CIBTech Journal of Biotechnology ISSN: 2319–3859 (Online)
An Open Access, Online International Journal Available at http://www.cibtech.org/jls.htm
2014 Vol. 3 (1) January-March, pp.26-30/Burhan et al.
Research Article

IN VITRO ASYMBIOTIC SEED GERMINATION LEADING TO PROTOCORM FORMATION IN *CEPHALANTHRA LONGIFOLIA* (LINNAEUS) FRITSCH AN ENDANGERED ORCHID OF KASHMIR HIMALAYA

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ABSTRACT
Among flower crops orchids contribute 10% share in international trade. They are also rich source of medicines due to presence of various phytochemicals. Present studies were undertaken to mass propagate *Cephalanthera longifolia* (Linnaeus) Fritsch, an orchid of considerable ornamental and medicinal importance. It is an extremely rare and threatened orchid of Kashmir Himalaya. In *Cephalanthera longifolia* (Linnaeus) Fritsch an efficient method of propagation has been developed via protocorms regeneration by culturing seeds from unripe fruits on half strength Murashige and Skoog (1962) medium supplemented with BAP and activated charcoal (AC).

Keywords: *Orchids, Murashige and Skoog (1962), BAP, Activated Charcoal (AC)*

INTRODUCTION
Orchids, more than other plants, exert a mysterious fascination for most people and all the wild orchids of tropical regions are highly puzzling and peculiar. Orchids exhibit an incredible range of diversity in size, shape and colour of their flowers. They have a wide ecological range of distribution and their growth is closely correlated with environmental conditions (Aravindhan *et al.,* 2011) In India, the states of Kerala, Karnataka, Tamil Nadu, Maharastra, Sikkim, West Bengal, Andaman, Hilly regions of Uttar Pradesh, Himachal Pradesh and entire north eastern region are suitable for commercial cultivation of orchids (Singh, 1991). The world orchid trade is estimated to be nearly US $ 44 billion with an annual growth rate of 10–20 percent. In Indian state of Jammu and Kashmir nearly 47 species have been listed, out of which one species*Listeria ovata* (Linn.) R. Br. Isendemic. Mannose specific lectins from Orchids like *Epipactis helleborine* (Linn.) Crantzand *Listeria ovata* (Linn.) R. Br. is targeted to inhibit virus cell fusion processes during chemotherapy of Human Immuno Deficiency Virus (HIV) Infections (Clercq 2000). Orchids like *Dactylorhiza hatagirea* (D. Don) Soo have aphrodisiacal qualities. ***Cephalanthera longifolia*** (Linnaeus) Fritschisa terrestrial orchid distributed in Europe, northern Africa and temperate Asia. In Kashmir it has been found growing in Erin nallah, Baltal, Pahalgam, Aru, Badwun, Dachigam National Park, Gulmarg and Baramulla over a range of 1500–2800 m above sea level. The plant flowers during May–July.

Generally, seed germination of terrestrial orchid species from temperate regions is difficult (Arditti *et al.,* 1982b; Rasmussen, 1995; Miyoshi and Mii, 1998). Information relating to the factors that complicate seed germination of these species remains fragmentary and obscure. For many orchid species, higher frequencies of germination have been achieved by culturing immature seeds than by culturing mature seeds (Withner, 1955; Linden, 1980; Arditti *et al.,* 1982a, b; Ballard, 1987; Mitchell, 1989; DePauw and Remphrey, 1993; Rasmussen, 1995; Light and Conaill, 1998). It has been postulated that dormancy is induced by some undefined changes during seed development and maturation, accumulation of some inhibitory substances such as phenolics in *Cymbidium goeringii* (Kako, 1976) and Abscisic acid in *Dactylorhiza maculata* and *Epipactis helleborine* (Linn.) Crantz (van der Kinderen, 1987), induction of a physiologically dormant state in embryos (Arditti *et al.,* 1982a) or by increasing impermeability of the

embryos during seed maturation (Miyoshi and Mii, 1988). No studies have elucidated the reasons for increased germination frequencies of immature seeds of the Orchidaceae.

MATERIALS AND METHODS

Green pods/capsule of *Cephalanthera longifolia* (Linnaeus) Fritschwere collected from the plants established at Kashmir University Botanical Garden (KUBG). Pods were surface sterilized by washing gently under running tap water. The pods were sterilized by dipping in 70% (v/v) ethanol for 1min followed by immersion in 2% Sodium hypochlorite solution for 10min with 5 drops of (Tween-20) and constant shaking for 5 min under Laminar air flow. Finally, the seed pod was gently rinsed 3 times with sterile double distilled water. The sterilized capsule was cut with sterilized surgical blade and the seeds were carefully scooped out and cultured asymbiotically on half-strength and full-strength inorganic salts of Murashige and Skoog medium (Murashige and Skoog, 1962) containing $30gL^{-1}$ sucrose and $8gL^{-1}$ agar supplemented with N6- benzyladenine and a-naphthaleneacetic acid. The pH of the media was adjusted to 5.7 with 1M KOH or HCl. The medium was autoclaved for 15 min at 121°C by maintaining 15lbs pressure.Nearly 10ml of medium was dispensed into culture vials. After 8 weeks of culture, the percentage of protocorm formation was recorded.

Figure 1: *Cephalanthera longifolia* habit

Figure 2: Green Pod

RESULTS AND DISCUSSION

Conservation and *in vitro* propagation of ornamental and medicinal orchid species is one of the biggest challenges as the demand is growing day by day. *Cephalanthera longifolia* (Linnaeus) Fritsch is a temperate orchid species of East Asia valued for its beautiful inflorescence, flowers and medicinal properties. The seeds studied are minute and dust-like. Multiplication of the species in nature is through seeds and only 0.3% of seeds germinate in the presence of suitable mycorhiza.

Since vegetative propagation methods are not available, development of *in vitro* methods are essential for conservation and commercialization of this species. The full strength of the salts of MS medium seems to be too high for the *in vitro* responses in case of temperate terrestrial orchids therefore in the present study, full and half strength MS medium was used whereby half strength MS medium showed positive response. As the terrestrial orchids are adapted to variable habitats, optimal *in vitro* culture conditions suitable for one species may differ for other species (Van Waes and Debergh, 1986b). During the present study *Cephalanthera longifolia* (Linnaeus) Fritsch a temperate Orchid species was subjected to *in vitro* studies. The seeds were inoculated on MS basal medium, half strength MS medium without and with varied concentrations of different auxins and cytokinins viz. 2, 4-D, NAA, IAA, IBA, BAP and Kinetin. Results were obtained on BAP supplemented medium.

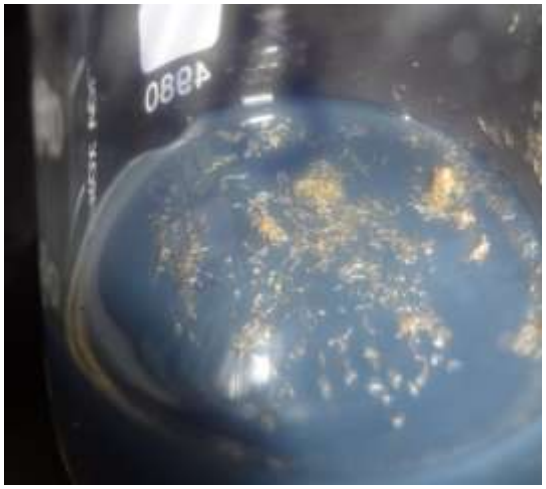

Figure 3: Seed inoculation

Figure 4: Protocorm formation

Table 1: Effect of Auxins

Phytohormone+ Activated Charcoal (AC)	Concentration	Response
MS Basal Control	_	_
2, 4-D(2, 4- Dichlorphenoxy Acetic Acid)	4µM	_
	8 µM	
	12 µM	
NAA (Napthylene Acetic Acid)	5 µM	_
	10 µM	
	15 µM	

Table 2: Effect of Cytokinins

Phytohormone + Activated Charcoal (AC)	Concentration	Frequency	Initation of Response
MS Basal Control		No response	
BAP (6-Benzyle Amino Purine)	4µM	-	
	8 µM	+	7 Weeks
	12 µM	++++	
Kinetin	4 µM	-	
	8 µM	++	7 Weeks
	12 µM	+	

Table 3: Combined Effect of Auxins and Cytokinins

Phytohormone + Activated Charcoal (AC)	Concentration	Frequency	Initation of Response
MS Basal Control	-	-	
2, 4-D +BAP	5µM+ 15 µM	+	7 Weeks
2, 4-D+ Kinetin	5µM+ 10 µM	+	7 Weeks
NAA+ BAP	5µM+ 15 µM	+++	7 Weeks
NAA+ Kinetin	5µM+ 10 µM	+	7 Weeks

The seeds of *Cephalanthera longifolia* (Linnaeus) Fritsch were inoculated on MS basal medium, half strength MS medium without and with varied concentrations of different auxins and cytokinins viz. 2, 4-D, NAA, Kinetin and BAP. Results were obtained on BAP supplemented medium. An efficient method of propagation in *Cephalanthera longifolia*(Linnaeus) Fritsch, has been developed via protocorm regeneration by culturing seeds from unripe fruits on half strength Murashige and Skoog medium supplemented with BAP 12 µM and activated charcoal (AC) $5gl^{-1}$. Seed germination of *Cephalanthera longifolia* (Linnaeus) Fritsch, on average began after 7 weeks of culture. By this time embryos had

enlarged by two times and filled the whole seed coat. Initially developing protocorms were elliptic or elongate, becoming clavate or pearshaped with a blunt apex. The colouration of protocorms varied from milkwhite at the beginning of germination to bright-green after 15 days. As a rule, three to four or more epidermal hairs were produced at this stage.Protocorms possess several centers of meristematic activity, but usually they develop only one shoot. Vegetative reproduction of specimens at protocorm stage is hardly ever observed in nature (Tatarenko and Vakhrameeva, 1998), whereas in culture *in vitro* protocorms are employed to obtain the greatest number of regenerants (Batygina and Shevtsova, 1985; Shevtsova *et al.,* 1986). Hence, this simple and efficient procedure for regenerating protocorm from seeds of *Cephalanthera longifolia* (Linnaeus) Fritsch could be used for large-scale propagation and ex situ conservation of this important threatened terrestrial orchid species.

ACNOWLEDGEMENT
The authors are highly thankful to the Head Department of Botany, University of Kashmir for providing the necessary laboratory facilities to carry out this work.

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