Journal of Biological Research **5**: 79 – 84, 2006
J. Biol. Res. is available online at http://www.jbr.gr

In vitro seed germination and plantlet regeneration of *Coelogyne mossiae* Rolfe

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Received: 28 August 2005 Accepted after revision: 18 April 2006

The effects of different nutrient solutions, organic supplements and plant growth regulators on *in vitro* seed germination and plantlet development of *Coelogyne mossiae* were studied. Seed germination was higher in C Knudson (1946) medium (95%) and lower in half-strength Murashige and Skoog (1962) medium (65%). After 5 months, when plantlets had produced 2-3 leaves, they were transferred to hormone-supplemented half-strength Murashige and Skoog medium and Knudson C medium. Half-strength Murashige and Skoog medium supplemented with 0.2 mgl^{-1} 6-benzylamino purine proved better for multiplication of protocorm and healthy shoot induction. After 12 months, to derive seedlings with a height of 7 cm, plantlets were transferred to auxin-supplemented half-strength Murashige and Skoog medium and basic half-strength Murashige and Skoog medium for root induction. High frequency of roots was observed in the medium fortified with 0.1 mgl^{-1} auxin. Then, rooted plantlets were transferred to a decomposed paper disposal cup containing garden soil, vermicompost, and brickgravals (1:1:1) used for successful hardening.

Key words: *in vitro* seed germination, protocorm-like bodies, orchid, *Coelogyne mossiae*.

Abbreviations: HMS, half-strength Murashige and Skoog medium; KnC, Knudson C medium; IBA, auxin; BAP, 6-benzylamino purine; KIN, kinetin.

INTRODUCTION

Generally, orchid seeds are characterized by lacking of storage tissues required for seed germination and seedling development. In nature, association with a specific fungal partner is a pre-requisite for orchid seed germination (Mitra, 1986). Although the exact role of mycorrhizal fungi is still questionable, it is generally considered that the associated endophyte provides to the germinating embryos and protocorms soluble carbohydrates, essential minerals, water, enzyme precursors and hormones (Rasmussen, 1992; De Pauw *et al*., 1995). This bizarre requirement for germination has always hindered rapid propagation of orchids, a plant group with great commercial importance. *In vitro* culture of orchid seeds has shown that different species require different and often specific medium composition for optimum germination and growth. The present study examines the effects of two different media and plant growth regulators on seed germination and protocorm multiplication of *Coelogyne mossiae*.

Coelogyne mossiae is an epiphyte, occurring often on trunks of trees, sometimes lithophytic on mossy rocks and is found in Kerala and Tamil Nadu in India. The species is commercially important for its fragrance of flowers. The flowers have a creamy white colour and are organized into a nodding inflorescence. *Coelogyne mossiae* is regarded as an endangered species due to its presence in declining habitats and to uncontrolled collection. It is necessary therefore, to develop an efficient method for rapid *in vitro* propagation.

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MATERIALS AND METHODS

Plant material and inoculation

Mature and undehiscent fruits (capsules) of *C. mossiae* Rolfe were collected from the Pambar shola in Kodaikanal Western ghats of Tamilnadu, India. For surface sterilization, the freshly collected undehiscent fruits were first rinsed in 90% ethanol for 50 sec, then in 0.1% (w/v) mercuric chloride for 10 min and finally were washed with sterile distilled water. The capsules were longitudinally opened with a sterilized knife and the seeds were aseptically transferred into 150 ml conical flasks containing 50 ml of culture medium.

Culture media

The germination media were based on inorganic salts of KnC and HMS. In all media, 2% (w/v) sucrose served as carbon source. In addition, plant growth regulators were supplemented in various combinations (Table 1). The pH was adjusted to 5.3 in the KnC and 5.7 in the HMS. The media were solidified with 0.9 (KnC) and 0.8% (HMS) agar and were autoclaved at 121˚C for 15 min. The cultures were maintained at 25 ± 2˚C under 12 h photoperiod provided by Philips white fluorescent lamps of 3.000 lux intensity. The chemicals used were of analytical grade (Himedia Laboratories, Mumbai, India).

TABLE 1. Effects of BAP and KIN in HMS and KnC media on protocorm multiplication and shootlet propagation of *Coelogyne mossiae*

Hormone	**Concentration (mgl^{-1})**	**% of response**	**% of protocorm multiplication**	**Shoot length (cm ± SE)**
		HMS medium		
Basic medium	–	60	–	5.16 ± 0.03^{ef}
BAP	0.1	80	90	7.28 ± 0.06^{ab}
	0.2	90	90	7.54 ± 0.07^{a}
	0.3	70	70	7.21 ± 0.07^{bc}
	0.4	60	50	6.44 ± 0.06^{d}
	0.5	40	30	5.18 ± 0.05^{e}
KIN	0.1	60	40	3.61 ± 0.05^{g}
	0.2	80	30	3.35 ± 0.03^{gh}
	0.3	50	20	2.75 ± 0.06^{i}
	0.4	40	20	2.16 ± 0.04^{j}
	0.5	30	–	1.95 ± 0.03^{jk}
		KnC medium		
Basic medium	–	70	60	4.89 ± 0.03^{e}
BAP	0.1	80	90	7.19 ± 0.01^{b}
	0.2	90	90	7.30 ± 0.06^{a}
	0.3	80	80	6.99 ± 0.04^{c}
	0.4	70	80	6.21 ± 0.06^{cd}
	0.5	60	70	4.82 ± 0.07^{ef}
KIN	0.1	50	60	3.37 ± 0.04^{g}
	0.2	40	60	3.20 ± 0.04^{h}
	0.3	30	50	2.56 ± 0.06^{i}
	0.4	30	20	1.81 ± 0.04^{j}
	0.5	20	–	1.60 ± 0.04^{k}

Protocorm growth and development

The cultures were examined at 10-day intervals for initiation of germination. Emergence of the embryo from the seed testa was considered as germination. Growth of protocorms was expressed in terms of protocorm length. The results of different morphogenetic responses were obtained after 3 months of culture. After 5 months, protocorms were transferred to hormone-supplemented KnC and HMS media (Table1). Hence, the whole treatment took 12 months.

In vitro rooting acclimatization

In vitro germinated plantlets were cultured on the appropriate medium for *in vitro* root induction. An HMS medium supplemented with IBA (0.1-0.5 mg l^{-1}) and a hormone-free medium were used for root induction. After 4 weeks of culture on the rooting medium, the plantlets were removed from the culture tubes and were washed thoroughly with sterile double distilled water to remove any traces of the medium. They were then treated with 0.1% (w/v) Bavistin (fungicide) and again washed with sterilized double distilled water. The rooted plantlets were planted in decomposed paper disposable cups filled with a mixture of sterile garden soil, vermicompost, and brick-gravals (1:1:1). The plants were covered with plastic bags for 30 days and maintained under humidity (67%). Plants became acclimatized to a reduced relative humidity by gradually opening the plastic cover and after 40 days they were completely uncovered and hardened to greenhouse conditions. The percentage of rooting, number of roots per plantlets and length of roots were recorded periodically.

Statistical analysis

Protocorm multiplication and shoot-root induction were achieved using the HMS medium and the KnC medium supplemented with cytokinin (BAP and KIN) and IBA. The multiplication of the protocorm and the shoot elongation were assessed using the Duncan's multiple range tests. The means ± standard errors (SE) are presented in each column. Means sharing at least one letter are no significantly different at $p \leq 0.05$.

RESULTS AND DISCUSSION

In vitro seed germination

The percentage of seed germination in the two media was periodically recorded at 10-day intervals after initial inoculation. Germination of seeds began after 6 weeks of inoculation. The initial stages of germination were typical for most orchids (Arditti, 1977). Since orchid seeds are unique due to the presence of an unorganized embryo and the absence of a functional endosperm, usually asymbiotic methods are used for easy germination (Ernst, 1975; Yam & Weatherhead, 1988; Hoshi *et al*., 1994). About 95% of the seeds were germinated in the KnC medium (Fig. 1a) and 65% in the HMS medium. After 12 weeks, the photosynthetic leaves emerged from the protocorm. In many terrestrial orchids like *Cypripedium reginae* (Harvis, 1982), *Cypripedium candidum* (De Pauw *et al*., 1995) and *Cypripedium calceolus* (van Waes & Debergh, 1986), a definite cytokinin preference for germination and protocorm growth has been reported. In all these species, a low level of cytokinin has been found to be optimum. Although the efficient amount of 0.2 mgl^{-1} BAP in *C. mossiae* in the HMS resulted in a preferable growth and development of the plantlets (Fig.1b, c), in the KnC medium the presence of BAP resulted in decreased development. Therefore, the growth promoting effect observed in *C. mossiae* could be attributed to the supply of exogenous amino acids present in the HMS medium.

Plantlet development

After 5 months, the shoots developed plantlets and were transferred to hormone-supplemented HMS and KnC media. The KnC medium supplemented with the hormones BAP (0.1-0.5 mgl^{-1}) and KIN (0.1-0.5 mgl^{-1}) showed slow responses when compared to the HMS medium. This effect may be due to the presence of vitamins, amino acids and other regulatory compounds in the MS medium. Jonojit & Nirmalya (2001) have also observed that addition of BAP to the HMS medium stimulated protocorm development and shoot bud initiation in *Geodorum densiflorum*. The HMS medium with 0.2 mgl^{-1} BAP, highly induced protocorm multiplication and seedling growth in *C. mossiae* and seedlings grew to a length of 7 cm (Fig. 1d). The cytokinins were essential for the initiation of shoots. Concentrations of BAP above 0.2 mgl^{-1} were found to inhibit protocorm multiplication and shoot growth (Table 1) (Hasegawa *et al*., 1965; Shimasaki & Uemoto, 1990; Chang & Chang, 2000). There was a gradual decrease in rhizogenesis with increased BAP concentration. Kinetin at 0.1-0.5 mgl^{-1} resulted in reduced protocorm multiplication and shoot development in the two media.

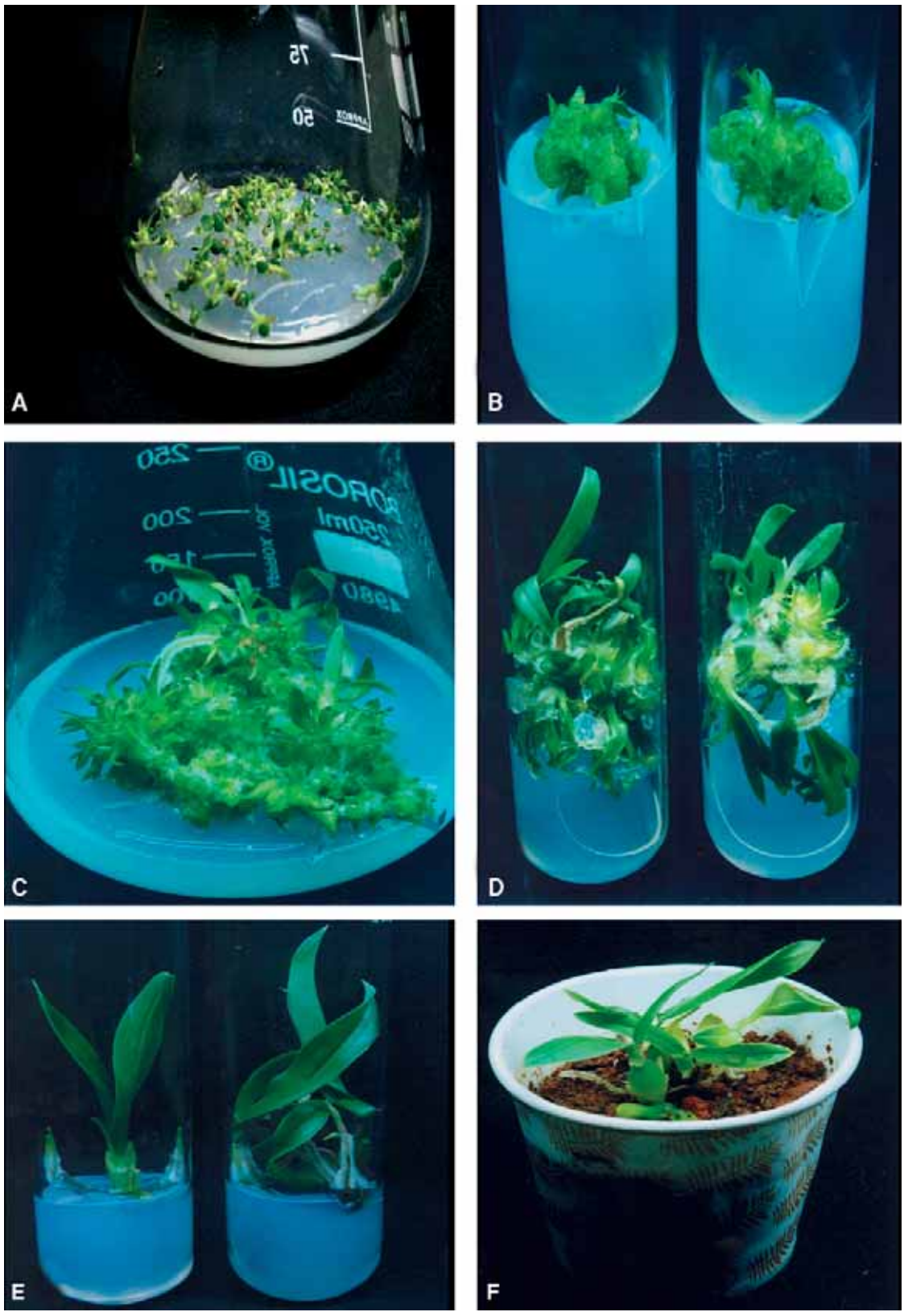

FIG. 1. *In vitro* seed germination and plantlet regeneration of *Coelogyne mossiae*. (A) *In vitro* seed germination in KnC medium; (B, C) Protocorm multiplication in HMS medium supplemented with 0.2 mgl^{-1} BAP; (D) Shoots allowed to grow in the hormone-free HMS medium; (E) Root induction in the HMS medium supplemented with 0.2 mgl^{-1} IBA; (F) Hardening into a mixture of garden soil, vermicompost and brickgravals.

TABLE 2. Effects of IBA in HMS and KnC media on root induction of *Coelogyne mossiae*

Hormone	Concentration (mgl^{-1})	% of root induction	No of roots per plantlet (mean ± SE)	Root length (cm ± SE)
HMS medium				
Basic medium	–	20	2.30 ± 0.20^{gh}	3.52 ± 0.06^{i}
IBA	0.1	80	4.90 ± 0.22^{b}	5.39 ± 0.04^{ab}
	0.2	90	5.80 ± 0.23^{a}	5.53 ± 0.04^{a}
	0.3	60	3.90 ± 0.22^{d}	5.17 ± 0.02^{cd}
	0.4	30	2.40 ± 0.15^{g}	4.42 ± 0.03^{fg}
	0.5	20	1.60 ± 0.20^{jk}	3.10 ± 0.35^{jk}
KnC medium				
Basic medium	–	20	2.00 ± 0.14^{hi}	3.25 ± 0.06^{ij}
IBA	0.1	60	3.90 ± 0.17^{de}	5.22 ± 0.03^{bc}
	0.2	80	4.80 ± 0.23^{bc}	4.95 ± 0.04^{de}
	0.3	50	3.00 ± 0.20^{f}	4.50 ± 0.04^{f}
	0.4	30	1.80 ± 0.18^{ij}	3.97 ± 0.05^{h}
	0.5	20	1.30 ± 0.14^{kl}	2.78 ± 0.04^{l}

The well shooted plantlets were removed from the culture tubes and transferred to the rooting media. In our study, 90% of the shoots rooted well after transfer to the HMS medium and the KnC medium supplemented with 0.2 mgl^{-1} IBA (Fig. 1e, Table 2). Rooting also took place in the two basic media, but duration was longer compared with the hormone-supplemented media. The rooted plantlets were carefully removed from the culture tubes and were treated with Bavistin 0.1% (w/v) fungicide for 3 min. Then, plantlets were transferred into a mixture of garden soil, vermicompost and brickgravals (1:1:1) in decomposed disposable cups (Fig. 1f) and they were covered with plastic bags for 30 days under humidity. Plants were acclimatized to reduced relative humidity by gradual opening of the plastic cover and after 40 days they were completely uncovered and hardened to greenhouse conditions. Under these conditions they exhibited 80% survival. In conclusion, the protocol we established for plant regeneration from seeds of mature undehiscent capsules of *C. mossiae* is feasible and may be adopted as an effective propagation method for this species.

ACKNOWLEDGEMENTS

We thank the Departments of Science and Technology (Government of India, and Tamilnadu State) for financial support (ST (TN)/ST/2K1/191/2002).

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