Micropropagation and other in vitro techniques have become more widely used in commercial horticulture and agriculture for the mass propagation of crop plants and for conservation of genetic resources, particularly with those crops which are vegetatively propagated or have recalcitrant seeds which cannot be stored under conventional seed bank conditions (George, 1993; George and Sherrington, 1984). Likewise, in vitro culture is being used in an increasing number of threatened plant species. In addition to micropropagation in its strict sense, other techniques available include in vitro seed germination, regeneration of plants from callus cultures, dual culture with symbiotic fungi and micro grafting. The use of these techniques have allowed the propagation of many rare, threatened and endangered plant species which prove problematic with conventional horticultural methods and particularly useful with groups of plants which are difficult to propagate using conventional techniques.

Orchids are inherently slow growers and seeds germinate poorly in nature due to their nutritional complexities. Traditionally orchids are mainly propagated through vegetative means such as keikis, back-bulbs, division of shoots etc., and through seeds.
Vegetative propagation is very slow while the seed germination in nature is very poor (~0.2-0.3%) (Sungkumlong and Deb, 2008). Their regeneration in nature is limited due to suppressed endosperm and requirement of fungal stimulus. But this constraint to commercial production has been overcome by the development of tissue culture techniques which have also opened new possibilities in conservation and commercialization of orchids (Deb and Sungkumlong, 2009, 2010; Lal et al., 1988; Sungkumlong and Deb, 2008; Tandon et al., 1990; Temjensangba and Deb, 2005a, 2006). Following this technique, round the year propagation of genetically uniform, disease free, fast maturing and high yielding plants are made possible and enabled to exploit the regenerative competence more effectively than the conventional method. Ever since Knudson (1922) demonstrated the ability to by-pass the fungal requirements for germination of *Cattleya* seeds/embryos *in vitro* by using appropriate level of carbohydrates in the culture medium, remarkable progress has been made in micropropagation of orchids (Arditti et al., 1982; Arditti and Ernst, 1993; Bejoy et al., 2004; Bhadra and Hossain, 2003; Chen et al., 2004; Kalimuthu et al., 2007; Kannan, 2009; Kaur and Bhutani, 2009; Kosir et al., 2004; Li and Xu, 2009; Martin, 2007; Mitra et al., 1976; Seeni and Latha, 1992; Sunitibala and Kishor, 2009; Temjensangba and Deb, 2005a; 2006; Vij and Pathak, 1990; Yam and Weatherhead, 1988). The immature embryos and the shoot meristem are the most commonly used explants for *in vitro* propagation of orchids. But the response of orchid seeds to physio-chemical factors differ from species to species (Arditti et al., 1982; Arditti and Ernst, 1993; Bejoy et al., 2004; Chen et al., 2004; Mitra, 1986; Seeni and Latha, 1992; Temjensangba and Deb, 2005a, 2006; Vij and Pathak, 1990; Yam and Weatherhead, 1988).

Seed germination represents the most efficient method for orchid propagation for mass multiplication and conservation purposes (Arditti et al., 1982; Stewart and
Kane, 2006; Temjensangba and Deb, 2005a). The immature seeds of various developmental stages can also germinate in vitro prior to reaching maturity and the technique is variously referred as ovule/embryo/green pod/green fruit culture (Bhadra and Hossain, 2003; Deb and Temjensangba, 2006b; Deb and Sungkumlong, 2009; Kannan, 2009; Li and Xu, 2009; Sagawa, 1963; Sungkumlong and Deb, 2008; Temjensangba and Deb, 2005a, 2006). However, orchid seed germination studies are often viewed as unreliable or unrealistic since little is known concerning the germination and in vitro seedling developmental requirements of many orchids. Compounding this difficulty, many workers (Temjensangba and Deb, 2005a, c, 2006; Deb and Sungkumlong, 2009) found that different species requires different developmental age for in vitro response for germination and most of the orchid species have a very short window period of developmental stage when they germinate in vitro. The in vitro germination potential of fertilized ovule/immature seeds has been positively tested in several commercially viable and/or threatened orchid taxa (Deb and Temjensangba, 2006b; Deb and Sungkumlong, 2008, 2009; Johnson et al., 2007; Kannan, 2009; Stewart and Kane, 2006; Sungkumlong and Deb, 2008; Temjensangba and Deb, 2005a, 2006).

Non-symbiotic seed germination of orchids is greatly influenced by several factors like green pod age/developmental stage of embryos, different nutrient media with adjuvant, plant growth regulators (PGRs) etc. Apart from immature seeds/embryos other explants like aerial roots, foliar explants, nodal segments, inflorescence could be used successfully for in vitro propagation of orchids (Deb and Temjensangba, 2005, 2007a; Deb and Sungkumlong, 2010; George and Ravishankar, 1997; Kosir et al., 2004; Li and Xu, 2009; Nayak et al., 1997; Prasad et al., 2000; Sinha and Hegde, 1997; Vij and Pathak, 1999; Vij et al., 2000).
In vitro culture of orchids is greatly influenced by several factors like explants age/ developmental stage, source of explants, nutrient media, organic carbon sources, different adjuncts, and quality and quantity of PGRs, light etc. Apart from different factors, for popularization of tissue culture technique in terms of commercial scale production of orchids, it has included the development of low cost protocols including low cost alternative substratum to agar as agar is one of the costly components in tissue culture.

Preparation of Substratums

Different types of substratums including 'agar, betel-nut coir, coconut coir, polyurethane foam (thereafter called 'foam') and forest leaf litter' were selected for the present study. In the present study, plant tissue grade agar was used (make: Hi-media, India). 'Foam' was collected from the local market which is generally used for preparation of mattresses. Other substratums like 'betel-nut coir, coconut-coir were extracted from the dried fruits and chopped into small pieces, while, 'leaf litter' was collected from the forest floor before they are decayed. Except agar all other materials were soaked with 'Extron' (a commercial laboratory detergent, make: Merck, India) (1:100) (v/v) for about two hours followed by washing under running tap water till the water ran clear. The substratums were air dried and stored till used. The dried substratum except foam were chopped into small pieces (~0.5 cm size), while the foam was cut into disk (according to the culture vials). These substratums except agar were then autoclaved at 1.05 Kg cm\(^{-2}\) pressure and 121°C for one hour before putting them in the culture vials.

Plant Materials

When working with rare and endangered species, the amount of available plant material can be very small, and this can place restrictions on the choice of methods.
Seeds are preferred to vegetative material as the source of propagation so that a wider genetic base can be maintained. However, in some species, seed is not readily available and therefore vegetative material has to be used. Success to a great extent depends on the selection of the right explants, physiological age, media composition, exogenous growth regulators and culture conditions.

**Seeds:** The immature seeds/green pods of different developmental ages of *Cymbidium aloifolium* [7-12 months after pollination (MAP) at one month interval] and *Cymbidium iridioides* (6-16 MAP at two months interval) were collected from the garden. These green pods were used for the experimental purpose.

**Leaf:** For both the species, foliar explants from *in vitro* grown plantlets/axenic cultures were collected and used for the present study. Foliar explants (0.5-1.5 cm size) were collected after about 5 wk of emergence just before inoculation.

**Roots:** Aerial roots of ~5-6 wk old were harvested from axenic cultures of both the species.

**Nodal explants:** Nodal explants were collected from the *in vitro* raised plantlets. The etiolated plantlets with distinct nodes were selected and the leaves were removed. The defoliated shoots were cut into segments with one or two nodes in each segment. These nodal segments were used for the initiation of culture.

**Sterilization of Plant Materials**

**Seeds:** The green pods/capsule of different developmental stages of both the species (*Cymbidium aloifolium* and *C. iridioides*) were harvested and washed thoroughly with Extron (1:100) (v/v) which were then rinsed under running tap water. The pods were surface sterilized with aqueous solution of mercuric chloride (HgCl₂) (0.3%) (w/v) for 5 min and subsequently rinsed 4-5 times with sterile doubled distilled water. Prior to dissection of the embryos and inoculation, the pods were dipped in ethanol (70%) (v/v).
and flamed. This operation was carried out under aseptic condition inside the laminar flow cabinet.

The aerial roots, foliar explants and nodal explants were harvested from the axenic culture and inoculated on initiation media without sterilization.

**Tissue Culture**

**Media:** For initiation of embryo/seed cultures, various media like Gamborg or (B2) (Gamborg *et al.*, 1968), Knudson ‘C’ (Knudson, 1946), Mitra *et al.*, (Mitra *et al.*, 1976), Murashige and Skoog (MS) (Murashige and Skoog, 1962) and SH (Schenk and Hildebrandt, 1972) media were used. For immature seed culture of both the species, the basal media were fortified with coconut water (CW) (0-20%) (v/v), casein hydrolysate (0-200 mg/l²) and different organic carbon sources such as dextrose, glucose and sucrose (0- 4%) (w/v) along with different levels of plant growth regulators (PGRs) such as α-naphthalene acetic acid (NAA) and N⁶-benzyl adenine (BA) (0-9 µM for *C. aloifolium* and 0-12 µM for *C. iridioides*) singly or in combination.

Foliar explants of both *C. aloifolium* and *C. iridioides* were cultured on MS medium fortified with sucrose (0-4%) (w/v) and supplemented with different levels of PGRs such as NAA and BA (0-9 µM) singly or in combination. In case of aerial root cultures of *C. aloifolium* and *C. iridioides*, MS medium was fortified with sucrose (0-4%) (w/v), activated charcoal (AC) (0-200 mg/l²) (w/v). The basal medium was further fortified with different levels of PGRs like IAA, NAA, BA, and KN (0-9 µM) singly or in combination. While, for nodal explants culture of both *C. aloifolium* and *C. iridioides*, the MS medium was enriched with sucrose 3% (w/v), and NAA and BA (0-9 µM) singly or in combination.

The pH of the media was adjusted to 5.6 using 0.1 N NaOH and 0.1 N HCl. The media were gelled using agar (0.8%) (w/v) before autoclaving. About 15 ml of the
medium was dispensed into each test tube (size 150 mm x 25 mm) and 30 ml into 400 ml culture bottle (diameter: 70 mm). Besides agar, different pre-processed substratums like betel-nut coir, coconut coir, foam and leaf litter were used as supporting materials to which about 10-12 ml of the prepared liquid media were dispensed in each test tube. The media were autoclaved at 1.05 Kg cm\(^{-2}\) pressures and at 121°C for 20 minutes.

**Initiation of Cultures**

**Immature embryos/green pods:** The immature seeds/embryos of different developmental stages from both the species were scoped out from the sterilized green pods under a laminar flow chamber with the help of scalpel blade and cultured on different basal media containing different substratum such as agar, betel-nut coir, coconut coir, foam and leaf litter, fortified with different levels of various adjuncts. The cultured embryos were incubated under different light conditions viz., dark, diffused (20 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) and full light (40 \(\mu\)mol m\(^{-2}\) s\(^{-1}\)) at 12/12 hr light/dark photo cycle. For each treatment 20 culture vials were maintained.

**Leaf:** The leaves from the *in vitro* raised plants of both the species were carefully taken out inside the laminar flow cabinet and were cultured on the nutrient medium. The intact leaves were cultured on different initiation media and in each culture vial two leaves were cultured. To determine the effect of orientation, explants were placed in a slanted (\(-45^\circ\)) and horizontal position, also position with upside and upside down. For each treatment 20 explants were cultured.

**Root segment:** The aerial roots from the *in vitro* raised plants of both the species were carefully taken out inside the laminar flow cabinet and were cultured on the nutrient medium. The aerial roots from the *in vitro* source of about 0.5-1 cm long were cultured on the initiation medium. In each test tube one or two root segments were cultured and about 20 root segments were used for each treatment.
Nodal explants: The processed nodal segments were cultured on the nutrient medium. In each culture vial 2 nodal segments were inoculated and for each treatment 20 segments were maintained.

The foliar explants, aerial roots and nodal explants were cultured under full light (40 μmol m⁻² s⁻¹) condition at 12/12 hr light/dark photo cycle. All the cultures were maintained at 25±2°C and sub-cultured at 4-5 wk interval unless mentioned otherwise. The experimental design was completely randomized and all the experiments were repeated at least thrice.

The Protocorm-like bodies (PLBs)/shoot buds developed from the germinated seeds/embryos, foliar explants, aerial roots and nodal segments were maintained on the optimum initiation condition for 2 passages for further proliferation and differentiation. In the cultures with different alternative substratums, about 5 ml of fresh liquid medium was poured in the same culture vial after removing the exhausted medium with a pasture pipette.

Regeneration of Plantlets and Mass Multiplication

The PLBs/shoot buds/advanced stage PLBs (just before release of first set of leaflets) formed from the cultured immature embryos, leaf, aerial roots and nodal explants were maintained further for two passages on the optimum initiation conditions for further development and differentiation. The tiny plantlets so formed were separated from the clumps and transferred to three different basal media (Knudson ‘C’, Mitra et al and MS) containing various organic carbon sources such as dextrose, glucose and sucrose (0-4%), CW (0-20%), CH (0-0.2 g/l), different levels of plant growth regulators (PGRs) like IAA, NAA, BA and KN (0-9 μM) singly or in combination for regeneration of plantlets and mass multiplication. Apart from agar gelled media, the cultures were also maintained on the different alternative substratums as used for germination of immature
embryos, for regeneration of plantlets and mass multiplication. In every sub-culture the shoot buds formed were separated and transferred on fresh regeneration medium. The plantlets were maintained for 2-3 passages on regeneration medium before they were transferred to the hardening condition.

**Hardening of Regenerated Plants**

About 5-6 cm long well-rooted plantlets (with 2-3 roots) of both the species were hardened for considerable period prior to transferring in the potting mix. The plantlets were taken out from the regeneration medium and the traces of agar were washed off with a soft brush (for cultures raised on agar gelled media) and then transferred on culture vials containing highly reduced strength of MS liquid medium (1/10th strength) supplemented with sucrose (1%) (w/v) devoid of any PGR, a protocol developed by Deb and Imchen (2010). In the culture vials different types of supporting materials were incorporated which includes charcoal pieces, brick pieces and chopped mosses (at 1:1 ratio). All these materials were washed thoroughly under running tap water and autoclaved at 1.05 Kg cm$^{-2}$ pressure and 121°C for one hr before putting them in the culture vials. The cultures were maintained for 4-6 wk in 12/12 hr photoperiod with light provided at 40 µmol m$^{-2}$s$^{-1}$ at a temperature of 25±2°C before transferring to community potting mix (CPM).

**Potting Mix and Transplantation of Regenerates**

To transplant the hardened plantlets of both the orchid species (*C. aloifolium* and *C. iridioides*), the CPM was prepared by mixing different substrates like sand: brick pieces: coconut husk: charcoal pieces: decayed wood in different combinations in the ratio of 1:1:1:1:1 with a layer of moss. The hardened plants were transferred to CPM along with the content (substratum of the hardening medium) and were covered with holed
transparent poly bags. The potted plants were maintained in a shaded place and fed with MS liquid salt solution (1/10th strength) weekly for 2-3 wk. The potted plants were exposed to normal day light for about 1 hr in a day for initial one wk and subsequently increased the exposure period by 2 hr from the second wk and finally after one month the plantlets were left in the normal full day light condition which were kept for about 7-8 wk before transferring to the wild.