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RESEARCH ARTICLE

In vitro seed germination and development of protocorm like bodies (PLBs) in two orchid plant species- *Epipactis royleana* Lindl. and *Dactylorhiza hatagirea* (D.Don) Soo. growing in Kashmir Himalaya

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Abstract

Orchids belong to one of the two largest families of flowering plants, the Orchidaceae. The family comprises of about 800 genera and more than 25,000 species. Many of the species are epiphytes and some are terrestrial and saprophytic. Orchids are prized for their beautiful long lasting flowers exhibiting an incredible range of diversity in size shape and colour. Today growing Orchids is more than just a hobby, it is an international business covering around 8% of the world floriculture trade and has a potential to alter the economic landscape of a country. Large scale multiplication of exquisite and rare hybrids using tissue technique has helped Orchids to occupy position as one of the top ten cut flowers. The major investigation on explant based Orchid tissue culture started from the pioneering works of Rotor (1949) followed by Morel (1960). The Kashmir Himalaya is bestowed with a large number of orchids *viz. Dactylorhiza hatagirea , Epipactis royleana , Epipactis helleborine , Spiranthes sinensis, Liparis kashmiriana, Listera ovata, Cephlantherea longifolia,* etc, most of which are not yet cultivated. Hence for the large scale cultivation of these plants, protocols providing *in vitro* regeneration from various vegetative parts are needed. In the present study a successful protocol for *in vitro* seed germination and production of protocorm like bodies (PLBs) from the seeds of *Epipactis royleana* and *Dactylorhiza hatagirea* has been developed using seeds collected 3 weeks after pollination. 6-Benzylamine purine (BAP) in combination with α-Napthalene Acetic Acid (NAA) in MS medium enhanced seed germination in comparison to control. In *Epipactis royleana* BAP (2.5mg/l) and NAA (1.5 mg/l) was the optimum hormonal combination on which the maximum quantitative increase in the protocorm like bodies (PLBs) was achieved while in case of *Dactylorhiza hatagirea* , BAP (2.0 mg/l) and NAA (1.5mg/l) was the optimum hormonal combination on which maximum quantitative increase in protocorm like bodies (PLBs) was achieved.

Introduction

Orchids belong to one of the two largest families of flowering plants, the orchidaceae. The family is comprised of 800 described genera and 25,000 species. Orchids constitute an order of royalty in the world of ornamental plants. Today growing orchids is more than just a hobby, it is an international business covering around 8 % of the world floriculture trade and has the potential to alter the economic landscape of a country. Large scale multiplication of exquisite and rare hybrids using tissue culture techniques has helped orchids occupy a position as one of the top ten cut flowers (Chugh *et al.,* 2009). Orchids seem to attract a universal fascination, with orchid societies in every developed nation. Orchids are remarkable examples of speciation through natural selection. In India orchids form 9 % of flora and about 1300 species are found in Himalayas with others scattered in Eastern and Western Ghats (Jain, 1980). A single orchid capsule or pod contains millions of seeds. The seeds are minute, contain undifferentiated

embryo and lack endosperm. Orchids are mostly out breeders. Inspite of a very large number of seeds produced, only few seeds germinate that lead to the production of heterozygous plants. In certain orchids, self pollination is not possible and even if possible as in the case of various species of *Vanda*, one has to wait for 4 to 6 months for pod development (Fitch, 1981). Thus a reliable propagation method is needed, one of such methods is the *in vitro* seed germination and production of protocorm like bodies (PLBs) which develop from seeds to produce plants for re-establishment in the wild or for commercial production. PLBs are the germinated embryos (Bernard, 1909). In orchid cultures, protocorms develop from the leaf epidermal cells or from the seeds, which proliferate to form a cluster upto a dozen protocorms which are cut into 4 to 6 pieces, each one of which gave rise to 3 to 5 protocorms in a month. At this rate of multiplication several million plants can be propagated in a year. Infact shoot tip culture of *Cymbidium* produce a million plants from a single bud in a year (Morel, 1960). During the present investigation, successful protocol was developed for seed germination and protocorm development in *Epipactis royleana* and *Dactylorhiza hatagirea*, the two important orchids growing in Kashmir Himalayas having great commercial potential.

Materials and methods

The green pods were collected 4 weeks after anthesis from the plants introduced and growing in Kashmir University Botanical Garden (KUBG). After removing the dry petals, the pods were washed thoroughly by running tap water, followed by washing with liquid detergent (Labolene 1% V/V) and surfactant (Tween–20 1% V/V) for five minutes. Then the pods were washed with running tap water to remove the detergent and finally with double distilled water so as to remove the last traces of the detergent. After that were surface sterilized with 0.1% HgCl2 for 6 minutes followed by thorough wash in sterile double distilled water under laminar air flow hood (Fig.1). The pods were then dipped quickly in 70% alcohol and then washed again with sterile double distilled water. Each pod was then transferred to sterile petridishes.

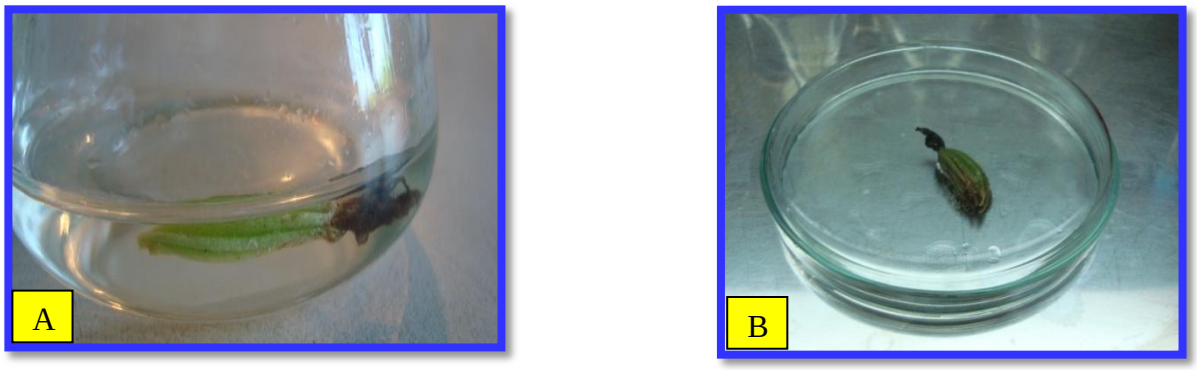

Fig. 1: Sterilized pods; **A**= *E. royleana*, **B**= *D. hatagirea*

The pods were cut longitudinally into 2 halves using a surgical knife, so as to expose the seeds. After careful separation from the green pods, the seeds were transferred in MS medium (1962) containing 3% w/v sucrose and gelled with 0.8% w/v agar.

The media was supplemented with varied concentrations of BAP and NAA. The pH of the medium was adjusted to 5.8 with 0.1N NaoH and autoclaved for 15 minutes at 121 ^{0}C.

All the cultures were maintained at 25 ± 2 ^{0}C under light provided by cool florescent tube light (3000 lux) with a photoperiod of 12 hrs daily.

Results and Discussion

Large scale production of ornamental plants have been achieved successfully using tissue culture techniques. Several workers have used various explants and culture media and introduced tissue culture methods for regeneration of orchids. The major investigation on explant based orchids tissue culture started from the pioneering works of Rotor (1949) followed by Morel (1960). Propagation of orchids through the production of protocorms

(PLBs) has been achieved in a number of orchids viz; *Oncidium* sp. (K. Kalimutha *et al*., 2006), *Cypripedium candidum* (Pauw *et al*., 1995), *Cypridedium acaule* (Barabe *et a*l., 1993), *Satyrium napalense* (Chauhan *et al.*, 2010).

In the present study a successful protocol has been developed for the seed germination and protocorm development in *Epipactis royleana* and *Dactylorhiza hatagirea* growing in Kashmir Himalaya.The sterile seeds were inoculated on a range of media containing varied concentrations of Cytokinins (BAP and Kinetin) and Auxins (NAA and IAA). The seeds did not germinate on such media. The seeds were then inoculated on media containing both auxins and cytokinins together. After that the seeds were inoculated on media containing BAP and NAA together germinated to produce PLBs in both the plant species. In case of *Epipactis royleana* the seeds germinated after three weeks of inoculation, while, in case of *Dactylorhiza hatagirea* germination occurs after seven months.The effect of the various concentrations of BAP and NAA was evaluated and it was observed that BAP (2.5 mg/l +NAA 1.5 mg/l) is the optimum concentration at which maximum protocorm development was achieved in case of *Epipactis royleana* (Table1 and Fig. 2). In case of *Dactylorhiza hatagirea* the seeds showed optimum germination and maximum protocorm development in MS medium containing BAP (2.0 mg/l +NAA 1.5 mg/l) (Table 2; Fig.3).

Table 1. *In vitro* seed germination and protocorm developtment in *E. royleana*

MS medium (BAP + NAA) mg/l	No. of green pods used per culture	Frequency of seed germination and protocorm development
MS basal (Control)	1	-
MS + 0.5 + 0.5	1	-
MS + 1.0 + 0.5	1	+
MS + 2.0 + 1.0	1	++
MS + 2.0 + 1.5	1	++
MS + 2.5 + 1.5	**1**	**+ ++**
MS + 3.0 + 2.0	1	+
MS + 3.5 + 2.5	1	-

N= 10
- No Response
+ Very small number of Protocorms
++ Small number of Protocorms
+++ High number of protocorms

Table2. *In vitro* seed germination and protocorm development in *D. hatagirea*.

MS medium (BAP + NAA) mg/l	No. of green pods used per culture	Frequency of seed germination and protocorm development
MS basal (Control)	1	-
MS + 0.5 + 0.5	1	-
MS + 1.0 + 0.5	1	+
MS + 2.0 + 1.0	1	++
MS + 2.0 + 1.5	**1**	**+++**
MS + 2.5 + 1.5	1	+ +
MS + 3.0 + 2.0	1	+
MS + 3.5 + 2.5	1	-

N= 10
-No Response
+ Very small number of Protocorms
++ Small number of Protocorms
+++ High number of protocorms

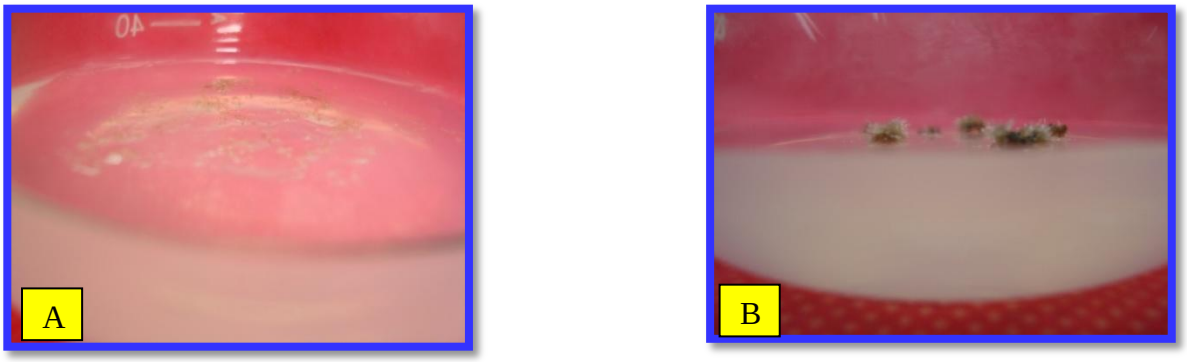

Fig.2: *In vitro* seed germination and protocorm like bodies(PLBs) development in *E. royleana*
A= Initation of seed germination; **B**= Development of PLBs

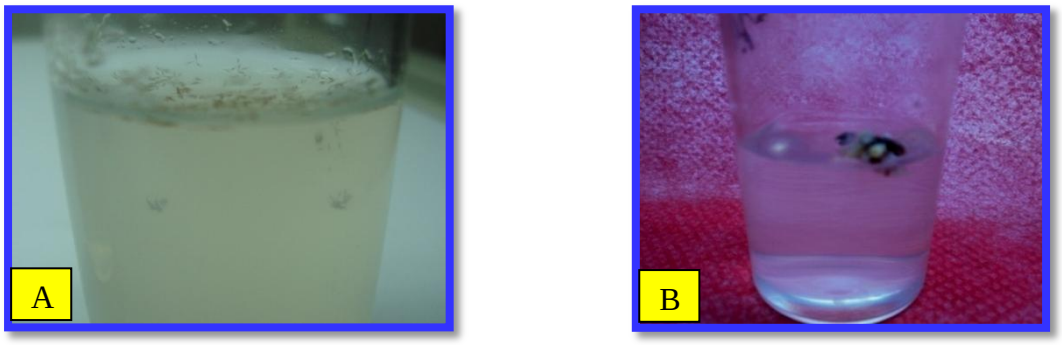

Fig.3: *In vitro* seed germination and protocorm like bodies(PLBs) development in *D. hatagirea*
A= Initation of seed germination; **B**= Development of PLBs

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