organogenesis in orchids and such behaviour can be judiciously exploited to achieve desirable response in many orchid taxa by altering nutrient regimen.

The PGRs marked a pronounced effect and elicit different responses in the seedling development depending on the concentration used. Inclusion of PGRs in the regeneration medium was obligatory for successful regenerations of plantlets and mass multiplication. In the absence of PGRs there was no regeneration in both the species. Thus both the auxins (IAA and NAA) used singly either impaired regeneration or failed to respond. However, when used in conjunction the response enhanced considerably. Different combinations of PGRs exhibited varied effect on differentiation of PLBs and regeneration of plantlets.

In *A. labrosa* the optimum response in terms of plantlet regeneration and mass multiplication was achieved on regeneration medium containing NAA-BA (10 + 8 µM) in combination, where as many as 17 shoot buds/PLBs were formed per explant after ~4 wk of culture. The medium containing NAA and Kn resulted in slender long and slightly etiolated plantlets while in IAA and BA rich medium supported some repetitive PLBs formation. The PLBs/shoot buds rose from all the three explant sources (i.e., immature embryos, foliar explant and root segments) differentiated on regeneration medium containing same medium supplements.

In *C. racemiferum*, the number of shoot buds formation from PLBs of immature seed origin was higher on MS medium containing NAA-BA (10 + 8 µM respectively) in combination but occasionally the plantlets were etiolated and leaves were thin. The medium containing NAA and Kn resulted stunted growth but on medium with NAA-Kn (10 + 16 µM respectively) in combination the plantlets were moderately healthy with few PLBs formation. In general the cultures maintained on medium enriched with IAA-BA combination resulted comparatively well rooted and healthy plantlets but cultures on IAA-Kn enriched medium produced well-rooted plantlets with moderate numbers of multiple shoot buds.

The inhibitory effect of IAA on seedling development has been reported in *Orchis purpurella* (Hadley and Harvais, 1968), *Dactylorhiza purpurella*, *Coeloglossum viride* and *Platanthera bifolia* (Hadley, 1970). On the other hand, IAA at 0.1 mgl^{-1} is reported to promote seed germination and seedling development of *Cymbidium punctulata* (Sharma and Tandon, 1986). But Vij and Aggarwal (2003) reported that NAA favoured the development of multiple shoots/PLBs in *Vanda coerulea*. Bhadra and Hossain (2004) reported highest number of multiple shoot buds

formation from nodal segment of *Micropera pallida* when medium was supplemented with 2.0 mgl^{-1} NAA and 2.0 mgl^{-1} BA. While the PGRs like BAP singly or in combination with IAA was best used for initiation of cultures and development of healthy plantlets there from leaf explants of *Saccolabium papillosum* (Kaur and Vij, 2000).

But the PLBs and shoot buds rose from foliar explants and aerial root segments were differentiated into plantlets and multiplied on MS medium containing IAA-Kn (2 μM each in combination) and IAA-Kn (2 + 6 μM in combination) respectively. With both the explants there was no or very poor response on medium containing singly auxin and or cytokinin and only the combination of auxin and cytokinin responded positively. The interactive and synergistic action of auxin and cytokinin is well recorded in several orchid taxa and species as well as hybrids (Seeni, 1988; Sinha and Hegde, 1999; Vij and Aggarwal, 2003; Vij *et al*, 2004a).

In the present study the incorporation of AC to the basal medium showed a considerable effect on the plantlet growth, development and regeneration in both the orchid species. Presence of AC in the regeneration medium of both the species triggered the formation of repetitive PLBs, which added a new dimension in rapid mass multiplication of the selected species. Amongst the different concentrations of AC a concentration of 0.15% was found suitable for *A. labrosa* while 0.1% AC in the regeneration medium was registered to be optimum for *C. racemiferum*. But at higher concentration of AC cultures degenerated. Earlier Vij *et al* (2000b) reported that the addition of AC to medium promoted multiple shoot bud formation in *Bulbophyllum careyanum* culture with as many as 28 plantlets/explants and replacement of callus phase by direct development of shoot buds in the additional presence of AC. The

benign effect of AC has been reported in many species (Paek *et al*, 1998; Chung *et al*, 1998; Chang and Chang, 2000; Nagaraju *et al*, 2004). All the workers reported that the incorporation of AC to the basal medium stimulated growth and produced healthy plantlets and significantly superior over the control. While, in *Cymbidium* hybrid culture presence of AC in the medium stimulated the early rooting (Nagaraju and Upadhyaya, 2001). However, AC in the regeneration medium proved inhibitory to regeneration in *Vanda* hybrid (Mathews and Rao, 1985), *Vanda teres* (Vij and Pathak, 1990) and *Vanda coerulea* (Vij and Aggarwal, 2003). It is argued that presence of AC in the medium absorbs exudates/growth inhibitors, enhance medium aeration and absorb light (Yam *et al*, 1989). While according to Van Waes (1987), it is due to its ability to established polarity by the darkening of the medium, and irreversibly absorbs inhibitory compounds etc, which may helps in culture proliferation.

The hardening of in *vitro* raised plantlets is essential for better survival and successful establishment. In other words the survival percentage is determined by the hardening of the plantlets. Losses of micropropagated plants after transferring to nature are attributed to low humidity, high levels of light and non-sterile condition of the *in vivo* environment (Paul, 1999). Conventionally the tissue-raised plants are hardened by transferring on a low nutrient medium having low organic carbon sources and maintained at high light intensity. Different matrix or substrates with manipulation in salt solution were employed for hardening of different angiospermic *in vitro* raised plants by various workers viz: soilrite for *Carica papaya* (Agnihotri *et al*, 2004), soaked cotton for *Saccharum offinarum* (Gill *et al*, 2004), etc. However, no report on the use of other substrate was recorded earlier in case of orchids.

During the present investigation the regenerates were hardened following the conventional method. Besides the conventional approach part of the regenerates were also maintained for hardening following a new approach. The tiny plantlets of both the species were taken out from the regeneration medium and transferred in $1/10^{th}$ strength of MS salt solution devoid of any sucrose and PGRs. In each culture tube small chips of charcoal, brick pieces and mosses were incorporated before the plantlets are transferred. Culture vials were maintained for ~12-16 wk under identical culture conditions. The hardened plants were then transferred to community potting mix-containing pieces of charcoal, brick, decayed wood (1:1:1) and moss. The potted plants were then transferred in poly-house and maintained for 2-3 months. The plants were fed with $1/10^{th}$ MS salt solution at one wk interval. The acclimatized plants were transferred to Departmental botanical garden and the performances of these transplanted plants were monitored regular interval. Feeding the plantlets with nutrient salt solution has been reported to be beneficial for the promotion of orchid survival and growth (Mukherjee, 1983 and Kumaria and Tandon, 1994).

In the current investigation, the plantlets hardened in conventional technique after its transfer to the community potting mix recorded high mortality rate while on the other hand new roots were found to develop from the plantlets hardened in the new technique. It is interesting to note that these newly developed roots get attached to the support medium with the passage of time and vigorous growth of the plantlets were observed. The attachment of roots to the support medium was mostly confined to the charcoal chips and moss, which strongly suggest the suitability of the material for the purpose. In other words, it resembles the velamenous root getting attached to the branch or the trunk of tree in natural habitat. A survival frequency of about 95% was registered in the potting mix after about two months of transfer in both species. While, following the conventional technique the survival frequency was 40% in *A. labrosa* and 45% in *C. racemiferum*. It was also observed that the transplanted

regenerates were dark green and healthy and new roots and leaves started emerging after one month of transfer in the potting mix.

Attempt was made to conserve the germplasm of both the species *in vitro*. The propagules of both the selected species were subjected to *in vitro* short to medium term storage using slow-growth method. The different types of propagules like advanced stage PLBs and young plantlets of both the species raised in culture were used for short to medium-term *in vitro* conservation. It was observed that among the different culture conditions studied, a diffused light (20 µmol m^{-2} s^{-1}) condition at 20°C was found to be suitable for both *A. labrosa* and *C. racemiferum*. The Cultures maintained in the dark mostly callused and in some cases cultures degenerated. The callused cultures failed to grow upon returning to normal light conditions and degenerated. While, the cultures maintained in normal light condition and 25°C temperature maintained a healthy culture growth and demanded rapid sub-culture. Amongst the different levels of MS medium, a 1/2 strength MS medium was found optimal over other concentration. Lower level of basal medium did not support plant growth and degenerated in subsequent subcultures. Generally sucrose (2%) was the ideal organic carbon source for *A. labrosa* but in *C. racemiferum* a similar response were registered with ½MS + sucrose (2%), ¼MS + mannitol (2%), ¼MS + glucose (3%) and ¾MS+ fructose (1%). In both the species the cultures could be successfully sub-cultured at 15-16 wk interval. Withers (1991) reported that reducing the growth rate *in vitro* short to medium term storage provide a convenient option but not suited for long term programmes as it poses risk of selection due to stress imposed on the cultures during storage. Accordingly, the reduction of medium nutrient status had been reported by Malaurie *et al* (1993) and the addition of activated charcoal to culture medium has also been reported as beneficial in minimal growth condition

(Paul, 1999). Slow growth method is a widely followed method but there is a continued concern about the level of somaclonal variation under slow growth conditions (Jaret and Gawel, 1991). In the present study the cultures were maintained under slow growth condition for more than 3 years and are still being maintained under slow-growth conditions. The normal plantlets were raised from the stored cultures when they were maintained on normal regeneration medium. No visible abnormality was registered in the regenerated plantlets and could be successfully potted in potting mix.

During the present investigation protocols were established for culture initiation from immature seeds/embryos, foliar explants and aerial roots of *Arachnis labrosa* and *Cleisostoma racemiferum*, regeneration of plantlets and mass multiplication. These techniques opens new routes for *in vitro* mass multiplication of these two rare and threatened orchids of North-East India in general and Nagaland in particular. The use of *in vivo* sourced explants offers several advantages especially for monopodial orchids. The protocol established for culture initiation from *in vivo* sourced aerial roots of *C. racemiferum* indicates the possibility of using alternative explants for monopodial orchids and which demands further research to exploit this explant source. The techniques developed for *in vitro* short to medium-term storage of germplasm will ensure the presence of these species in nature in days to come. The protocols may be used by the local commercial orchid growers, NGOs and Government agencies associated with floriculture, which will add to economic development of Nagaland.

Chapter-5

Summary

The state of Nagaland is endowed with great natural biotic resources including orchids. Nagaland is represented by 366 species belonging to 88 genera including endemic species like *Coelogyne hitendrae, Sunipia jainii,* etc. But these natural resources are fast depleting due to removal of forest cover and their natural habitat from unplanned developmental activities, logging and traditional agricultural practice "Slash and burn cultivation" prevailing in the state. As a result of these, many valuable floristic resources are lost, threatened and becoming extinct without realising their potential. So there is an urgent necessity to develop protocols for *in vitro* multiplication and conservation of the rare and threatened species. With a view to multiply and conserve two rare and threatened species (viz: *Arachnis labrosa* and *Cleisostoma racemiferum*) of North East India in general and Nagaland in particular the present study was undertaken. The cultures were initiated from different explants sources like immature embryos/seeds, foliar explants and aerial roots of two rare and threatened orchids of Nagaland viz. *Arachnis labrosa* and *Cleisostoma racemiferum.*

In both the species cultures were initiated from immature embryos of different developmental stage (8-20 week after pollination, WAP). In *A. labrosa* the seed

cultured from green pod of 18 WAP resulted optimum germination while, in *C. racemiferum* seeds of 16 WAP were found suitable for non-symbiotic germination. Seeds from <14 and <12 WAP old pods of *A. labrosa* and *C. racemiferum* respectively showed delayed response and supported poor germination while the older seeds of both the species showed a decline in germination frequency, also an increase in fungal contamination. The first sign of successful germination was noted as nodular swelling of seeds after 25 and 52 days in *A. labrosa* and *C. racemiferum* respectively.

About 81% seed germination in *A. labrosa* was achieved on Mitra *et al* medium containing sucrose (3%) (w/v), coconut water (CW) (15%) (v/v) followed by MS medium (~66%) containing sucrose (3%) (w/v), CW (15%) (v/v). While in *C. racemiferum* optimum seeds germination (~90%) was registered on MS medium containing sucrose (3%) (w/v), CW (15%) followed by on Mitra *et al* medium (~80%) containing sucrose (2%) (w/v) and CW (15%) (v/v). Amongst the different plant growth regulators used in different combinations in *A. labrosa* optimum germination was accomplished on medium containing NAA-BA (20 +16 µM respectively in combination). But in *C. racemiferum* a combination of NAA-BA (10 + 16 µM respectively in combination) was found optimum. With both the species cultures maintained in diffused light condition (20 µmol m^{-2} s^{-1}) and at 12/12 hr photoperiod and 24±1°C supported better germination followed by full light condition (40 µmol m^{-2} s^{-1}). The *p*H of the initiation medium showed a marked effect on successful germination and a *p*H of 5.6 was found suitable in both the species.

The regeneration from foliar explant of both the species and root explant of *A. labrosa* remained recalcitrant to *in vitro* morphogenetic response. While ~15 wk old the aerial roots from *in vivo* source of *C. racemiferum* showed a poor morphogenetic

response. About ~20% of the explants produced PLBs after 75 days of culture on MS medium containing sucrose (3%), CH (200 mgl^{-1}) and IAA-Kn (2 μM each in combination). The direct shoot buds were obtained from the basal part of foliar explants of ~15 wk axenic culture in *A. labrosa* on MS basal medium enriched with sucrose (3%), CH (500 mgl^{-1}) and BA (4 μM) singly. After 7-8 wk of culture initiation ~85% cultures responded positively where as many as 21 shoot buds were developed per explants. But in *C. racemiferum*, optimum regeneration from foliar explants was recorded from ~15 wk old in MS medium containing Sucrose (3%), CH (100 mgl^{-1}) and BA (2 μM) singly. After ~40 days of culture 80% of the culture responded positively.

Better morphogenetic response from the aerial root explants of *in vitro* source of *A. labrosa* was recorded (~80% response) in explants placed in slanted position (~45°). Among the different media and other adjuvant used better response was achieved on MS basal medium containing sucrose (3%), CH (500 mgl^{-1}) and IAA (4μM) + Kn (6 μM) in combination after 5 wk of culture. MS medium enriched with sucrose (3%), CH (200 mgl^{-1}) and IAA (1 μM) and kinetin (1 μM) in combination supported ~100% morphogenetic response from *in vitro* grown roots of *C. racemiferum*.

The PLBs/shoot buds/seedlings developed from non symbiotic seed germination of both orchid species were cultured on Mitra *et al* and MS basal medium with different combinations of PGRs (NAA, IAA, BA and Kinetin). Better growth, development and differentiation were recorded for both the species in MS basal medium fortified by sucrose (3%) and coconut water (15%). The presence of plant growth regulators was obligatory for regeneration of plantlets. A combination of

NAA-BA (10 + 8 μM respectively) in the regeneration medium registered to be optimum for plantlet regeneration and multiple shoot bud induction of *A. labrosa*. The PLBs and shoot buds raised from all the three explant sources differentiated on the same regeneration medium containing identical adjuvant. There were as many as 17 shoot bud formations per explants per subculture. The medium containing NAA-Kn combination resulted in slender long and slightly etiolated plantlets while in IAA-BA combination cultures produced some repetitive PLBs.

In case of *C. racemiferum* the number of shoot bud/PLBs formation was highest on MS medium enriched by NAA (10 μM) and BA (8 μM) in combination but occasionally the plantlets were etiolated and leaves thin. NAA-Kn supplemented medium resulted in stunted growth but in NAA-Kn combination, the plantlets were moderately healthy with few PLBs formation.

The propagules developed from foliar explants of *C. racemiferum* differentiated into plantlets and multiple shoots on MS medium conjunct with sucrose (3%), IAA-Kn (2 μMeach) in combination. After 8-10 wk of culture as many as, 10-12 shoot buds developed per explant. But the propagules from the root segment differentiated into rooted plantlets and multiple shoots on MS medium containing sucrose (3%), IAA-Kn (4 μMeach) in combination.

The incorporation of activated charcoal in the regeneration medium showed a pronounced effect on mass multiplication by producing repetitive PLBs and multiple shoot buds. A concentration of AC (0.15%) was found optimum for *A. labrosa* while, for *C. racemiferum* AC (0.1%) in the regeneration medium supported optimum repetitive PLBs formation. In both the species incorporation of higher concentration of AC found to be inhibitory.

The regenerated rooted plantlets were maintained for ~2-3 passages for further growth followed by transferring them in hardening media. Two different hardening techniques were followed in the present study viz. a. conventional hardening and b. a new approach. In new approach the plants were transferred in culture vials containing $1/10^{th}$ MS liquid medium freed from plant growth regulators. Small pieces of bricks, charcoal and mosses were incorporated in the hardening medium and cultures were maintained for 10-12 wk. The hardened plants were transferred to community potting mix containing charcoal pieces, brick pieces, coconut husk and decayed wood (1:1:1:1 ratio) with a layer of moss. The potted plants were maintained in poly house for 2-3 months before transferring to the Botanical garden. The potted plants were fed with $1/10^{th}$ MS salt solution at one wk interval. It was observed that the regenerates hardened following the new technique developed during the present investigation performed better compare to the conventional method. It was observed that following this technique survival frequency of potted plants was enhanced significantly to about 95% in both the species compared to 40% in *A. labrosa* and 45% in *C. racemiferum* following conventional technique/control after two months of transfer. It was also observed that the transplanted regenerates were dark green and healthy and new roots started emerging after one month of transfer in the potting mix.

The *in vitro* short to medium term conservation of germplasm using slow growth method was registered better in diffused light (20 µmol m^{-2} s^{-1}) condition at 20°C temperature. The optimum slow-growth with *A. labrosa* was achieved on ½MS medium containing sucrose (2%). At this condition, slow and retarded growth of plants was observed without any gross abnormality in external morphology except the stunted growth. While, in *Cleisostoma racemiferum* a similar optimum slow-growth

of culture were recorded on ½MS medium + sucrose (2%), 1/4thMS medium + mannitol (2%), 1/4thMS medium+ glucose 3%) and 3/4thMS medium+ fructose 1%). In general at lower concentration of organic carbon sources cultures degenerated while, at higher levels of organic carbon supported normal growth of the culture in both the species. The cultures could be successfully at 15-16 wk interval. The cultures from slow-growth conditions resumed the normal growth on culturing on normal regeneration medium and conditions.

The protocols established opens new routes for *in vitro* mass multiplication of these two rare and threatened orchids of North-East India in general and Nagaland in particular. The use of *in vivo* sourced explants offers several advantages especially for monopodial orchids. The protocol established for culture initiation from *in vivo* sourced aerial roots of *C. racemiferum* indicates the possibility of using alternative explants for monopodial orchids and which demands further research to exploit this explant source. The techniques developed for *in vitro* short to medium-term storage of germplasm will ensure the presence of these species in nature in days to come. The protocols may be used by the local commercial orchid growers, NGOs and Government agencies associated with floriculture, which will add to economic development of Nagaland.

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Nationality	:	Indian
Category	:	Schedule Tribe (ST)

Academic qualification

Exam Passed	Board/ University	Year	%of mark obtained	Div/Class
H. S. L. C.	NBSE	1993	34.87	3rd
P. U.	NU	1995	46.88	2nd
B.Sc.	NU	1998	54.12	2nd
M.Sc.	NU	2001	66.68	1st
NET	CSIR/UGC	2004		
Ph.D.	NU	2006 (Thesis submitted)		

Specialization Ph. D	:	Plant Tissue culture
Thrust area of Research	:	Conservation Biotechnology
Current research works	:	Micropropagation and *in vitro* conservation of some rare and threatened orchids of Nagaland.
Title of the PhD thesis	:	"*In vitro* mass multiplication and conservation of two rare and threatened orchids in Nagaland".
Research Experience	:	1. JRF & SRF in DBT sponsored R&D project W.e.f 17th June, 2002- 27th March 2005.

Conf./Workshop attended	:	1. 91st session of Indian Science Congress Association, Panjab University, Chandigarh. Jan 3-7, 2004. 2. MoEF, Govt. of India sponsored workshop on 'Conservation and sustainable utilization of medicinal plants of North-East India. NEHU, Shillong. 27th & 28th May, 2004.
Scholarship and Fellowship	:	JRF & SRF from DBT under R&D Project (June 17,2002- 27th March,2005), Dept. of Botany, Nagaland University, Lumami.
Academic Award	:	CSIR-UGC NET (Lectureship)
List of publication	:	EnclosureI

I Temjensangba do hereby declare that the information's given above are true to the best of my knowledge and belief.

Dated: June 01, 2006

Sincerely yours'

(Temjensangba)

Enclosure I

List of Publications

1. Deb CR and Temjensangba. 2003. Non symbiotic seed germination and micropropagtion of *Cleisostoma racemiferum* (Lind.) Garay – A threatened orchid of North-East India (Abstract). *In*: National Seminar on "Impact of increasing human population on natural resources", BHU, Varanasi. October 16- 18. Pp 18.
2. Deb CR, Jamir NS and Temjensangba. 2003. Orchid diversity of Nagaland – A revised status. *J. Orchid Soc. India*, 17 (1&2): 5-15.
3. Deb CR and Temjensangba. 2004. Regeneration of plants through immature seed culture of *Arachnis labrosa* (Lindl. Ex Paxt.) Reichb: A rare and threatened orchid of North- East India (Abstract), *In*: 74th annual session of NASI, University of Rajasthan and Birla Institute of scientific Research, Jaipur, December 2 – 4. Pp. 21-22.
4. Temjensangba and Deb CR. 2005. Regeneration of plantlets from *in vitro* raised leaf explants of *Cleisostoma racemiferum* (Lindl.) Garay. *Indian J. Exp. Biol.*, 43:377- 81.
5. Deb CR and Temjensangba. 2005. *In vitro* regenerative competence of *Cleisostoma racemiferum* (Orchidaceae) aerial roots. *J. Pl. Biochem. Biotech.*, 14: 35- 38.
6. Deb CR and Temjensangba, 2005. Green pod culture and regeneration of *Arachnis Labrosa* (Lindl. Ex. Paxt) Reichb: A rare and threatened orchid. *Curr. Sci.*, 88 (12): 1966-1969.
7. Temjensangba and Deb CR. 2005. Factors regulating Non-Symbiotic seed germination of some rare orchids of Nagaland. *Nagaland University Res. J.*, 3:48-54.
8. Deb CR and Temjensangba. 2006. *In vitro* mass multiplicatuion of a rare and threatened monopodial orchid, *Arachnis labrosa* (Lindl. Ex Paxt.) Reichb. using alternative explant sources (Abstract). *In*: 8th National seminar "Orchid conservation, improvement and commercialization & Satellite symposium on

orchids: Why and how?, The Orchid Society of India, Panjab University, Chandigarh, India, March 18-20, 2006. Pp. 43.

9. Temjensangba and Deb CR. 2006. Effect of different factors on non-symbiotic seed germination, formation of protocorm-like bodies and plantlet morphology of *Cleisostoma racemiferum* (Lindl.) Garay. *Indian J. Biotech.*, 5:223-228.

10. Deb CR and Temjensangba. Direct shoot bud regeneration from foliar Explants of a rare and threatened monopodial orchid, Arachnis *labrosa* (Lindl. ex Paxt.) Reichb. *J. Orchid Soc. India.* (*In Press*).

11. Deb CR and Temjensangba. On the regeneration potential of *Arachnis labrosa* (Lindl. ex Paxt.) root segments: a study *in vitro*. *Phytomorphology.* (*In Press*).

12. Deb CR and Temjensangba. *In vitro* propagation and mass multiplication of *Malaxis khasiana* Soland ex Swartz through seed germination. *Indian J. Exp. Biol.*, (*In press*).

Manuscript under process

1. Deb CR and Temjensangba. A note on some new orchids from Nagaland. ***For*** The *J. Orchid Soc. India.*

2. Temjensangba and Deb C R. Encapsulation of *in vitro* raised propagules and regeneration of plantlets of some rare orchids. *For J. Orchid Soc. India.*

3. Deb CR and Temjensangba. A new and efficient technique of *in vitro* hardening tissue culture regenerated orchids. *For Pl. Cell Tiss. Org. Cult.*

Popular article

1. Temjensangba. 2003. Orchids: How to grow them. North East Herald, October 10, 2003, Pp 5.