Indian Journal of Biotechnology
Vol 5, April 2006, pp 223-228

Effect of different factors on non-symbiotic seed germination, formation of protocorm-like bodies and plantlet morphology of *Cleisostoma racemiferum* (Lindl.) Garay

Temjensangba and Chitta Ranjan Deb*

Department of Botany, Nagaland University, Headquarters: Lumami, Mokokchung 798 601 (University Branch Office), India

Received 31 August 2004; revised 3 May 2005; accepted 2 July 2005

Cleisostoma racemiferum, an epiphytic orchid of primary forest under threat in their natural habitat, was studied. Immature seeds of different developmental stages [(8-20 week after pollination (WAP)] were cultured on Knudson 'C', Mitra *et al* and MS media supplemented with sucrose (0-3%, w/v), coconut water (CW, 0-20%, v/v) and NAA (0-30 μ*M*) + BA (0-8 μ*M*) singly or in combination. After 7 week of culture, first sign of germination was recorded as nodular swelling of seeds. Amongst the three different basal media tested, better germination was supported by MS medium, followed by Mitra *et al* and Knudson 'C' media containing sucrose (3%) and NAA (10.0 μ*M*) + BA (8.0 μ*M*). Of various developmental stages of the seed, better germination was obtained from green pods of 16 week after pollination. Younger seeds did not show any sign of germination, while mature seeds exhibited delayed and deformed germination. Though incorporation of CW in the medium did not show much influence on seed germination, but 15% (v/v) CW in the initiation medium enhanced the early differentiation of protocorm-like bodies (PLBs) into plantlets. Within 14-16 week of culture on germination medium, the PLBs started releasing the first set of leaflets. The advanced stage PLBs were converted into rooted plantlets on MS medium containing IAA (10.0 μ*M*) + Kn (9.0 μ*M*). Although the medium containing NAA (10.0 μ*M*) + BA (8.0 μ*M*) resulted in multiple shoot buds, but the leaves were thin and etiolated. Further, the medium enriched with NAA (10.0 μ*M*) + Kn (9.0 μ*M*) resulted in stunted growth of plantlets, while presence of IAA (10.0 μ*M*) + BA (8.0 μ*M*) resulted in plantlets with poor roots.

Keywords: *Cleisostoma racemiferum*, green pod culture, plant growth regulators, seed age
IPC Code: Int. Cl.[8] A01H4/00

Introduction

Orchid seeds are microscopic and non-endospermous with undifferentiated embryos. They are produced in large numbers and their germination in nature depends upon a suitable association with mycorrhizal fungus, which provides an essential physico-chemical stimulus for initiating germination[1]. The orchids are propagated through vegetative means as well as seeds. However, the rate of vegetative propagation is very slow and the seed germination in nature is very poor, i.e. 0.2-0.3%[2]. *In vitro* germination of seeds is an important part in the orchid multiplication and conservation programmes since the 'dust seeds' are tiny and contain few food reserves. Knudson[3] demonstrated the possibility of by-passing the fungal requirement of orchid seeds during *in vitro* germination and since then non-symbiotic seed germination has been accepted as an important tool for propagating orchids[4]. The non-symbiotic seed germination potential of fertilized ovules has been positively tested in several commercially viable and/or threatened orchid taxa[5,6]. However, non-symbiotic seed germination of orchids is greatly influenced by several factors, like seed age, different nutrient media with adjuvants and plant growth regulators[3,5-8]. Moreover, none of these basal nutrient media with different adjuvants fulfills the requirements of the entire orchidaceous group.

Cleisostoma racemiferum (Lindl.) Garay is an epiphytic orchid and native of primary forests. The species is under threat due to removal of their natural habitat for 'Slash and Burn' cultivation, unplanned developmental activities, etc. In this communication, we describe the effect of different factors like green pod age, culture media and plant growth regulators on non-symbiotic seed germination, formation of protocorm-like bodies (PLBs) and plantlet morphology of *C. racemiferum*.

*Author for correspondence:
Tel: 91-369-2268221; Fax: 91-369-2268204
E-mail: debchitta@rediffmail.com

Materials and Methods

In Vitro Seed Germination

C. racemiferum green pods/capsules were harvested at 2 week interval starting from 8 week after pollination (WAP) up to 20 WAP. The pods were surface sterilized with 0.5% (w/v) mercuric chloride for 5 min and rinsed 3-4 times with sterilized distilled water. Thereafter, the pods were flamed on gas burner and seeds were scooped out by making longitudinal slit and inoculated on different media, viz. Knudson 'C'[9], MS[10] and Mitra *et al*[11]. These media were fortified with coconut water (CW; 0-20%, v/v) and sucrose (0-3%, w/v) and then supplemented with different plant growth regulators, like NAA (0, 10.0, 20.0 and 30.0 μM) and BA (0, 8.0, 16.0 and 24.0 μM) singly or in combination.

PLBs and Multiple Shoot Formation

The PLBs developed from germinating seeds were transferred on optimum culture conditions and maintained for 2 more passages at 4 week interval. The advanced stage PLBs (with first set of leaflets) were transferred on both MS and Mitra *et al* media containing sucrose (3%, w/v), CW (10%, v/v) and the following plant growth regulator for plantlet regeneration: (i) IAA (0-20.0 μM) and Kn (0-18.0 μM), (ii) IAA (0-20.0 μM) and BA (0-16.0 μM), (iii) NAA (0-20.0 μM) and BA (0-16.0 μM), and (iv) NAA (0-20.0 μM) and Kn (0-18.0 μM), single or in combination.

Before autoclaving at 121°C and 1.05 kg cm^{-2} for 20 min, 0.8% agar was incorporated as gelling agent and *p*H of the media was adjusted to 5.6 using 0.1 *N* NaOH and HCl. Cultures were maintained at 25±2°C under cool white fluorescent light at 40 µmole m^{-2} s^{-1} light intensity and 12/12 h photo cycle. For seed germination, 10 culture vials were maintained for each treatment. The cultures were sub cultured at 4 week intervals both for seed germination and plantlet regeneration, and data were recorded at 1 week interval. Cultures on regeneration media were monitored for plantlet formation and morphology. All the experiments were repeated thrice and the experimental design was completely randomized.

Results and Discussions

In Vitro Seed Germination

After 7 week of inoculation, the first sign of germination was observed as yellowish nodular swelling of seeds (Fig. 1a), which subsequently converted into PLBs. Amongst three different basal nutrient media studied in the present investigation on *C. racemiferum*, better germination was supported by MS medium followed by Mitra *et al* and Knudson 'C'. The developmental stage of green pods was found to be the most crucial factor for both nodular swelling of seeds and PLBs formation from the cultured seeds. Seeds from the pods of 16 WAP initiated germination after 52, 62 and 68 d after initiation of culture on MS, Mitra *et al*, and Knudson 'C' media, respectively; while for PLBs formation, another additional 40, 53 and 56 d were required on the respective media (Table 1). Green pods of age >16 WAP required longer duration and, in some cases, did not show any sign of germination. The seed sown at relatively early stage of development (<14 WAP) did not germinate at all; though, in some cases, showed nodular swelling, but did not form PLBs and degenerated subsequently. It may be due to the fact that embryos have not been reached proper stage of

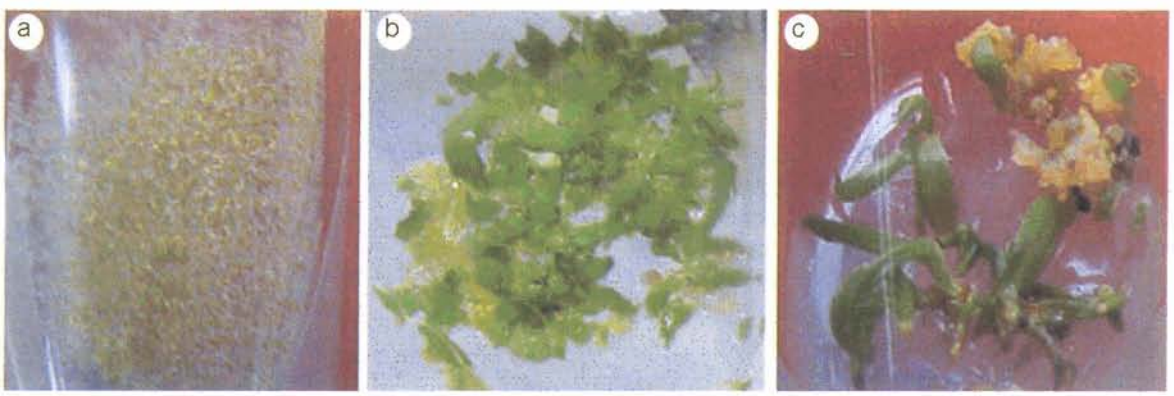

Fig. 1—Different stages of seed germination and plantlets formation in *C. racemrferum*: a. Nodular swelling of seeds in culture, b. First set of leaflets are formed in PLBs, & c. Rooted plantlets and repetitive PLBs are formed in culture

maturation as reported in *Vanda coerulea*[12], *Cymbidium iridioides* and *Cy. lowianum*[8]. The importance of time interval between pollination and fertilization has also been stressed[13]. The relative time taken by ovules after pollination for successful germination seems to vary with species[14]. Other workers also reported the effect of green pod age on *in vitro* germination of orchid seeds[8,15-17]. In *Dactylorhiza hatagirea*[17], seeds of 16 WAP exhibited better germination over younger and older seeds but in case of *Cy. macrorhizon* better response was recorded from seeds of 12 WAP and seeds from mature pods (20 WAP) did not germinate[15]. While Jamir *et al*[8], reported better germination from 120 days old pods in *Cy. iridioides*.

Previous studies revealed that no single nutrient medium is universally suitable for asymbiotic seed germination of all or most orchid taxa. For example, Mitra *et al* medium was found suitable over other nutrient media for *Cy. macrorhizon*[15] and *Goodyera biflora*[16]; Knudson 'C' medium for *Cy. elegans*, *Coelogyne punctulata*[5] and *D. hatagirea*[17]; VW medium[6] for *V. coerulea*; Nitsch medium[8] for *Cy. iridioides*; and Knudson 'C', VW and MS media for *Aerides rosea*[18]. In the present study, however, MS medium was found most suitable over Mitra *et al* and Knudson 'C' media for asymbiotic seed germination of *C. racemiferum* (Tables 1 & 2).

Besides seed age and basal nutrient media, other factors, like organic carbon concentration, CW and plant growth regulators, have also shown marked effect on asymbiotic seed germination and subsequent differentiation in *C. racemiferum*. The sucrose concentration (3%) supported better germination (~90%) on MS[10] medium; while on Mitra *et a1* medium, 2% sucrose concentration found to be superior over other concentration and exhibited 80% germination (Table 2). At other concentrations,

Table 1—Effect of green pod age and basal nutrient media* on asymbiotic seed germination and PLBs formation of *C. racemiferum*

Basal nutrient media	Age of capsule (WAP)	Time taken to respond (d)	
		Nodulation	PLBs formation
Knudson 'C'[9]	8	-	-
	10	-	-
	12	90	-
	14	84	60 (degenerated)
	16	68	56
	18	75	70
	20	70	-
Mitra *et al*[11]	8	-	-
	10	-	-
	12	80	degenerated
	14	63	42
	16	50	53
	18	58	60
	20	60	degenerated
MS[10]	8	-	-
	10	-	-
	12	120	68
	14	70	47
	16	52	40
	18	55	40
	20	70	60

* Media containing sucrose (3%, w/v), CW (15%, v/v) and NAA (10.0 μ*M*) + BA (8.0 μ*M*) in combination.

Table 2—Effect of sucrose concentration in different media* on asymbiotic seed germination of *C. racemiferum*

Basal media	Sucrose conc. (%)	Germination rate (%) (±SE)**	Type of response
Knudson 'C'[9]	0	0	No germination
	1	40 (± 3.0)	Germinated seeds degenerated subsequently
	2	40 (± 2.0)	Very few germinated seeds converted into PLBS
	3	60 (± 2.0)	Germinated seeds formed PLBs and browning of PLBs observed in some cases
Mitra *et al*[11]	0	0	No germination
	1	45 (± 1.0)	Very few germinated seeds converted into PLBS
	2	80 (± 2.0)	Germinated seeds formed healthy PLBs
	3	50 (±2.0)	Conversion of PLBs was comparatively poor
MS[10]	0	0	No germination
	1	50 (± 2.0)	Germinated seeds mostly degenerated and very few converted into PLBs
	2	60 (± 3.0)	PLBs were not healthy
	3	90 (± 2.0)	Germinated seeds formed healthy PLBs

* Media containing CW (15%, v/v) and NAA (10.0 μ*M*) + BA (8.0 μ*M*) in combination
**Standard error

germination was poor. Sharma and Tandon[5] reported the effect of different organic carbon sources on *in vitro* seed germination and found 2-3% sucrose, D-fructose and D-glucose suitable for seed germination of *Cy. elegans* and *Co. punctulata*[5].

Incorporation of CW in the medium did not show much influence on seed germination in *C. racemiferum*. It, however, enhanced the early differentiation of PLBs into plantlets when incorporated at 15% (v/v) concentration in the germination medium (data not presented). Devi *et al*[6] and Vij *et al*[19] also reported the promotory effect of CW on seed germination. Amongst the different concentrations and combinations of plant growth regulators studied for asymbiotic seed germination of *C. racemiferum*, a combined treatment of NAA (10.0 μ*M*) + BA (8.0 μ*M*) exhibited better PLBs formation, followed by single treatment of both NAA (10.0 μ*M*) and BA (8.0 μ*M*) (data not presented). Devi *et al*[6] reported the promontory effect of NAA (0.1-0.5 mg L^{-1}) + Kn (1-2 mg L^{-1}) in *V. coerulea*, while Sinha *et al*[18] reported inhibitory effect of NAA, BA and Kn in the medium. In the present study, the first set of leaflets started appearing in 14-16 week of cultures on germination medium (Fig. 1b).

PLBs and Multiple Shoot Formation

On regeneration media, the advanced stage PLBs started converting into young rooted plantlets and repetitive PLBs within 3-4 weeks (Fig. 1c). Table 3 shows the effect of plant growth regulators on PLBs formation and regeneration of plantlets. Amongst the two media tested, it was noted that MS medium supported the optimum growth and differentiation into rooted plantlets. While, out of different plant growth regulator combinations, IAA (10.0 μ*M*) + Kn (9.0 μ*M*) was found to be optimum, where well rooted plantlets developed on both the media. The number of multiple shoot formation was significantly higher on medium containing NAA (10.0 μ*M*) + BA (8.0 μ*M*) but leaves were thin and etiolated (Fig. 2a). The medium containing NAA (10.0 μ*M*) + Kn (9.0 μ*M*) resulted in stunted growth of plantlets; very few shoot buds and callusing were observed at the base, though roots were very long (Fig. 2b). In case of IAA (10.0 μ*M*) + BA (8.0 μ*M*) combination, multiple shoot with stunted growth and poor roots (Fig. 2c) were obtained, while IAA (10.0 μ*M*) + Kn (9.0 μ*M*) resulted in well rooted plantlets (Fig. 2d) with moderate numbers of developed multiple shoots.

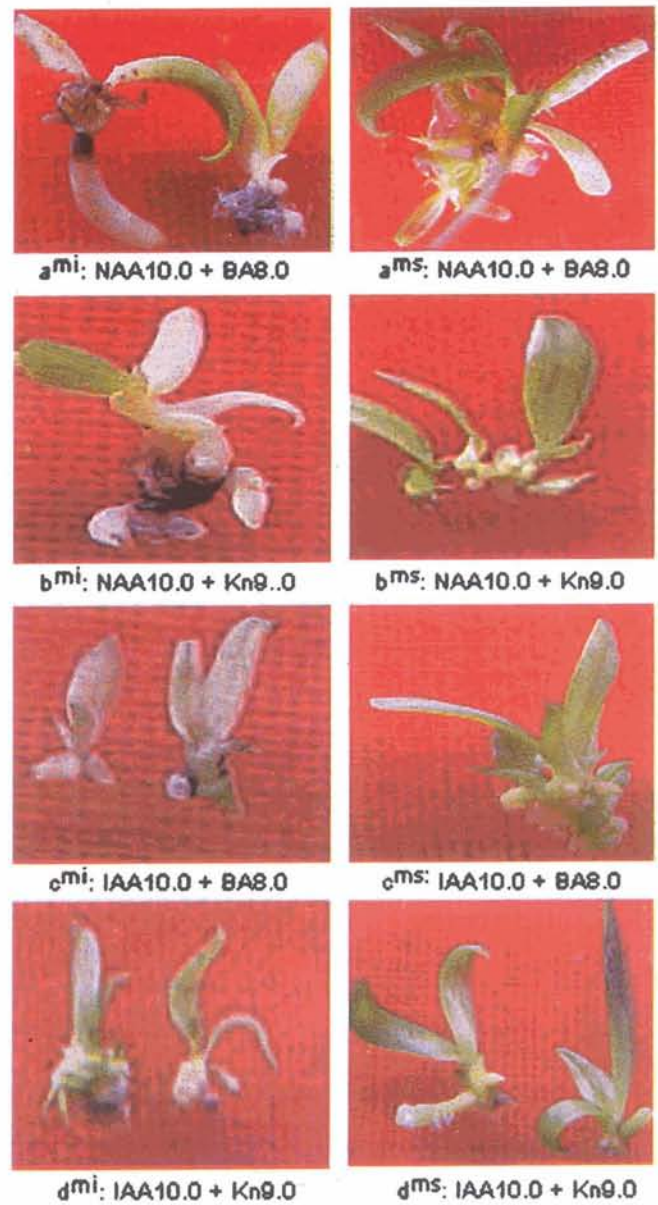

Fig. 2—Effect of culture media and plant growth regulators* on multiple shoot formation and plantlet morphology of *C. racemiferum* (figures are from the optimum growth regulator combinations only). * **mi**: on Mitra *et al*[11] medium and **ms**: on MS[10] medium (figures in subscript indicate the concentrations of plant growth regulators in μ*M*)

The protocol described here reveals the different factors controlling immature seed germination of *C. racemiferum* and could also be used for clonal mass multiplication of this threatened species facing rapid denudation. However, further work on *in vitro* mass multiplication and short- to medium-term conservation of the species is in progress.

Table 3—Effect of different plant growth regulators* on PLBs development and plantlet regeneration of *C. racemiferum*

Conc. of growth regulators (μM)	Time taken to respond (d)	Type of response
$NAA_{10.0}$	40	Plantlets were short, stout with broad leaves
$NAA_{20.0}$	44	Plantlets slightly elongated and leaf long and linear
$NAA_{30.0}$	42	As above
$IAA_{10.0}$	36	Growth retarded, stunted and etiolated
$IAA_{20.0}$	40	Growth healthy, leaf broad and long but no multiple shoot
$IAA_{30.0}$	38	Slightly stunted growth and leaf linear and elongated
$BA_{8.0}$	43	Growth healthy with few PLBs from the explants
$BA_{16.0}$	43	As above
$BA_{24.0}$	42	Moderate plant growth but no multiple shoot buds
$Kn_{9.0}$	41	Healthy growth with broad leaf
$Kn_{18.0}$	40	As above
$Kn_{27.0}$	40	Plant growth retarded and slightly etiolated
$NAA_{10.0} + BA_{8.0}$	**38**	**Multiple shoots but leaves are thin and etiolated**
$NAA_{20.0} + BA_{16.0}$	40	Plant growth normal, leaf thin and short
$NAA_{30.0} + BA_{24.0}$	40	Plantlets thin, elongated and etiolated
$IAA_{10.0} + BA_{8.0}$	**42**	**Multiple plantlets, leaf long but poor rooting**
$IAA_{20.0} + BA_{16.0}$	40	Plantlets growth slightly retarded with poor or no root
$IAA_{30.0} + BA_{24.0}$	45	As above
$IAA_{10.0} + Kn_{9.0}$	**43**	**Well rooted multiple plantlets with repetitive PLBs**
$IAA_{20.0} + Kn_{18.0}$	42	Growth slightly stunted, leaf small and linear
$IAA_{30.0} + Kn_{27.0}$	40	Plant growth stunted and etiolated dies after sometime
$NAA_{10.0} + Kn_{9.0}$	**40**	**Well differentiated rooted plantlets with stunted growth**
$NAA_{20.0} + Kn_{18.0}$	47	Well developed plantlets but growth slightly retarded and stunted
$NAA_{30.0} + Kn_{27.0}$	45	Well differentiated plantlets but retarded and stunted growth, and etiolated

*In MS medium containing sucrose (3%, w/v) and CW (15%,v/v)

Acknowledgement

Authors are thankful to the Department of Biotechnology, Ministry of Science and Technology, Government of India, New Delhi for financial assistance through a research grant to Dr Chitta Ranjan Deb.

References

1 Harley JL, *The biology of mycorrhiza* (Leonard Hill, London), 1959.

2 Vij S P, Orchids and tissue culture: Current status, in *Role of plant tissue culture in biodiversity conservation and economic development*, edited by S K Nandi, L M S Palni & A Kumar (Gyanodaya Prakashan, Nainital, India) 2002, 491-502.

3 Knudson L, Non-symbiotic germination of orchid seeds, *Bot Gaz*, 73 (1922) 1-25.

4 Arditti J, Clements M A, Fast G, Hadley G, Nishimura G *et al*, Orchid seed germination and seedling culture—A manual, in *Orchid Biology – Reviews and perspectives*, vol II, edited by J Arditti (Cornell University Press, Ithaca, New York) 1982, 243-370.

5 Sharma S K & Tandon P, Asymbiotic germination and seedling growth of *Cymbidium elegans* Lindl. and *Cioelogyne punctulata* Lindl. as influenced by different carbon sources, *J Orchid Soc India*, 12 (1990) 83-87.

6 Devi C G, Damayanti M & Sharma G J, Aseptic embryo culture of *Vanda coerulea* Grief, *J Orchid Soc India*, 12 (1998) 83-87.

7 Pathak P, Mahant K C & Gupta A, *In vitro* propagation as an aid to conservation and commercialization of Indian orchids: Seed culture, in *Orchid: Science and commerce*, edited by P Pathak, R N Sehgal, N Shekhar, M Sharma & A Sood (Bishen Singh Mahendra Pal Singh, Dehradun, India) 2001, 319-362.

8 Jamir C, Devi J & Deka P C, *In vitro* propagation of *Cymbidium iridioides* and *C. lowianum*, *J Orchid Soc India*, 16 (2002) 83-89.

9 Knudson L, A new nutrient solution for germination of orchid seeds, *Am Orchid Soc Bull*, 15 (1946) 214-217.

10 Murashige T & Skoog F, A revised medium for rapid growth and bioassay with tobacco tissue cultures, *Physiol Plant*, 15 (1962) 473-497.

11 Mitra G C, Prasad R N & Roy Chowdhury A, Inorganic salt and differentiation of protocorms in seed callus of an orchid and correlated changes in its free amino acid content, *Indian J Exp Biol*, 14 (1976) 350-351.

12 Sharma J, Studies on *Vanda*: Effect of age capsules (pods) on *in vitro* seed germination, *J Orchid Soc India*, 12 (1998) 43-45.
13 Valmayor H L & Sagawa Y, Ovule culture in some orchids, *Am Orchid Soc Bull*, 36 (1967) 766-769.
14 Vij S P, Genetic resources of orchids, in *Advances in horticulture (Ornamental Plants I)*, vol 12, edited by K L Chadha & S K Bhattacharjee (Malhotra Publishing House, New Delhi, India) 1995, 153-181.
15 Vij S P & Pathak P, Asymbiotic germination of the saprophytic orchid, *Cymbidium macrorhizon*: A study *in vitro*, *J Orchid Soc India*, 2 (1988) 25-32.
16 Pathak P, Vij S P & Mahant K C, Ovule culture in *Goodyera biflora* (Lindl.) HK. F.: A study *in vitro*, *J Orchid Soc India*, 6 (1992) 49-53.
17 Vij S P, Pathak P & Mahant K C, Green pod culture of a therapeutically important species *Dactylorhiza hatagirea* (D. Don) Soo, *J Orchid Soc India*, 9 (1995) 7-12.
18 Sinha S K, Singh L S & Hegde S N, *In vitro* multiplication of *Aerides rosea* Loddiges ex. Paxt. through asymbiotic seed germination, *Arunachal For News*, 16 (1998) 38-44.
19 Vij S P, Kher A & Pathak P, Regeneration competence of *Bulbophyllum careyanum* (Hook) Spreng. pseudobulb segments, *J Orchid Soc India*, 14 (2000) 47-55.