Indian Journal of Experimental Biology
Vol. 52, June 2014, pp. 658-663

In vitro regeneration of *Coelogyne nervosa* A.Rich. and *Eria pseudoclavicaulis* Blatt., threatened orchids of Western Ghats, India

B Sahaya Shibu[1], P Servin Wesley[1], Sarmad Moin[2] & B Chitra Devi[*]

Department of Botany, Karpagam University, Coimbatore 641 021, India

Received 18 December 2012; revised 1 April 2013

The seeds of *C. nervosa* and *E. pseudoclavicaulis* were germinated asymbiotically on Knudson C (KC) and Schenk and Hildebrandt basal medium (SH). Growth regulators such as 2,4-Dichlorophenoxyacetic acid (2,4-D) individually and in combinations with benzyladenine (BA) and kinetin were used for callus induction from the protocorm like bodies. *Coelogyne nervosa* showed maximum (90%) callus induction in Knudson C medium supplemented with 2,4-D (2.26 µM) and *Eria pseudoclavicaulis* showed 60% callus induction in Schenk and Hildebrandt medium supplemented with 2,4-D (2.26 µM). Calli developed a route of production of protocorm-like bodies and eventually developed into plantlets on transfer to growth regulator free half strength basal medium. The well rooted plants were hardened successfully in the potting mixture containing coconut husk, charcoal, and brick pieces in the ratio 2:1:1.

Keywords: Callus induction, *Coelogyne nervosa*, *Eria pseudoclavicaulis*, *In vitro* regeneration, Protocorm like bodies

Orchidaceae having 20,000-30,000 species[1] is the largest family in the plant kingdom. Orchids are one of the most fascinating groups of ornamental plants and numerous novel cultivars have been produced by interspecific, as well as intergeneric, hybridization of wild plants with exotic and elegant flowers. Many orchid species in the wild are endangered as a consequence of environmental disruption, succession of natural habitats, and overexploitation for horticultural purposes[2]. However, wild orchids are nowadays steadily decreasing due to over collection of orchid hunters, shifting cultivation, extension of crop cultivation and urban development[3]. Procedures must be developed to conserve their germplasm and the continuous utilization of plant materials for medicine.

Although *in situ* conservation and preservation of natural habitats is the most suitable method for sustaining these threatened species, the development of techniques for their mass propagation and reintroduction to natural habitat is also becoming popular[4]. *In situ* conservation by preservation and enhancement of dwindling populations of endangered orchid species is very difficult because of the relatively low germination rates and slow growth of orchids which requires symbiotic relationships with mycorrhizal fungi in natural habitats[5-7]. Thus, in recent years, the maintenance of living collections has been considered to be an important aspect of conservation[2]. *In vitro* germination can be a powerful tool for preserving rare and native orchid species. By using this method, large number of orchids can be produced while maintaining a more variable gene pool that can be obtained through clonal propagation[4]. Since many years ago, *in vitro* techniques have been found to be useful in the propagation of large number of threatened plants and conservation of their germplasm[8,9]. The application of *in vitro* propagation techniques to rare wild orchid species is undoubtedly a powerful tool for *ex situ* conservation[3].

Coelogyne nervosa A.Rich. an endangered medicinal lithophyte [Synonyms: *Coelogyne corrugata* Wight., *Pleione nervosa* (A.Rich.) Kuntze., *Pleione corrugata* (Wight) Kuntze] is distributed in the Western Ghats of Karnataka, Kerala and Tamil Nadu. *Eria pseudoclavicaulis* Blatt. is classified as vulnerable according to the IUCN status in 1994 and 2000[10]. Only a few plantlets can be regenerate from calli in an undefined medium where the calli are difficult to maintain and eventually fail to survive during subcultures[11]. Effective plant regeneration from callus

*****Correspondent author
Mobile: +919789212939
E-mail: shibubt@gmail.com, chitrasaraswathy@gmail.com
Present address:
[1]Department of Biotechnology, SAFI Institute of Advanced Study, Malappuram 673 633, India
[2]Department of Biotechnology, Thanthai Hans Roever College, Perambalur 621 212, India

of orchids was achieved previously using protocorm-like-bodies PLBs[12-14]. However, there is no report on plant regeneration of *C. nervosa* and *E. pseudoclavicaulis.* This communication is the first one on the plant regeneration in *C. nervosa* and *E. pseudoclavicaulis*.

Materials and Methods

Plant source—The immature capsules of *C. nervosa* and *E. pseudoclavicaulis* obtained from the National Orchidarium & Experimental Garden, Botanical Survey of India (Southern Regional Centre), Yercaud, Tamil Nadu, were germinated on Knudson[15] C (Hi-Media) and Schenk and Hildebrandt[16] basal medium (Hi-Media) with 3% sucrose. The 80 days old protocorms, (before the emergence of first leaves) were used as explants for plant regeneration studies.

Preparation of media—Knudson C medium and Schenk and Hildebrandt medium with growth regulators such 2,4-dichlorophenoxyacetic acid (2,4-D; 2.26, 4.52 and 9.03 µM) either individually or in combinations with benzyladenine (BA; 2.22, 4.44 and 8.88 µM) and kinetin (2.32, 4.64 and 9.29 µM) were prepared. Half strength basal medium without growth regulators was used for the differentiation of callus into complete plantlets. Sucrose (3%) was added and the *p*H was adjusted to 5.5-5.8 with either 1*N* NaOH or 1*N* HCl. Agar (0.8%) (extra pure gelling point 32-35 °C, Hi-Media) was added and melted. Around 10-15 mL of the medium was dispensed into 250 mm × 150 mm culture tubes (Borosil) sealed with aluminium foil before being autoclaved at 1.06 kg pressure for about 20 min at 121 °C. Ten replicates were used for each treatment.

Culture condition—The cultures were maintained at 40 µmol cm^{-2} s^{-1} light intensity provided with cool white fluorescent light at 16/8 h (dark/light) photoperiod and at 25±2 °C with 60% RH. The cultures were monitored regularly and the data were noted at 1 week interval.

Acclimatization—The regenerated shoots with well developed roots after 90 days (from the first subculture) were transferred to the potting mixture containing coconut husk, charcoal, brick pieces in the ratio 2:1:1 (Fig. 1E and 2E).

Results

Callus induction—Eighty days old protocorms were inoculated on the basal medium supplemented with 2,4-D alone or in combination with kinetin and BA. Callus initiated from the protocorms became visible within 45 days for *C. nervosa* and 60 days for *E. pseudoclavicaulis*. Two morphologically distinct types of callus were observed. The first type was pale yellow in colour and compact in texture. The second type of callus was appeared slightly later and developed more rapidly than the first with translucent in colour and friable.

Callus induction was observed directly from seed derived protocorms of *C. nervosa* and *E. pseudoclavicaulis.* KC and SH medium supplemented with various concentrations and combinations of 2,4-D (2.26, 4.52 and 9.03 µM), BA (2.22, 4.44 and 8.88 µM) and kinetin (2.32, 4.64 and 9.29 µM) were found to be efficient in inducing callus from protocorms of both the orchids (Tables 1 and 2). 2,4-D (2.26 µM) supplemented KC basal medium promoted 90% callus induction (Table 1) in *C. nervosa* within 45 days (Fig. 1B). SH medium supplemented with 2,4-D (2.26 µM) shows 60% callus induction (Table 2) in *E. pseudoclavicaulis* in 60 days (Fig. 2B). The callus induction frequency decreased with increasing concentrations of 2,4-D (4.52 and 9.03 µM) in both the orchids.

Addition of kinetin and BA to KC and SH medium supplemented with 2,4-D suppress the effects of 2,4-D in callus induction and the time required for callus induction was longer in *C. nervosa* and *E. pseudoclavicaulis*. Among the cytokinins tested, the kinetin (2.32 µM and 4.64 µM) combined with 2,4-D (2.26 µM) in KC medium shows the better results with an average maximum of 50% callus induction in *C. nervosa* whereas in *E. pseudoclavicaulis*, BA (2.22 µM and 4.44 µM) induced callus at the maximum of 25% callus in the presence of 2,4-D on SH medium but kinetin failed to induce callus in combined with 2,4-D.

Plantlet conversion—The well developed calli were transferred to half strength basal medium (KC for *C. nervosa* and SH for *E. pseudoclavicaulis*) for further differentiation. After 90 days with an intermediate subculture, the calli transformed into protocorms like bodies (PLBs). These PLBs eventually develop into plantlets. The callus induced by 2,4-D at higher concentrations (4.52 and 9.03 µM) and in combination with BA and kinetin exhibited necrosis on transfer to half strength hormone-free KC and SH medium. The protocorm derived calli of *C. nervosa* induced in the lower concentration of 2,4-D (2.26 µM) shows 70% frequency of plantlet conversion with the maximum of 10 plantlets

Table 1—Effect of plant growth regulators on callus induction from 80 days old protocorms of *C. nervosa*.

Hormone (µM)			Number of days taken for callus induction	Frequency of callus induction (%)	Frequency of plantlet conversion in basal medium (%)	Average number of plantlets ±SE mean
2,4,D	BA	Kinetin				
2.26	-	-	45	90	70	10±0.91
4.52	-	-	45	85	65	8±0.81
9.03	-	-	45	50	-	-
2.26	2.22	-	-	-	-	-
4.52	2.22	-	-	-	-	-
9.03	2.22	-	-	-	-	-
2.26	4.44	-	-	-	-	-
4.52	4.44	-	-	-	-	-
9.03	4.44	-	-	-	-	-
2.26	8.88	-	60	10	-	-
4.52	8.88	-	-	-	-	-
9.03	8.88	-	60	10	-	-
2.26	-	2.32	60	40	-	-
4.52	-	2.32	60	10	-	-
9.03	-	2.32	-	-	-	-
2.26	-	4.64	60	50	-	-
4.52	-	4.64	-	-	-	-
9.03	-	4.64	60	10	-	-
2.26	-	9.29	-	-	-	-
4.52	-	9.29	-	-	-	-
9.03	-	9.29	-	-	-	-

Only the significant treatments are computed here
Data represents the mean of ten replicates

Table 2—Effect of plant growth regulators on callus induction from 80 days old protocorms of *E. pseudoclavicaulis.*

Hormone (µM)			Number of days taken for callus induction	Frequency of callus induction (%)	Frequency of plantlet conversion in basal medium (%)	Average number of plantlets ±SE mean
2,4,D	BA	Kinetin				
2.26	-	-	60	60	100	12±0.53
4.52	-	-	60	50	92	9±0.66
9.03	-	-	-	-	-	-
2.26	2.22	-	90	20	62	3±0.56
4.52	2.22	-	-	-	-	-
9.03	2.22	-	90	10	-	-
2.26	4.44	-	-	-	-	-
4.52	4.44	-	90	25	40	2±0.49
9.03	4.44	-	90	10	-	-
2.26	8.88	-	-	-	-	-
4.52	8.88	-	-	-	-	-
9.03	8.88	-	-	-	-	-
2.26	-	2.32	-	-	-	-
4.52	-	2.32	-	-	-	-
9.03	-	2.32	-	-	-	-
2.26	-	4.64	-	-	-	-
4.52	-	4.64	-	-	-	-
9.03	-	4.64	-	-	-	-
2.26		9.29	-	-	-	-
4.52		9.29	-	-	-	-
9.03		9.29	-	-	-	-

Only the significant treatments are computed here
Data represents the mean of ten replicates

(Fig. 1C and 1D) whereas the calli of *E. pseudoclavicaulis* induced in the lower concentration of 2,4-D (2.26 µM) showed 100% conversion frequency with the maximum of 12 plantlets (Fig. 2C and 2D). The well rooted plantlets were acclimatized.

Discussion

Generally callus induction in orchids is rather difficult due to its slow growth and necrotic tendency[3]. In the present study, 80 days old protocorms were cultured on hormone containing medium. They became swollen, followed by initiation of a callus mass visible after 45 days in *C. nervosa* and 60 days in *E. pseudoclavicaulis*. Maximum frequency of callus induction from protocorms were obtained on the basal media supplemented with various concentrations of 2,4-D and kinetin in *C. nervosa* which is similar to the finding of Naing *et al*.,[3] on the orchid *Coelogyne cristata*. Recently, combinations of 2,4-D and Thidiazuron (TDZ) have been reported for the callus induction of ornamental plants including some orchid genera, *Cypripedium formosanum*[17], *Cymbidium*[14], *Vanda coerulea*[18]. However, BA alone or in combination with 2,4-D totally inhibited callus induction in *Paphiopedilum* hybrid[19]. Similarly Ishii *et al*.,[20] reported that the combination of 2,4-D and BA could not effectively induce callus from leaf segment in *Phalaenopsis*. This shows that 2,4-D at certain concentration induced the callus formation in orchids. Similarly

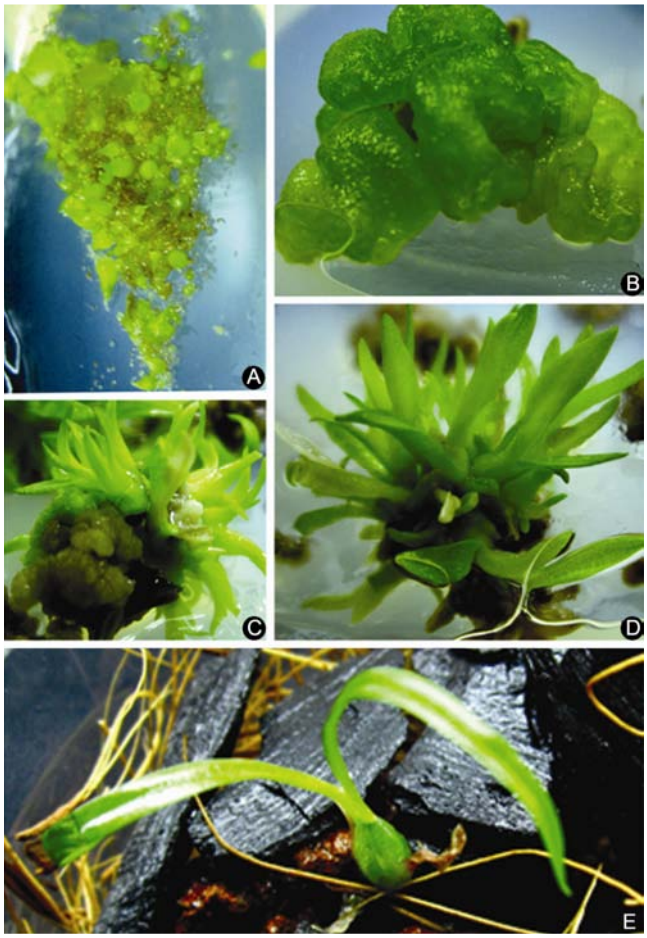

Fig. 1—Regeneration of *C. nervosa* on Knudson C medium. [A, 80 days old protocorms of *Coelogyne nervosa* in KC medium; B, Formation of callus on KC medium supplemented with 2,4-D (2.26 µM); C, Plantlet regeneration on hormone free KC medium; D, Conversion of complete plantlets; E, Plantlet after acclimatization].

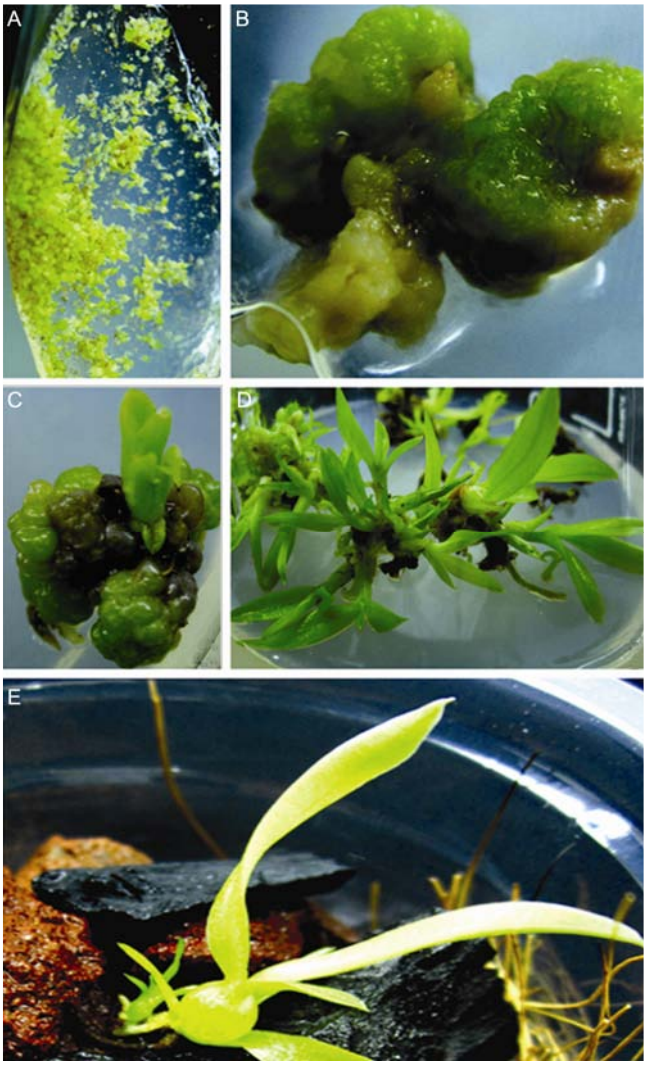

Fig. 2—Regeneration of *E. pseudoclavicaulis* on Schenk and Hildebrandt medium [A, 80 days old protocorms of *Eria pseudoclavicaulis* in SH medium; B, Formation of callus on SH medium supplemented with 2,4-D (2.26 µM); C, Plantlet regeneration on hormone free SH medium; D, Conversion of complete plantlets; E, Plantlet after acclimatization].

the ratios between concentrations of 2,4-D, BA and kinetin were significantly associated with the percentage survival of protocorm and callus formation. Of the combinations tested, the lower concentration of 2,4-D (2.26 µM) was found as optimal concentrations for the best callus induction in both the orchids. Finally it revealed that addition of exogenous hormones to the medium is quite important for callus induction. In the present study lower concentration of 2,4-D (2.26 µM) successfully induced callus from protocorms in both the orchids within a short period of time. However, the presence of BA and kinetin inhibited callus induction from protocorms.

When PLB were transferred into different kinds of basal media, formation of shoots appeared on all media but conversion frequencies of PLBs to shoot and average number of shoots were ultimately low[21]. In the present study, transfer of callus obtained from lower concentration of 2,4-D (2.26 µM) to hormone-free medium stimulated more PLB development and eventually allowed to produce plantlets in both the orchids. Similarly Chen *et al*.,[22] reported that once transferred the calli to the basal medium devoid of any hormonal factors, the PLBs and PLB-derived buds germinated and developed into normal plantlets which were transferred readily with a 100% survival rate whereas effect of each basal media (VW, HP, MS and half MS) on plant regeneration from PLB has been successfully reported[23-27]. The regenerated plants of *C. nervosa* and *E. pseudoclavicaulis* showed maximum survival of 60% on the potting mixture containing coconut husk, charcoal and brick pieces (2:1:1). The above protocol can be used for large scale production of these threatened orchid species.

Acknowledgement

Thanks are due to Prof. R. Gopalan, (former Scientist, BSI), Department of Botany, Karpagam University, Dr. G.V.S Murthy, Additional Director, Botanical Survey of India, Southern Regional Centre, Coimbatore and Dr. M. U. Sharief, Head, National Orchidarium and Experimental Garden, Botanical Survey of India, Yercaud, Tamil Nadu for support.

References

1 Koopowitz H, *Orchids and their conservation* (Batsford Press, London and Timber Press, Portland Oregon) 2001, 176.

2 Godo T, Komori M, Nakaoki E, Yukawa T & Miyoshi K, Germination of mature seeds of *Calanthe tricarinata* Lindl., an endangered terrestrial orchid, by asymbiotic culture *in vitro*, *In Vitro Cell Dev Biol Plant*, 46 (2010) 323.

3 Naing, A H, Chung J D & Lim K B, Plant regeneration through indirect somatic embryogenesis in *Coelogyne cristata* orchid, *Am J Plant Sci*, 2 (2011) 262.

4 Park S Y, Hosakatte N & Paek K Y, *In vitro* seed germination of *Calanthe sieboldii*, an endangered orchid species, *J Plant Biol*, 43(3) (2000) 158.

5 Bernard N, La germination des orchidées, *Revue Generale de Botanique*, 16 (1903) 458.

6 Warcup J H, Symbiotic germination of some Australian terrestrial orchids, *New Phytol*, 72 (1973) 387.

7 Rasmussen H, *Terrestrial orchids from seed to mycotrophic plant* (Cambridge University Press, Cambridge), 1995.

8 AmoMarco J B & Lledo MD. *In vitro* propagation of *Salix tarraconensis* Pau ex Font Quer, an endemic and threatened plant, *In Vitro Cell Dev Biol Plant*, 32 (1996) 42.

9 Dhar U, Upreti J & Bhatt I D, Micropropagation of *Pittosporum napaulensis* (DC.) Rehder & Wilson—a rare, endemic Himalayan medicinal tree, *Plant Cell Tissue Organ Cult*, 63 (2000) 231.

10 Conservation Assessment and Management Plan Workshop Report for Endemic Orchids of the Western Ghats, Wildlife Information Liaison Development Society Zoo Outreach Organisation, Coimbatore, 15-19 May, 2001.

11 Stewart J & Button J, Tissue culture studies in *Paphiopedilum*, *Am Orchid Soc Bull*, 44 (1975) 591.

12 Begum A, Tamaki M & Kako S, Formation of protocorm-like-bodies (PLB) and shoot development through *in vitro* culture of outer tissue of *Cymbidium* PLB, *J Japanese Soc Horticult Sci*, 63(3) (1994) 663.

13 Roy J & Banerjee N, Induction of callus and plant regeneration from shoot tip explants of *Dendrobium fimbriatum* Lindl. var. Oculatum Hk.f, *Sci Hort*, 97(3) (2003) 333.

14 Huana L V T, Takamura T & Tanaka M, Callus formation and plant regeneration from callus through somatic embryo structures in *Cymbidium* orchid, *Plant Sci*,166 (2004) 1443.

15 Knudson L, A nutrient for the germination of orchid seeds, *Am Orchid Soc Bull*, 15 (1946) 214.

16 Schenk R U & Hildebrandt A C, Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures, *Canadian J Bot*, 50 (1972) 199.

17 Lee Y & Lee N, Plant regeneration from protocorms derived callus of *Cypripedium Formosanmun*, *In Vitro Cell Dev Biol Plant*, 39(5) (2003) 475.

18 Lang N T & Hang N T, Using biotechnological approaches for *Vanda* orchid improvement, *Omonrice*, 14 (2006) 140.

19 Lin Y H, Chang C & Chang W C, Plant regeneration from callus culture of a *Paphiopedilum* hybrid, *Plant Cell Tissue Organ Cult*, 62 (2000) 21.

20 Ishii Y, Takamura T, Goi M & Tanaka M, Callus induction and somatic embryogenesis of *Phalaenopsis*, *Plant Cell Rep*, 17 (1998) 446.

21 Naing A H, Chung J D, Park I S & Lim K B, Efficient plant regeneration of the endangered medicinal orchid, *Coelogyne cristata* using protocorm-like bodies, *Acta Physiologiae Plantarum*, 33 (2011 B) 659.

22 Chen Y C, Chang C & Chang W C, A Reliable protocol for plant regeneration from callus culture of *Phalaenopsis*, *In Vitro Cell Dev Biol Plant*, 36 (2000) 420.

23 Lakshmanan P, Lob C S & Gob C J, An *in vitro* method for rapid regeneration of a monopodial orchid hybrid *Aranda deborah* using thin section culture, *Plant Cell Rep*, 14 (1995) 510.

24 Park S Y, Murthy H N & Peak K Y, Mass multiplication of protocorm-like bodies using bioreactor system and subsequent plant regeneration in *Phalaenopsis*, *Plant Cell Tissue Organ Cult*, 63 (2000 B) 67.

25 Shimura H & Koda Y, Micropropagation of *Cypripedium macranthos* var. rebunense through protocorm- like bodies derived from mature seeds, *Plant Cell Tissue Organ Cult*, 78 (2004) 273.

26 Sheelavanthmath S S, Murthy H N, Hema B P, Hahn E J & Paek K Y, High frequency of protocorm like bodies (PLBs) induction and plant regeneration from protocorm and leaf sections of *Aerides crispum*, *Sci Hort*, 106 (2005) 395.

27 Yi-xun Y, Ling L, Juan-xu L & Jing W, Plant regeneration by callus-mediated protocorm-like body induction of *Anthurium andraeanum* hort., *Agricultural Sciences in China*, 8(5) (2009) 572.