CHAPTER I
INTRODUCTION

1.1 Background

Orchidaceae is the largest family of flowering plants found in the world. There are about 25,000-30,000 species of orchids in the world (Hossain, 2011). It contributes about one-tenth of the world's total flowering plants. They are found in all regions of the world from the tropics to high alpine except the icy Antarctica and hot deserts, but their greatest diversity occurs in the tropical and sub-tropical regions. They are notably abundant in the moist tropics where majorities are epiphytic in the forest. Most of the temperate and almost all of the alpine genera are terrestrial, while some are lithophytes. Taxonomically, they represent the most highly evolved family amongst monocotyledons (Dressler, 1993).

Nepal, the nature's paradise, represents just 0.03% of the total global area but accounts for 2.7% of the total flowering plants of the world. Orchidaceae is the largest family in terms of species of approximately 6,500 species of flowering plants found in Nepal (Press *et al*., 2000; Bista *et al.*, 2001). Nepal is home to about 400 species of native orchids belonging to 102 genera of which 12 are endemic to Nepal. Thus, orchids contribute about 6% of the total flora of Nepal (Raskoti, 2009).

In Nepal, orchids are found at an altitudinal range of 100 to 5200 m asl but are highly abundant at 1600 m asl. Orchid diversity is high in the central and eastern Nepal which account for 218 and 300 species respectively. In the western Nepal, orchid species are sparsely populated and represented by only 65 species (Acharya *et al*., 2011). It may be due to scanty rainfall, abundance of coniferous forest and less orchid exploration. Wild orchid is one of the integral parts of Nepal's floral heritage. Orchids are popularly known as "sunakhari", "sungava", "chandigava" and "jivanti" in Nepal.

The term "Orchids" gets their name from the Greek word "orchis" meaning "testicles", from the appearance of subterranean tuberoids of the genus *Orchis*. The word "orchis" was first used by Theophrastus, father of Botany (370-285 BC), in his book "*de historia plantarum"*. Orchids are perennial or rarely annual herbs with roots having multi-layered spongy tissue. They are classified as epiphytes,

terrestrial and lithophytes on the basis of their habitat. Vegetatively, they are either single stemmed (monopodial) or many stemmed (sympodial).

Orchid, a beautiful gift of nature, is very distinct plant group amongst angiosperm. They are one of the unique plants group and very popular in every corner of the world due to their various shape, size, habit, habitat, colorful flowers, shinning green leaves and variously shaped pseudobulbs etc. They are important aesthetically, medicinally and also regarded as ecological indicators (Joshi *et al*., 2009). They are valued by many different cultures and tribes in different parts of the world (Arditti, 1992; Kasulo *et al.*, 2009).

Orchids are valued as potted plants, foliage plants as well as cut flowers because of their exotic beauty and long lasting bloom (Hew, 1994). The most beautiful ornamental orchids of Nepal belong to following genera: *Aerides, Bulbophyllum, Calanthe, Coelogyne, Cymbidium, Dendrobium, Phalaenopsis, Pleione, Rhynchostylis, Thunia and Vanda* (Rajbhandari and Bhattarai, 2001).

Besides floricultural value, some orchids are highly used as medicinal plants to cure different diseases. A total of 90 species of orchids of Nepal have been reported to have medicinal value by Pant and Raskoti (2013). Subedi (2011) has reported that 60 species of the wild orchids of Nepal have been used for local therapeutic purpose. Whole plants as well as different parts, viz., roots, rhizomes, pseudobulbs, stems and leaves of these orchids are used as medicine. These orchids have been used by the native people of Nepal for treating different diseases such as general debility, stomachache, bone fractures, colds, wound healing, general weakness and to cure various other diseases. Chinese and Japanese were the first to describe orchids for medicinal use (Reinikka, 1995; Bulpitt *et al.*, 2007). Medicinal orchids belong mainly to the following genera: *Anoectochilus, Bletilla, Calanthe, Coelogyne, Cymbidium, Cypripedium, Dactylorhiza, Dendrobium, Ephemerantha, Eria, Galeola, Gastrodia, Gymnadenia, Habenaria, Ludisia, Luisia, Nervilia and Thunia* (Szlachetko, 2001; Pant and Raskoti, 2013).

Due to these valuable properties, the demand of orchid is increasing day by day in the national and international markets. Today growing orchids is an international business and is a million dollar industry in many countries (Griesbach, 2002). This

has led to illegal collection of orchids from their natural habitats even from the protected areas (Acharya and Rokaya, 2010). Practice of illegal trade is rampant to the neighbouring countries, especially China and India, as they buy raw material of orchids to use in different traditional medicines (Subedi *et al*., 2013). The greatest threat to orchids is due to habitat loss, degradation and fragmentation but the unsustainable use of orchid resources and illegal export of commercially important species causes biodiversity erosion and revenue loss to Nepal (Bhattarai *et al*., 2002).

Orchids as a whole are cited under Appendix II of CITES, except *Paphiopedilum insigne* and *Paphiopedilum venustrum* which are cited under Appendix I, as potentially threatened or endangered species in the natural habitat, where trade of wild orchids in national and international market for commercial use is strictly prohibited. Habitat loss, deforestation and over exploitation of orchids having medicinal and ornamental values have depleted the orchid wealth of Nepal. As a consequence the orchid species are disappearing day by day and restricted to narrow pocket areas in their natural habitat. So, effective strategies should be implemented to conserve these precious gems of nature before it gets extinct.

Orchid seeds are extremely small and dust like; a single capsule may contain 1,000-4,000,000 seeds depending upon species and the size of fruit. They are acotyledonous with little or no endosperm. They are 1-2 mm long and 0.5-1.0 mm wide. The embryo has a round or spherical form, consisting of relatively undifferentiated isodiametric cells with dense granulated cytoplasm and conspicuous nuclei (Arditti, 1967).

Orchids require a combination of different factors for reproduction in nature. Orchid seeds are unique and are poorly developed even at maturity. They have unique physiology of germination. They require the presence of suitable fungus for germination in nature as they lack their own food reserves i.e. endosperm. When an orchid seed land on a substrate, the germination process is initiated only when the fungus penetrates the seed testa and invades the embryo (Gutierrez-Miceli *et al.,* 2008). Fungus is believed to augment the carbohydrate, auxin and vitamin transport in the orchid (Mohanraj *et al.,* 2009). Most of the fungal partners of orchid have been classified as the genus *Rhizoctonia* and *Rhizoctonia* like fungi. *Rhizoctonia* include a variety of teleomorph eg. *Ceratobasidium*, *Sebacina*, *Tulasnella*, *Thanatephorus,* etc.

and *Rhizoctonia* like fungi include the anamorphic genera like *Ceratorhiza*, *Ephulorhiza*, *Moniliopsis*, etc. (Ovando *et al.*, 2005).

The germination rate of orchid seeds in nature is only 2-5% (Rao, 1977) even if they do so, the seeds take a long time for their germination and any disturbance in the habitat or physical environment destroys the whole population. Also, the seedlings take 12 years to become an adult plant (Basker and Narmatha Bai, 2006). They are highly heterozygous and the propagation of orchids through conventional means is a very sluggish process and laborious. Vegetative propagation of orchids through division of clumps of rhizomes, bulbs or by the rooting of off-shoots is slow and difficult to obtain desired number of orchids. These difficulties in natural germination and vegetative propagation drive some of the indigenous species to extinction. It is therefore important to take initiative for mass propagation of orchid and to establish in nature. The discovery of *in vitro* germination and micropropagation contribute immensely to alleviate their scarcity. This technique can also reduce the length of time required for introduction and conservation of new varieties. Hence, tissue culture technique is a potential alternative method for mass scale propagation and conservation of rare, endangered and threatened orchid population in their natural habitat.

1.1.1 Plant Tissue Culture

In vitro plant tissue culture is the most commonly used biotechnological tool for mass propagation. It is widely used for the improvement of field crops, forest, horticulture and plantation crops for increased agricultural and forestry production. *In vitro* culture is highly applicable to maintain the same clone as the mother stock and production of superior clone. It is also widely used for the production of secondary metabolites which have high demand in the drug industry. This technique has been globally recognized for the improved production of high quality and disease free plants in short time.

The *in vitro* propagation, often called as micropropagation, is a scientific technique widely used for the mass production of desired plants by multiplication of genetically identical copies of individual plants in artificial nutrient medium under aseptic condition in short time irrespective of physiological and climatic barriers.

The *in vitro* propagation is carried out by using the various parts of plants as an explant such as root, stem, leaf, seed, apical shoot, auxiliary bud, meristem, embryo, single cell, protoplast, pollen grain, ovule, etc.

The micropropagation technique is highly preferred over the conventional asexual propagation methods as millions of plants can be produced in a short duration from a very small amount of tissue using this technique while it takes longer time and favourable season is required in the conventional methods. Furthermore, the micropropagation is highly useful in mass propagation of specific clones, production of virus free plant via meristem culture, clonal propagation of parental stock for hybrid seed production and germplasm preservation of rare, endangered and threatened plant species. Thus, this technique is more significant for the introduction and conservation of new varieties and production of disease free plants.

Vegetative propagation of plant by conventional breeding method is time consuming and results variation in the out-coming genera while genetically pure elite population can be obtained through the *in vitro* tissue culture technique. This technique has been accepted as potential approach for the mass scale propagation of valuable plant species, particularly those species that have recalcitrant seeds and slow rate of vegetative propagation. So, the *in vitro* culture is highly fruitful for orchids as their seeds have low rate of germination in the nature and slow process of vegetative propagation.

Under the *in vitro* culture all required nutrient materials and external environmental facilities are provided artificially. This technique is independent to seasons and can be carried out throughout the year which is highly useful for *ex-situ* conservation of rare and endangered species. *Ex-situ* conservation is one of the most effective ways of conservation (Falke, 1990). The culture initiation depends on the physiological status of explants and the chemical stimulus present in the nutrient medium. Orchids are the very first horticultural plant cloned by tissue culture method on commercial scale (Pant, 2006).

1.1.2 Plants and their Morphology

***Coelogyne stricta* (D. Don) Schltr.**

Synonyms:

1. *Coelogyne elata* Lindl.
2. *Cymbidium strictum* D. Don
3. *Pleione elata* (Lindl.) Kuntze

C. stricta are commonly known as 'The Rigid Coelogyne Pseudobulbs'. They are epiphyte on tree trunks of lithophytes on mossy rocks, forms large colonies, warm to cold growing, likes partial shade, occurs in primary subtropical forest at elevations of 1400-2000 m asl. Found in Eastern and Central Nepal, Bhutan, China, India, Laos and Myanmar. Flower blooming time is April- June.

Pseudobulbs oblong-cylindrical, polished, sheathed at the base, 7.6 to 12.7 cm long and 2.5-5.0 cm in diameter, rising at distances of about 5 cm from a very stout sheathed rhizome. Leaves in pairs, thinly coriaceous, narrowly elliptic-oblong, acute, narrowed at the base to the long petiole; length of blade 17.8-35.6 cm, breadth 3.8-7.0 cm, of petiole 3.2 to 7.6 cm. Peduncle from the apex of the adult pseudobulb between the leaves and rather shorter than them, naked below, bearing many short imbricate sheaths just under the raceme. Raceme 10.2-15.2 cm long; distichous, laxly many flowered. Flowers 3.2 cm across; floral bract sub-rhomboid, acute, conduplicate, slightly longer than the stalked ovary, caducous. Sepals sub-equal, oblong, sub-acute, spreading. Petals as long as the sepals but much narrower, sub-acute. Lip elongate, the lower part oblong and with narrow entire side lobes, separated from the sub-orbicular anterior lobe by an erose-edged sinus; anterior lobe irregularly erose, undulate, obtuse; the disc with two erose-crenulate lamellae from the base to near the apex. Column winged in its upper half, the wing erose at the apex; pollinia dimidiately ovate, capsule clavate.

The flowers are ochraceous white in colour, the lip having a broad spot of yellow and pink on the lamellae on its anterior lobe, the lower part of the lamellae being yellow; the column is white and the ovary pinkish-brown. The flowers have rather an unpleasant smell (King and Prantling, 1970).

***Coelogyne flaccida* Lindl.**

Synonyms:

1. *Coelogyne lactea* Rchb.f
2. *Pleione flaccida* (Lindl.) Kuntze
3. *Pleione lactea* (Rchb.f.) Kuntze

C. flaccida are commonly known as 'The Loose Coelogyne Pseudobulbs'. They are epiphyte on tree trunks, warm growing, like partial shade, occurs in tropical to subtropical forest at elevations of 900-1100 m asl. Found in central Nepal, China, India, Laos and Myanmar. Flower blooming time is April.

Pseudobulbs ovoid-cylindrical, with large fibrous brown sheaths at the base, 5.0 -15.2 cm long and 2.0-2.5 cm in diameter. Leaves thinly coriaceous, in pairs, narrowly oblong, acuminate, narrowed into a long channeled petiole, length 10.2-15.2 cm, breadth 2.0-3.2 cm, the petiole 3.8-5.0 cm. Raceme from the base of the pseudobulb, decurved, many flowered, lax, as long as the leaves; its peduncle short and with large lax sheaths at the base. Flowers 3.8 cm across (vertically); bracts ovate-cymbiform, acute, 2.0-2.5 cm long, caducous. Sepals sub-equal, oblong-lanceolate, acute, spreading. Petals narrowed and shorter than the sepals, acute. Lip oblong, 3-lobed; the side lobes long, with acute apices; the terminal lobe oblong, reflexed; the disc with three yellow flexuose ridges between the side lobes. Column long, broadly and erosely hooded at the apex. Pollinia oblong. Capsule ellipsoid, tapering to both ends but especially to the lower, winged, 3.8- 4.4 cm long.

The sepals and petals are of a pale brown colour; the lip is of a darker brown, the terminal lobe however being white with a yellow patch near its base; the lamellae are yellow. The column is white with a broad yellow streak in front and the anther is buff-coloured. The flowers have a disagreeable smell (King and Prantling, 1970).

1.1.3 Importance of plants

- Both *Coelogyne stricta* and C. *flaccida* are important from conservation point of view as both are listed under Appendix II of CITES.
- Both orchids have high aesthetic values so are used as ornamental plants in different gardens, nurseries, hotels, etc.

- Both orchids have medicinal values. Paste of pseudobulb of *C. stricta* is applied to the forehead against headache and fever (Baral and Kurmi, 2006) and the paste of pseudobulb of *C. flaccida* is applied to the forehead to treat headache and the juice is taken for indigestion (Manandhar, 2002).
- Both orchids fetch high prices in the national and international markets.

1.2 Hypothesis

Under similar conditions of light, temperature and medium, *in vitro* growth rates are different for the intra-generic species.

1.3 Objectives

1.3.1 Broad objective

To develop the tissue culture protocol for the seed germination and seedlings growth of *Coelogyne stricta* (D. Don) Schltr. and *Coelogyne flaccida* Lindl. from immature seeds and their acclimatization in different substrates.

1.3.2 Specific objectives

- To determine the appropriate condition for *in vitro* mass propagation of *C. stricta* and *C. flaccida.*
- To compare and determine the effect of each combination of MS medium on *in vitro* seed germination of *C. stricta* and *C. flaccida*.
- To find-out the best acclimatization substrate for the *in vitro* grown plantlets of *C. stricta* and *C. flaccida* for their *ex-situ* conservation.

1.4 Rationale

High market price of orchids in the national and international markets has led to its rampant collection from its natural habitat. Many orchids are vanishing from their natural habitat due to their reckless collection by orchid enthusiasts, illegal trade, deforestation, habitat destruction and natural disasters. As a consequence, they are restricted to narrow pocket areas in their natural habitat. So, from both conservation and commercial point of view it is imperative to develop a quick method for the propagation of orchids.

Orchid seeds lack functional endosperm so the germination of seeds requires a long period of time and an aid of suitable fungus. Its vegetative propagation is slow and any disturbance to the soil or physical environment destroys the whole population. So, *in vitro* multiplication provides the best solution to this problem. Therefore, the development of tissue culture protocol of different orchids is the demand of time and an essential pre-requisite, especially for large scale commercial propagation and their conservation in their natural habitat.

Coelogyne stricta and *C. flaccida* selected for the present study are highly ornamental plants having high market values. Furthermore, both orchids have medicinal values and are highly threatened in nature; cited in Appendix II of CITES. A perusal of available literature revealed that no tissue culture protocol for these plants have been developed using immature seeds in MS medium and medium supplemented with different combinations of BAP and NAA. Thus, the present investigation is undertaken to develop an efficient protocol for the *in vitro* micropropagation of these orchids using immature seeds for their *ex-situ* conservation and germplasm preservation.

CHAPTER II

LITERATURE REVIEW

Till date lots of research has been done on tissue culture in the national and international laboratories of the world. Some of the relevant literatures reviewed are:

Bopaiah and Jorapur (1986) germinated seeds of *Cymbidium aloifolium* on modified Knudson C (MKC) medium supplemented with 10% coconut milk (CM) and 3 mg/l each of peptone (P) and casein hydrolysate (CH). The protocorm like bodies obtained from the 8 weeks old primary cultures were sub-cultured on fresh medium containing all the above nutrients besides 200 g/l banana pulp. The additional presence of vitamins (thiamine HCl, niacin, pyridoxine), auxins (NAA, 2,4-D), cytokinin (KN) and amino acid (glycine) in different concentration on the nutrient medium comprising MKC + CM + P + CH + banana pulp was found to be the most suitable for the growth of healthy seedlings.

Mitra (1986) cultured orchid seeds obtained from green pods of different species which germinated readily after 8-12 weeks of culture. Of the various media used for seed germination the one used by Mitra *et al.* (1976) with lesser amount of ammonia, and of nitrates of potassium and calcium and of phosphate ions along with several vitamins was found to be the most suitable for a large number of orchid species. The additional presence of amino acids, casein hydrolysate, yeast extract, coconut milk, auxin, cytokinin, adenine sulphate, gibberellin, urea and peptone in the media yielded better germination of embryo and protocorm formation.

Reddy *et al*. (1992) studied *in vitro* seed germination and seedling development of four different orchids on four different media. The germination percentage, protocorm development and the extent of seedling growth varied with species and culture medium. MS (Murashige and Skoog, 1962) and RL (Rosa and Laneri, 1977) media yielded better results than Kn (Knudson, 1946) and VW (Vacin and Went, 1949). The ground orchid *Spalthaglottis plicata* responded better on MS medium while epiphytic *Epidendrum radicans, Dendrobium crepidatum* and *Cymbidium aloifolium* on RL medium.

Pyati and Murthy (1995) studied *in vitro* seed germination and seedling development of *Dendrobium ovatum* on four different basal media, viz., Knudson C (KC), Vacin and Went (VW), Murashige and Skoog (MS) and Burgeff (N_3f). KC medium was found to provide the optimum condition for the seed germination. KC medium supplemented with 100 mg/l coconut milk, 100 mg/l cane juice, 200 mg/l yeast extract, 200 mg/l casein hydrolysate, 200 mg/l peptone and 0.5 mg/l nicotinic acid was found to be the best for seed germination.

Sharma and Chauhan (1995) studied the acclimatization of *in vitro* grown seedlings of *Dendrobium chrysanthum* and *Paphiopedilum spicerianum* in different potting mixture. *D. chrysanthum* showed the best response in the potting mixture comprising brick, charcoal, tree fern, bark pieces, leaf mould and dry sphagnum moss in the ratio of 1:1:1:1:1:2 while *P. spicerianum* showed the best hardening in the mixture of leaf mould, perlite, vermiculate and dry sphagnum moss in the ratio of 1:1:1:2.

Pant and Gurung (2005) cultured seeds of *Aerides odorata* on MS medium and medium supplemented with different combinations of BAP and NAA. Hormone-free MS medium was found to be the best for the early germination, higher number of protocorms formation and their further differentiation. Combination of BAP (2 mg/l) and NAA (1 mg/l) proved to be the best for seed germination among all other hormonal combinations. For the seed germination BAP was more effective than NAA and lower concentration of BAP and NAA were more effective than their respective higher concentration.

Shrestha (2005) successfully achieved asymbiotic germination of *Coelogyne ovalis.* MS medium supplemented with 1 ppm NAA was found to be the most favourable combination for the germination, growth and development of seedlings. The maximum number of shoots was obtained on media supplemented with 1 ppm BAP while MS media with 2 ppm IBA showed best rooting.

Stewart and Kane (2006) successfully germinated seeds of *Habenaria macroceratitis* on six different asymbiotic media, viz., Murashige and Skoog, Knudson C, Vacin and Went, Modified Lucke, Modified Malmgren and Lindemann medium and obtained germination within 7 weeks of inoculation with varying

germination percentage on different media. *In vitro* seedlings cultured under 16/8 L/D produced the highest number of tubers per seedling (1.06).

Franco *et al.* (2007) studied the hardening of *Cattleya trianae* developed from *in vitro* culture. They evaluated 10 substrates, some organic (pine bark, coconut fibre and wood shavings), some inert icopor (polystyrene foam), vegetable coal and their combinations, and the effects these have on morphometric and phenotypic traits in the hardening phase of 250 plants of *C. trianae* cultivated *in vitro*. Coconut fiber alone or mixed in equal parts with bark and coal was found to the most effective substrate when percentage survival (80%), biomass and leaf length were evaluated. Under greenhouse conditions, plants grew better with filtered light, relative humidity bordering on 80%, permanent aeration, misting with water and an average temperature of 25±2°C.

Shadang *et al*. (2007) studied the effects of four different media, viz., Murashige and Skoog (MS), Modified Knudson C (MKC), Vacin and Went (VW) and Ichihashi and Yamashita (IY) and their half strength on seed germination and subsequent *in vitro* development of protocorms of *Hygrochilus parishii*. Seeds germinated within 15-20 days of culture on all media with varying percentage and developed into greenish protocorm after 80-100 days. The best germination percentage was observed on half strength of MKC medium.

Pradhan and Pant (2009) cultured immature seeds of *Cymbidium elegans* and *Dendrobium densiflorum* on MS medium. *C. elegans* showed the best response on MS medium supplemented with 1 mg/l BAP where seeds germinated after 9 weeks of culture whereas *D. densiflorum* showed the best response on hormone-free MS medium with germination after 5 weeks of culture.

Basker and Narmatha Bai (2010) studied the seed germination of *Eria bambusifolia* on two different media, viz., Murashige and Skoog (MS) and Knudson C (KC). MS was found to be the best medium with germination percentage of 48 and protocorms developing after 7 weeks of culture.

Rajkarnikar (2010) cultured seeds of *Dendrobium amoenum* on MS medium supplemented with 0.1% casein hydrolysate, 10% coconut milk and 3% sucrose. MS

medium supplemented with BAP (1 mg/l) and NAA (1 mg/l) was found to be the best combination for the further multiplication of PLBs and healthy seedlings growth.

Nongdam and Chongtham (2011) studied the *in vitro* seed germination of *Cymbidium aloifolium* in two different media, viz., Murashige and Skoog (MS) and Mitra (M), and media supplemented with different combinations of BAP, NAA and IBA. MS medium supplemented with 0.5 mg/l NAA was found to be the best combination for the seed germination and seedling growth among the MS combinations. Protocorms, first shoot and seedlings were obtained after 5-6 weeks, 6-7 weeks and 11-12 weeks of culture respectively.

Pant and Swar (2011) cultured seeds of *Cymbidium iridioides*. The growth and development of seedlings were best observed on MS medium supplemented with BAP (1 ppm) and NAA (1 ppm). Protocorms vigorously obtained on this condition developed root and shoot and complete seedlings were obtained just after 8 weeks of primary culture.

Pant *et al.* (2011) studied the *in vitro* seed germination and seedling development of *Phaius tancarvilleae* on hormone-free MS medium and medium supplemented with different combinations of BAP and NAA. MS medium supplemented with 0.5 mg/l BAP was found to be the ideal condition for the early seed germination, protocorm formation and development of seedlings. The germination started after 7 weeks and complete seedlings were obtained after 24 weeks of culture.

Rajkarnikar (2011) cultured seeds of *Cymbidium aloifolium* on MS medium supplemented with 0.1% casein hydrolysate and 3% sucrose. MS medium supplemented with BAP (2 mg/l), NAA (1.5 mg/l) and coconut milk (10%) was found to be the best combination for the further multiplication of PLBs and micro-shoots.

Abraham *et al.* (2012) cultured mature seeds of *Coelogyne nervosa* on 1/2 MS (Murashige and Skoog), MS, Kn (Knudson) and VW (Vacin and Went) media to evaluate the seed germination response. Of the four basal media used, MS medium favoured the maximum seed germination. Further experiments to enhance seed germination were done on MS medium supplemented with various concentrations

(10, 20, 30 and 40 %) of coconut water (CW). 30% CW gave the highest response in terms of seed germination (96%), fresh weight (7.2 mg/seedling) and seedling length (15.2 mm).

Gogoi *et al*. (2012) studied the *in vitro* seed germination of *Cymbidium eburneum* on five different media, viz., Murashige and Skoog (MS), Gamborg (B5), Mitra (M), Knudson C (KC) and Nitsch (N). The highest germination rate was observed on Mitra medium whereas the development of the protocorms were found be the best on MS medium with protocorms developing after 9 weeks of culture.

Paudel *et al.* (2012) studied *in vitro* seed germination and seedling growth of *Esmeralda clarkei* on hormone-free MS medium and medium supplemented with different combinations of BAP and NAA. Hormone-free MS medium was found to be the best combination for the formation of protocorm and development of seedlings. The protocorms and seedlings were obtained after 16 weeks and 25 weeks of culture respectively.

Prakash *et al.* (2012) studied the effect of different media, viz., Murashige and Skoog (MS), Vacin and Went (VW), Knudson C (Kn C) and Raghavan and Torrey (RT) on *in vitro* seed germination and protocorm formation of *Vanda tessellata*. They obtained the highest seed germination (95%) and protocorm formation (90%) on MS medium while seed germination and protocorm formation on VW, Kn C and RT was found to be (40%, 37%), (65%, 50%) and (30%, 20%) respectively.

Wesley *et al.* (2013) studied the asymbiotic seed germination of three important orchids, viz., *Coelogyne breviscapa, Dendrobium aqueum* and *Flickingeria nodosa* on five different basal media, viz., Murashige and Skoog, Linsmaier and Skoog, Lindemann Orchid, Schenk and Hildebrandt and Knudson C. On MS medium the seeds of *C. breviscapa* germinated after 60 days with only 19% germination and *D. aqueum* took 60 days for germination with only 17% germination. *F. nodosa* did not germinate at all on MS medium.

CHAPTER III

MATERIALS AND METHOD

3.1 Plant materials

The plant materials used for the present study were 8 weeks old immature capsules of *Coelogyne stricta* (D. Don) Schltr. and *Coelogyne flaccida* Lindl. The capsules were collected from the orchid house of National Botanical Garden, Godawari, Kathmandu.

3.2 Methodology

3.2.1 Preparation of stock solution for MS medium

The solidified Murashige and Skoog (1962) medium was used as the basal medium for the present research that consists of macronutrients, micronutrients, iron source, vitamins, sucrose and agar. The composition of the MS medium is as follow:

Table 1: Stock solution of nutrient salts

Table 1a: Stock solution of macronutrients (Stock A)

Chemical components	Composition of MS (final conc.) mg/l	(10X) g/l stock concentration	Volume to be taken for 1 liter medium
Potassium nitrate (KNO_3)	1900	19.0	100 ml
Ammonium nitrate (NH_4NO_3)	1650	16.5	
Calcium chloride ($CaCl_2.2H_2O$)	440	4.4	
Magnesium sulphate ($MgSO_4.7H_2O$)	370	3.7	
Potassium dihydrogen phosphate (KH_2PO_4)	170	1.7	

Table 1b: Stock solution of micronutrients (Stock B)

Chemical components	Composition of MS (final conc.) mg/l	(100X) mg/100 mlstock concentration	Volume to be taken for 1liter medium
Boric acid (H_3BO_3)	6.2	620	1 ml
Manganese sulphate ($MnSO_4.4H_2O$)	22.3	2230	
Zinc sulphate ($ZnSO_4.7H_2O$)	8.6	860	
Sodium molybdate ($Na_2MoO_4.2H_2O$)	0.25	25	
Cobalt chloride ($CoCl_2.6H_2O$)	0.025	2.5	
Copper sulphate ($CuSO_4.5H_2O$)	0.025	2.5	
Potassium iodide* (KI)	0.83	83	

* 100 ml stock solution of KI was made separately and used as 1 ml/l in MS medium.

Table 1c: Stock solution of iron source (Stock C)

Chemical components	Composition of MS (final conc.) mg/l	(10X) mg/100 ml stock concentration	Volume to be taken for 1liter medium
Sodium Ethylene diamminetetraacetate (Na_2EDTA)	37.3	373	10 ml
Ferrous sulphate ($FeSO_4.7H_2O$)	27.8	278	

Table 1d: Stock solution of vitamins (Stock D)

Chemical components	Composition of MS (final conc.) mg/l	(100X) mg/100 ml stock concentration	Volume to be taken for 1liter medium
Glycine	2.0	200	1 ml
Nicotinic acid	0.5	50	
Pyridoxine HCl	0.5	50	
Thiamine HCl	0.1	10	
Myo-inositol**	100	10,000	

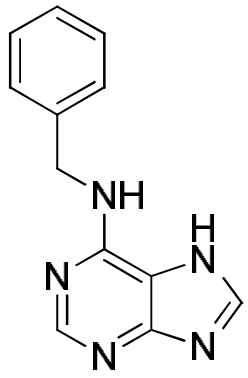
** Myo-inositol was freshly made at the time of media preparation (100 mg/l).

Sucrose (30 g/l) was used as a carbon source and agar (8 g/l**)** was used for the solidification of the medium.

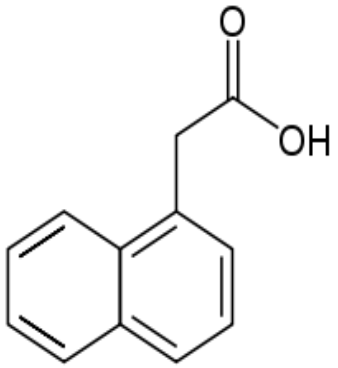
During the preparation of each stock solution, the above mentioned chemicals were weighed with precise accuracy using digital balance (0.001 g) and dissolved completely one by one in a conical flask containing sterile water with the help of magnetic stirrer according to the protocol of Bhattarai (2000). The final volume was made to 1 liter for stock A and 100 ml each for stock B, C and D by adding sterile water. The stock solution of potassium iodide (KI) was prepared separately because it causes the precipitation in the stock solution B. All the stock solutions were kept in sterile dark bottles and preserved in the refrigerator at 4°C due to light sensitivity.

3.2.2 Hormones used for the experiment

Hormones are organic substances which are naturally produced in plants and also found in synthetic form. These are of various types and differ in their activity but most of them stimulate the growth and development of plants. In the present experiment, 6-Benzylaminopurine (BAP) as a cytokinin and α- Naphthalene acetic acid (NAA) as an auxin was used.



BAP (6-Benzylaminopurine)



NAA (α-Naphthalene acetic acid)

3.2.3 Preparation of Hormones stock solution

10 mg of NAA and BAP were weighed carefully and dissolved in 2.5 ml of 1N NaOH in separate beakers and 50 ml of sterile water was added. The pH of the hormones was maintained 5.8 using 0.2N NaOH and 0.2N HCl. Final volume of solution was made to 100 ml by adding sterile water making stock of 100 ppm. Both

stock solutions were then kept in clean dark brown bottle and preserved in the refrigerator at 4°C.

3.2.4 Preparation of nutrient medium

Firstly, the media preparation table was sterilized by cleansing with cotton soaked with 70% ethyl alcohol and the all required stock solutions, sterile water, jars, conical flasks, measuring cylinder and heating volumetric flask were set aside on the table. Then, required amount of each stock solution i.e. stock A, 100 ml/l; stock B, 1 ml/l; stock C, 10 ml/l and stock D, 1 ml/l were poured one by one in sterile flask accordingly for 6 liter media and mixed homogenously using magnetic stirrer. After that 6 ml of potassium iodide and 600 mg of myo-inositol was added to the mixture followed by 180 g of sucrose. The final volume of the medium was made up to 6 liter by adding required amount of sterile water. The pH of the medium was adjusted to 5.8 using 0.2N NaOH and 0.2N HCl. The medium was then poured in 15 different conical flasks; each containing 400 ml of medium and different combination of hormones were added to each flask. They were then solidified with 0.8% agar and boiled on heater till the agar got completely dissolved. The 15 different combinations of media prepared were poured into 180 different jars; each jar containing about 33 ml media. Jars were then covered tightly with its plastic cover. The jars containing medium were then labeled with permanent marker and sterilized using an autoclave at pressure of 15 psi and temperature of 121°C for 20 minutes. After cooling down, the jars were taken out from the autoclave and kept in an incubation room (25±2°C).

3.2.5 Method of Sterilization

3.2.5.1 Sterilization of glasswares and metallic instruments

During the experiment all the necessary glasswares and metallic instruments were subjected to dry heat sterilization before its use. Glasswares such as beakers, conical flasks, measuring cylinder, pipettes, petridish were dipped in detergent water for a whole day, rinsed with the help of bottle brush and cleaned by tap water. Those cleaned glasswares were sterilized by moist heat in an autoclave at 15 psi and 121°C for 20 minutes. The metallic instruments like forceps, spatula and

surgical blade handle were wrapped with aluminum foil before keeping inside the hot air oven for sterilization at 180°C for 2 hours.

3.2.5.2 Sterilization of Inoculation chamber

Before inoculation of explants, the laminar air flow chamber was sterilized by cotton soaked with 70% ethyl alcohol. All the requirements for inoculation i.e. 70% ethyl alcohol, sterile water, petriplates, beaker, forceps, spatula, surgical blade with its handle, jars with media except the plant material were exposed under ultraviolet (UV) radiation for 45 minutes. Then UV light was turned off and air blower switched on. After 20 minutes, the laminar air flow chamber was ready for inoculation.

3.2.5.3 Sterilization of plant materials

The young capsules, kept in a beaker covered with muslin cloth, were first washed under running tap water to remove the dust and soil particles present on their surface. After that few drops of tween-20 solution was added and run under tap water for about 50 minutes until water in the beaker became clear. Then it was transferred to pre-sterilized laminar air flow chamber. Capsules were rinsed in 70% ethyl alcohol for 2 minutes and then dipped in 1% solution of sodium hypochlorite for 10 minutes. Finally the capsules were washed with sterile water for 5 times.

3.2.6 Inoculation of seeds

Capsules of both orchids were put on sterile petridish containing sterile filter paper to absorb the traces of water present on its surface. Forceps and spatula to be used were incinerated by dipping in 95% ethyl alcohol and flaming at least 3 times over burning spirit lamp and allowed to cool. Each capsule was then cut longitudinally into two equal halves using sterile surgical blade. Then seeds of orchids were scrapped with the help of sterile spatula and inoculated on control MS medium and the medium supplemented with different combination of growth hormones in the vicinity of burning Bunsen burner. The inoculated jars where then tightly closed using its labeled plastic cover. After that the jars were transferred to the incubation room where temperature was maintained at 25± 2°C under photoperiod of 16/8 hours light/dark cycle.

3.2.7 Data recording

After the jars were transferred to the incubation room jars were regularly observed for germination and after the initiation of germination data was taken at regular intervals of one week. The data for initiation of germination, protocorm formation, 1st shoot formation and 1st root formation for each 15 combination of both species were taken. The shoot lengths of 10 randomly taken samples of plantlets of each 15 combination of both species were recorded on the 25th week of culture. The photographs of distinct phases of germination and their growth and development were also taken accordingly.

3.2.8 Acclimatization

The *in vitro* grown plantlets having well developed roots and measuring more than 2.5 cm were then acclimatized according to the following steps:

- The jars containing well rooted plants were opened and kept at room temperature for one week.
- The plantlets were then picked out and washed with running water to remove traces of agar attached to it.
- The plantlets were dipped in fungicide bapistine (0.1%) for 5 minutes to minimize the chances of infection.
- After that the plantlets were washed with distilled water and dried using blotting paper.
- The plantlets were then transferred to cleaned plastic trays containing different potting mixtures as follow:
    - Coco-peat and sphagnum moss in the ratio of 2:1
    - Coco-peat, sphagnum moss and sand in the ratio of 2:1:1
    - Coco-peat, sphagnum moss, sand and soil in the ratio of 2:1:1:1
    - Coco-peat and sand in the ratio of 2:1
    - Coco-peat, sand and soil in the ratio of 2:1:1

- NPK solution (5%) was sprayed once a week for fastening their growth.
- The plants were then covered using transparent polythene sheets, with holes made for aeration, to control humidity.
- The plants were kept in green house for 3 months and finally transferred to the natural environment.

3.2.9 Statistical Analysis

Statistical analysis was done using SPSS 16.0. The data obtained were analysed using Microsoft Excel 2010. Normality test (Shapiro-Wilk test as $n < 100$) was done of the shoot length data of different combinations of MS medium of both species. Paired T-test was done for the normal data and Wilcoxon test for the non-normal data of the shoot lengths taken. Furthermore, Kruskal Wallis test was done to compare the shoot length growth of both species since the data obtained were non-parametric.

CHAPTER IV

RESULTS

The germination of seeds and growth and development of seedlings were markedly influenced by the presence of growth regulators in the medium. The results of *in vitro* seed germination of *Coelogyne stricta* and *C. flaccida* have been described under different headings according to different combinations of hormones used, growth rates of shoots of *C. stricta* and *C. flaccida* and acclimatization of plantlets.

Table 2: Effect of growth regulators supplemented to MS medium on seed germination and seedling growth of *Coelogyne stricta* (D. Don) Schltr.

Medium combination	Observation taken in weeks				Remarks
	Initiation of germination	Protocorm formation	1st shoot formation	1st root formation	
MS	5	8	13		Average
MS + 0.5BAP	5	9	16		Poor
MS + 1BAP	8	14	19		Poor
MS + 1.5BAP	8	14	19		Poor
MS + 2BAP	7	13	21		Poor
MS + 0.5NAA	5	8	12	25	Good
MS+0.5BAP+0.5NAA	5	8	11	24	Good
MS+1BAP+0.5NAA	6	9	14	29	Good
MS+1.5BAP+0.5NAA	5	9	14	25	Good
MS+2BAP+0.5NAA	6	9	13	25	Good
MS + 1NAA	8	10	13		Average
MS+0.5BAP+1NAA	7	9	12	26	Good
MS+1BAP+1NAA	7	9	13	23	Best
MS+1.5BAP+1NAA	7	10	13	24	Good
MS+2BAP+1NAA	7	10	13	25	Good

Culture conditions: 25± 2˚C, 32 weeks, 16 hours photoperiod and 6 replicates were used in each combination.

Table 3: Effect of growth regulators supplemented to MS medium on seed germination and seedling growth of *Coelogyne flaccida* Lindl.

Medium combination	Observation taken in weeks				Remarks
	Initiation of germination	Protocorm formation	1st shoot formation	1st root formation	
MS	5	7	9		Average
MS + 0.5BAP	6	7	10		Poor
MS + 1BAP	7	10	15		Poor
MS + 1.5BAP	8	11	14		Poor
MS + 2BAP	7	9	14		Poor
MS + 0.5NAA	7	9	13		Average
MS+0.5BAP+0.5NAA	6	8	10	22	Best
MS+1BAP+0.5NAA	6	7	10	24	Good
MS+1.5BAP+0.5NAA	7	8	11		Average
MS+2BAP+0.5NAA	7	8	11		Average
MS + 1NAA	6	7	9	24	Good
MS+0.5BAP+1NAA	7	8	10	23	Good
MS+1BAP+1NAA	6	7	9	23	Good
MS+1.5BAP+1NAA	6	7	10	25	Good
MS+2BAP+1NAA	6	7	10	25	Good

Culture conditions: 25± 2˚C, 32 weeks, 16 hours photoperiod and 6 replicates were used in each combination.

After inoculation of seeds different medium combinations gave different response and at different time. Seedling development showed different developmental stages, viz., initiation of germination, protocorm formation, shoot initiation and root formation. The medium combination that produced the 1st well developed seedling was regarded as the best combination, the medium combinations that produced well developed seedlings within 32 weeks of culture were regarded as good, the medium combinations with only well-developed shoots but no root within 32 weeks of culture were regarded as average and the medium combinations with only poorly developed shoot and no root within 32 weeks of culture were regarded as poor.

1. MS Basal Medium

On hormones free MS medium, *C. stricta* required 5 weeks for seed germination. The protocorms developed in the 8th week while shoot development was observed in the 13th week of culture (Fig. 8g).

In *C. flaccida*, the germination was observed in the 5th week and the protocorms were developed in the 7th week (Fig. 9e). The shoot was developed in the 9th week.

2. MS + 0.5 BAP

On MS medium supplemented with 0.5 mg/l BAP, the seeds of *C. stricta* germinated in the 5th week. The protocorms formation followed by shoot development was observed in the 9th and 16th week respectively (Fig. 8e, 8h).

C. flaccida showed initiation of germination in the 6th week. The protocorms and shoot were observed in the 7th and 10th week respectively.

3. MS + 1 BAP

Seed culture on MS medium supplemented with 1 mg/l BAP required 8 weeks for germination in *C. stricta*. The protocorms were developed in the 14th week and the first shoot formation was observed in the 19th week.

In *C. flaccida*, the seed germination was observed in the 7th week. The protocorms and shoot were developed in the 10th and 15th week respectively.

4. MS + 1.5 BAP

C. stricta showed initiation of germination in the 8th week on MS medium supplemented with 1.5 mg/l BAP. The protocorm were developed in the 14th week and the shoot development was observed in the 19th week.

Seed germination of *C. flaccida* was observed in the 8th week. The protocorms were developed in the 11th week followed by the shoot development in the 14th week.

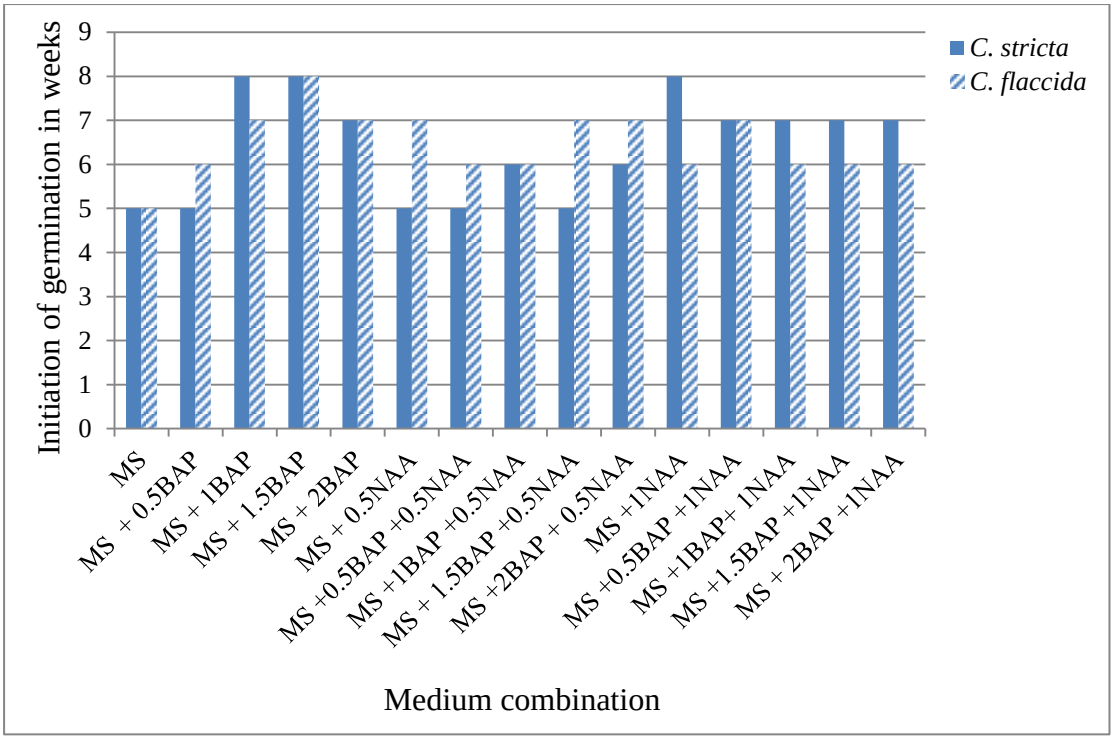


Figure 1: Comparative number of weeks taken for initiation of germination in different MS combinations of *Coelogyne stricta* and *C. flaccida.*

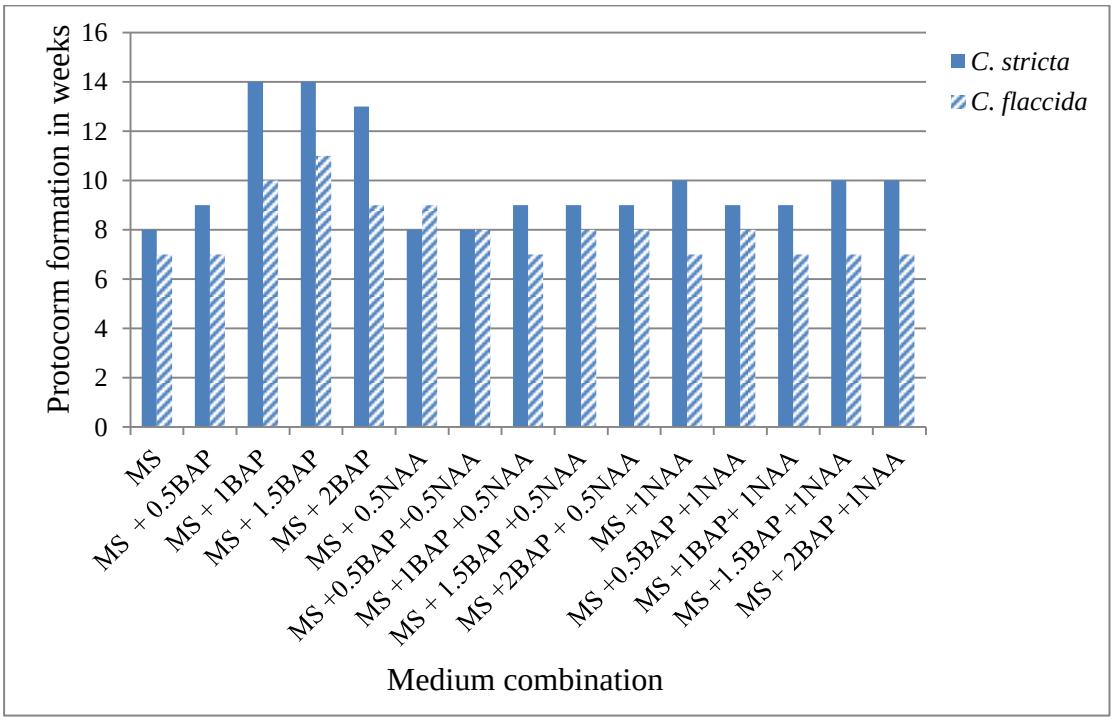


Figure 2: Comparative number of weeks taken for protocorm formation in different MS combinations of *Coelogyne stricta* and *C. flaccida.*

5. MS + 2 BAP

Seed culture on MS medium supplemented with 2 mg/l BAP required 7 weeks for seed germination in *C. stricta*. The protocorms were developed in the 13th week and the shoots were developed in the 21st week.

In *C. flaccida*, seed germination was observed in the 7th week. The protocorms formation and shoot development were observed in the 9th and 14th week respectively.

6. MS + 0.5 NAA

Seed culture on MS medium supplemented with 0.5 mg/l NAA required 5 weeks for germination in *C. stricta*. The protocorms were developed in the 8th week. The initial shoot and root development were observed in the 12th and 25th week respectively (Fig. 8j).

The germination in *C. flaccida* was obtained in the 7th week. The protocorms were developed in the 9th week and shoot was observed in the 13th week.

7. MS + 0.5 BAP + 0.5 NAA

In *C. stricta*, the initiation of seed germination was obtained in 5th week on MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA. The protocorms were developed in the 8th week followed by shoot formation in the 11th week (Fig. 8f). The first root was developed in the 24th week.

C. flaccida showed initiation of germination in the 6th week. The protocorms and shoot were first observed in 8th and 10th week respectively (Fig. 9f, 9h). The first root developed in the 22nd week and was the best condition for seedling growth of *C. flaccida*.

8. MS +1 BAP + 0.5 NAA

C. stricta showed initiation of seed germination in the 6th week on MS medium supplemented with 1 mg/l BAP and 0.5 mg/l NAA followed by protocorm development in the 9th week. The shoot and root development were obtained in the 14th and 29th week respectively.

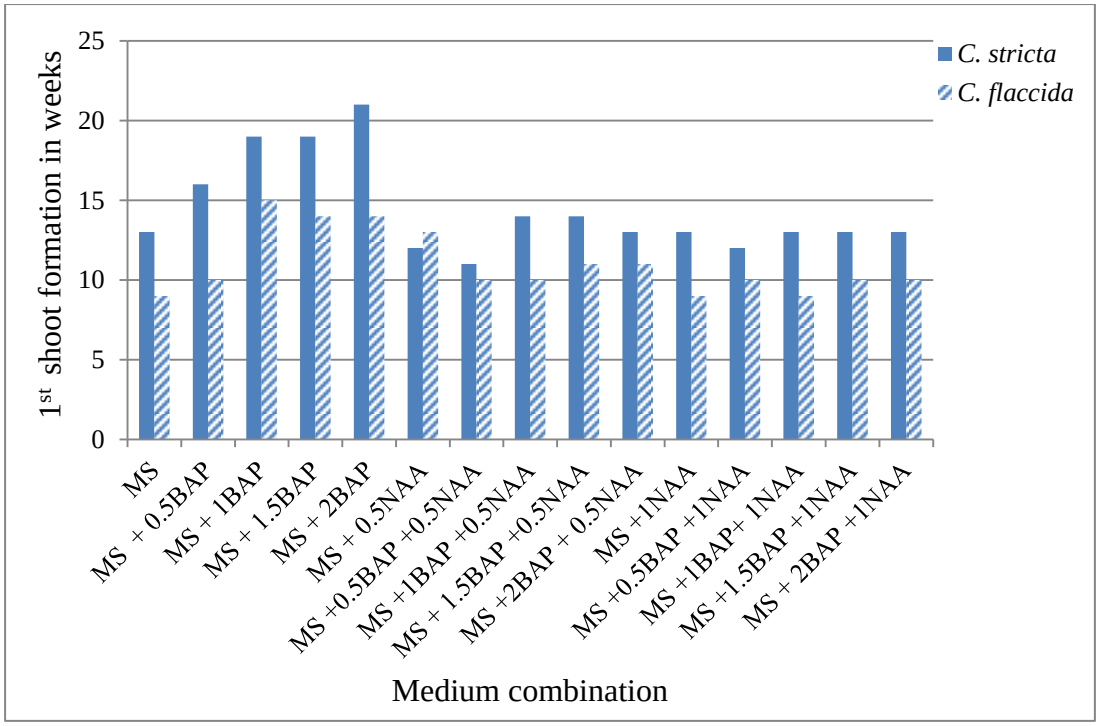


Figure 3: Comparative number of weeks taken for 1st shoot formation in different MS combinations of *Coelogyne stricta* and *C. flaccida.*

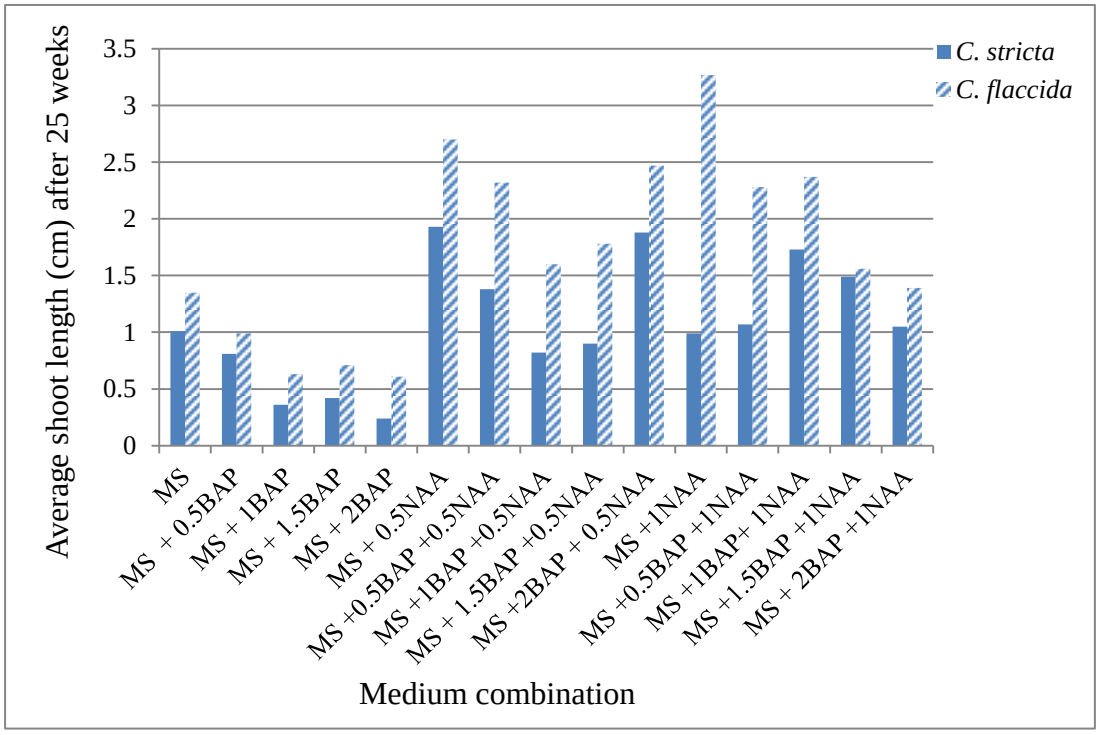


Figure 4: Comparison of average shoot length after 25 weeks of culture in different MS combinations of *Coelogyne stricta* and *C. flaccida.*

In *C. flaccida* seed germination was obtained in the 6th week. The protocorms were developed in the 7th week followed by shoot development in the 10th week. The root initial was observed in the 24th week.

9. MS + 1.5 BAP + 0.5 NAA

Seed culture on MS medium supplemented with 1.5 mg/l BAP and 0.5 mg/l NAA required 5 weeks for germination of *C. stricta*. The protocorms were developed in the 9th week and shoot was developed in the 14th week. The first root developed in the 25th week.

C. flaccida showed initiation of germination in the 7th week. The protocorms and shoot were developed in 8th and 11th week respectively.

10. MS + 2 BAP + 0.5 NAA

Seed germination required 6 weeks for *C. stricta* on MS medium supplemented with 2 mg/l BAP and 0.5 mg/l NAA. The protocorms were developed in the 9th week followed by shoot formation in the 13th week. The first root initial was observed in the 25th week (Fig. 8k).

Seed germination of *C. flaccida* was observed in the 7th week. The protocorms were developed in the 8th week followed by shoot development in the 11th week.

11. MS + 1 NAA

C. stricta showed initiation of germination in the 8th week on MS medium supplemented with 1 mg/l NAA. The protocorms were developed in the 10th week and the shoot development was obtained in the 13th week.

In *C. flaccida*, seed germination was observed in the 6th week. The protocorms and shoot were developed in the 7th and 9th week respectively (Fig. 9g, 9k). The first root was developed in the 24th week.

12. MS + 0.5 BAP + 1NAA

Seed culture on MS medium supplemented with 0.5 mg/l BAP and 1 mg/l NAA required 7 weeks for germination of *C. stricta*. The protocorms were developed in the

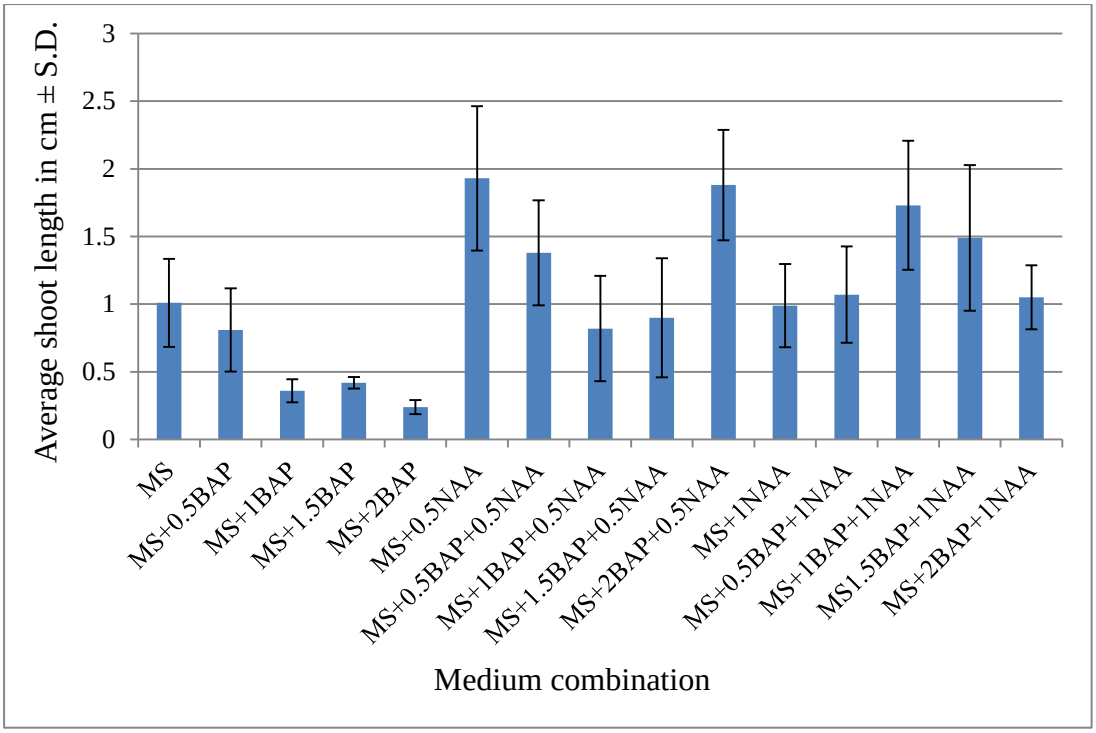


Figure 5: Average shoot length with standard deviation after 25 weeks of culture in different MS combinations of *Coelogyne stricta*.

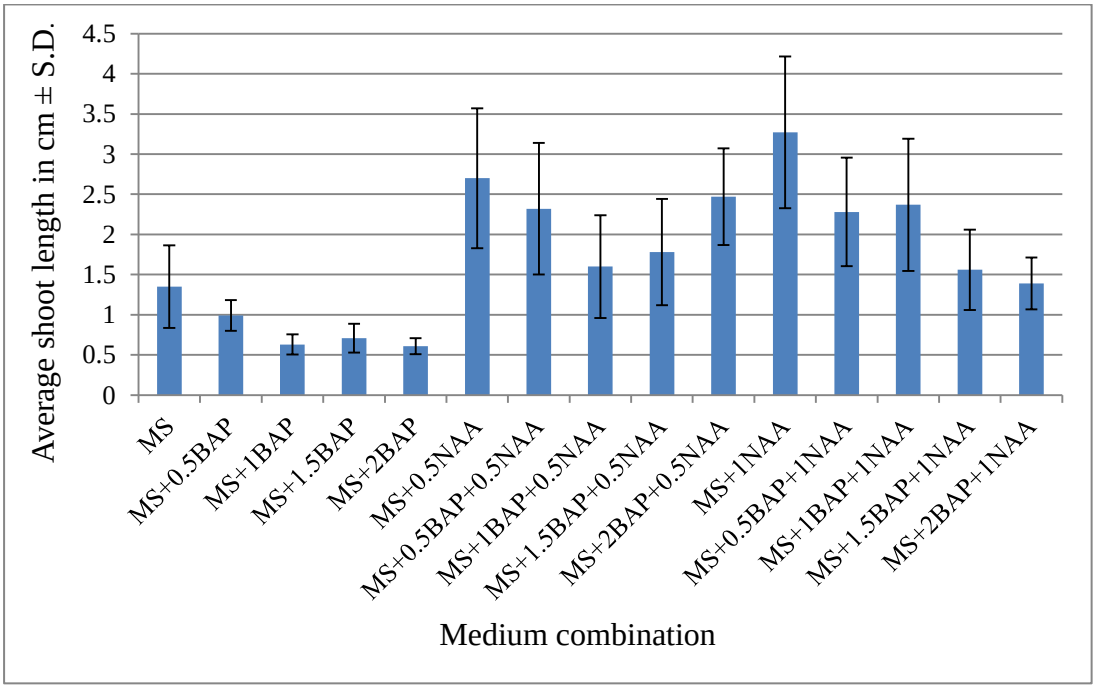


Figure 6: Average shoot length with standard deviation after 25 weeks of culture in different MS combinations of *Coelogyne flaccida*.

9th week and the first shoot and root development were observed in the 12th and 26th week.

C. flaccida showed initiation of germination in the 7th week. The protocorms were developed in the 8th week while shoot was obtained in the 10th week. The first root developed in the 23rd week (Fig. 9j).

13. MS + 1 BAP + 1 NAA

C. stricta showed initiation of germination in the 7th week on MS medium supplemented with 1 mg/l BAP and 1 mg/l NAA. The protocorms were developed in the 9th week and the shoot development was obtained in the 13th week (Fig. 8i). The first root developed in the 23rd week and was the best condition for seedling growth of *C. stricta*.

In *C. flaccida* germination was observed in the 6th week. The protocorms were developed in the 7th week followed by shoot development in the 9th week (Fig. 9i). The root initial was observed in the 23rd week.

14. MS + 1.5 BAP + 1 NAA

C. stricta showed initiation of germination in the 7th week on MS medium supplemented with 1.5 mg/l BAP and 1 mg/l NAA. The protocorms were developed in the 10th week followed by shoot development in the 13th week. The first root was observed in the 24th week.

C. flaccida showed initiation of germination in the 6th week. The protocorms were developed in the 7th week. Shoot and root were observed in the 10th and 25th week respectively.

15. MS + 2BAP + 1 NAA

C. stricta required 7 weeks for the initiation of seed germination on MS medium supplemented with 2 mg/l BAP and 1 mg/l NAA. The protocorms were developed in the 10th week. The shoot and root development were observed in the 13th and 25th week respectively.

In *C. flaccida*, the initial germination was observed in the 6th week. The protocorms and shoot were obtained in the 7th and 10th week respectively. The first root was developed in the 25th week.

Growth rates of shoots of *C. stricta* and *C. flaccida*.

Shoot lengths of 10 samples were taken after 25 weeks of culture from each MS combination of *C. stricta* and *C. flaccida* (Appendix 1). It showed significant difference in the growth rates of the two intra-generic species, viz., *C. stricta* and *C. flaccida* grown under similar conditions of light, temperature and medium (Appendix 5).

Acclimatization of plantlets

Plantlets having well developed roots and shoots and measuring more than 2.5 cm were acclimatized in five different combinations of potting mixture. Potting mixture consisted of coco-peat, sphagnum moss, sand and soil in different ratio. *Coelogyne stricta* and *C. flaccida* both showed best response of 90% and 70% respectively in coco-peat, sphagnum moss and sand in the ratio of 2:1:1.

Table 4: Acclimatization of *Coelogyne stricta* (D. Don) Schltr. in different potting mixture

Potting mixture	Ratio of potting mixture	Total number of plantlets	No. of plantlets hardened after				% of acclimatization
			2nd week	4th week	6th week	8th week	
Coco-peat + sphagnum moss	2:1	10	8	8	7	7	70
Coco-peat + sphagnum moss + sand	2:1:1	10	10	9	9	9	90
Coco-peat + sphagnum moss + sand + soil	2:1:1:1	10	6	6	5	4	40
Coco-peat + sand	2:1	10	6	6	6	6	60
Coco-peat + sand + soil	2:1:1	10	6	6	4	4	40

Table 5: Acclimatization of *Coelogyne flaccida* Lindl. in different potting mixture

Potting mixture	Ratio of potting mixture	Total number of plantlets	No. of plantlets hardened after				% of acclimatization
			2nd week	4th week	6th week	8th week	
Coco-peat + sphagnum moss	2:1	10	7	6	6	6	60
Coco-pea t+ sphagnum moss + sand	2:1:1	10	7	7	7	7	70
Coco-pea t+ sphagnum moss + sand + soil	2:1:1:1	10	6	6	5	5	50
Coco-peat + sand	2:1	10	7	6	6	5	50
Coco-pea t+ sand + soil	2:1:1	10	6	5	4	4	40

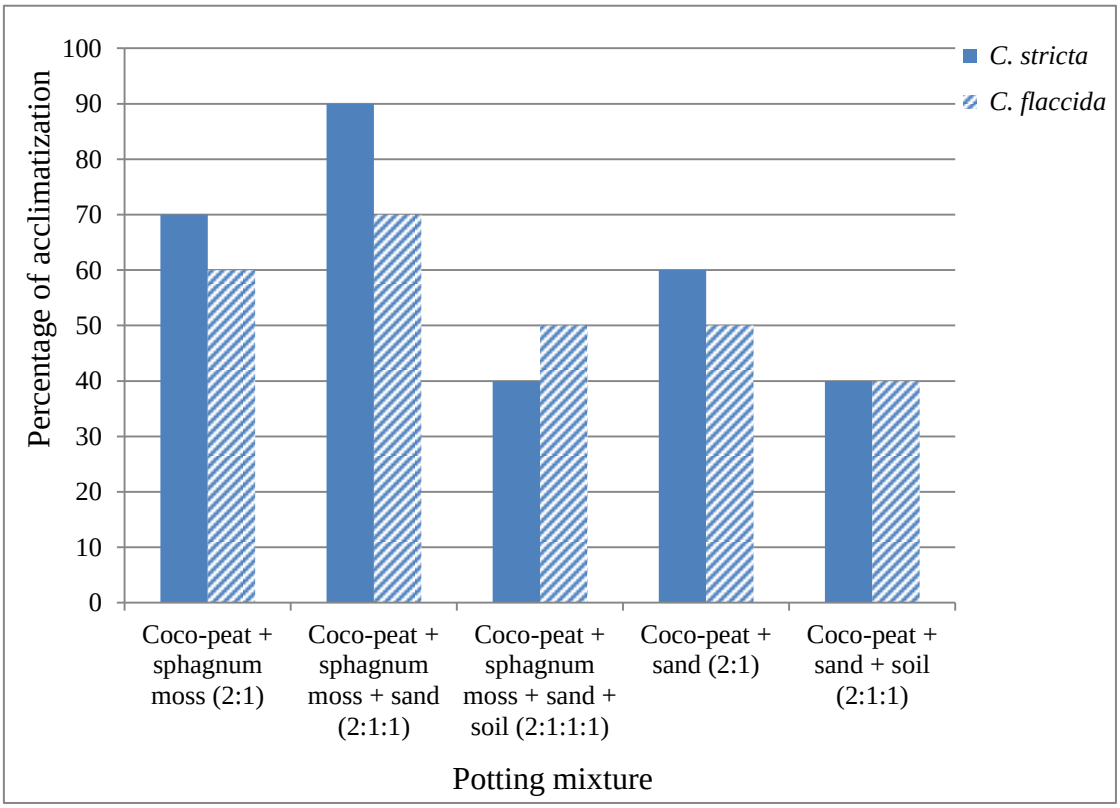
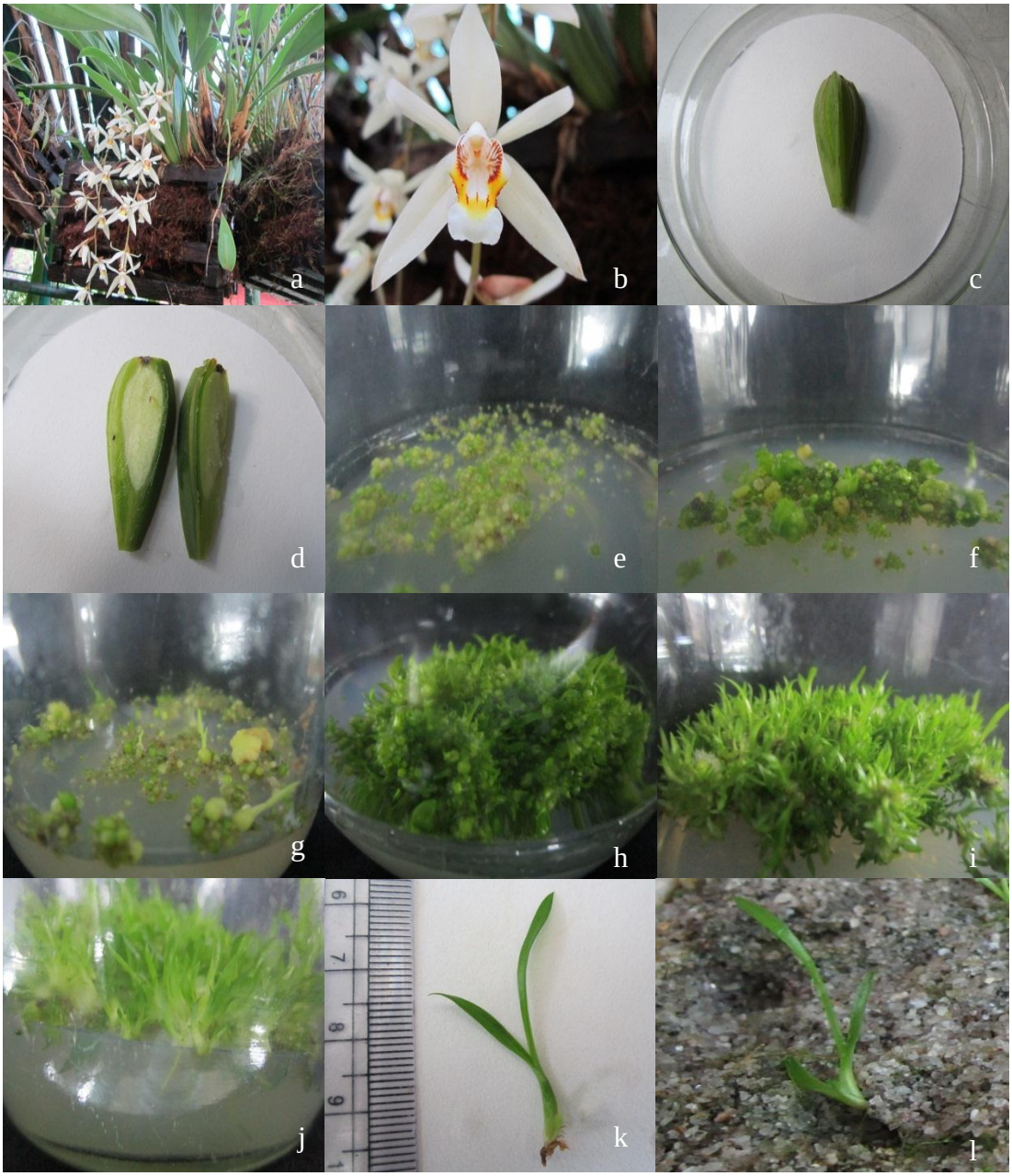


Figure 7: Comparison of percentage of acclimatization in different potting mixture of *C. stricta* and *C. flaccida*.

PHOTO PLATES OF *COELOGYNE STRICTA*

Figures 8(a-l): (a) Plant of *Coelogyne stricta* with immature capsules; (b) A flower; (c) An immature capsule; (d) Capsule cut longitudinally into two halves; (e) Clumped protocorms on MS medium supplemented with 0.5 mg/l BAP after 10 weeks of culture; (f) Initiation of shoots on MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA after 11 weeks of culture; (g) Initiation of shoots on hormone-free MS medium after 13 weeks of culture; (h) Formation of shoots on MS medium supplemented with 0.5 mg/l BAP after 20 weeks of culture; (i) Shoots on MS medium supplemented with 1mg/l BAP and 1 mg/l NAA after 20 weeks of culture; (j) Initiation of root on MS medium supplemented with 0.5 mg/l NAA after 25 weeks of culture; (k) Shoot length of seedling obtained from MS medium supplemented with 2 mg/l BAP and 0.5 mg/l NAA after 25 weeks of culture; (l) Acclimatized plant.

PHOTO PLATES OF *COELOGYNE FLACCIDA*



Figures 9(a-l): (a) Plant of *Coelogyne flaccida* with immature capsules; (b) A flower; (c) An immature capsule; (d) Capsule cut longitudinally into two halves; (e) Formation of protocorms on hormone-free MS medium after 7 weeks of culture; (f) Swelling of protocorms and initiation of shoots on MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA after 10 weeks of culture; (g) Initiation of shoots on MS medium supplemented with 1 mg/l NAA after 9 weeks of culture; (h) Formation of shoots on MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA after 12 weeks of culture; (i) Shoots on MS medium supplemented with 1 mg/l BAP and 1 mg/l NAA after 15 weeks of culture; (j) Development of root on MS medium supplemented with 0.5 mg/l BAP and 1 mg/l NAA after 24 weeks of culture; (k) Shoot length of seedling obtained from MS medium supplemented with 1 mg/l NAA after 25 weeks of culture; (l) Acclimatized plant.

CHAPTER V

DISCUSSION

In the present study two intra-generic species of orchids were taken for *in vitro* seedlings growth. Seeds from immature capsules of *Coelogyne stricta* and *C. flaccida* were cultured on hormone-free MS medium and medium supplemented with different combination of growth hormones. Immature capsules were selected for this research as it shows better germination response and saves time (Pant, 2006). The most effective germination response for *C. stricta* was found to be on MS medium supplemented with BAP (1 mg/l) and NAA (1 mg/l) whereas *C. flaccida* showed best response on MS medium supplemented with BAP (0.5 mg/l) and NAA (0.5 mg/l). The quantity and nature of growth regulators have significant effect on the germination of orchid seeds (Arditti, 1992).

The most appropriate medium was selected on the basis of time taken for germination of seeds and their growth and development. Initiation of seed germination was observed after five weeks of culture in both species. This was supported by the findings of Reddy *et al*. (1992), who studied the seed germination and seedling growth in four different species of orchids (*Cymbidium aloifolium, Dendrobium crepidatum, Epidendrum radicans* and *Spathoglottis plicata*). Hoshi *et al*. (1994) also reported similar findings in their study on the seed germination of four species of *Cypripedium*. It was also supported by the findings of Stewart and Kane (2006) on the seed germination of *Habenaria macroceratitis* and Pradhan and Pant (2009) on *Dendrobium densiflorum*.

Protocorms were obtained after 7 weeks and 8 weeks of culture in *C. flaccida* and *C. stricta* respectively. This was supported by the findings of Basker and Narmatha Bai (2010) in the seed germination of *Eria bambusifolia* which took 7 weeks for protocorms formation and Nongdam and Chongtham (2011) on *Cymbidium aloifolium* which took 5-6 weeks. Similar findings were also reported by Pradhan and Pant (2009) on *Dendrobium densiflorum* which took 6 weeks for protocorm formation, Pant *et al*. (2011) on *Phaius tancarvilleae* which took 9 weeks and Gogoi *et al*. (2012) on *Cymbidium eburneum* which took 9 weeks.

The first shoot initials were obtained after 9 weeks and 11 weeks of culture in *C. flaccida* and *C. stricta* respectively. This was supported by the findings of Pradhan and Pant (2009) on *Dendrobium densiflorum* which took 8 weeks for the first shoot formation, Pant *et al*. (2011) on *Phaius tancarvilleae* which took 12 weeks and Nongdam and Chongtham (2011) on *Cymbidium aloifolium* which took 6-7 weeks.

The seed germination with respect to protocorm formation and shoot formation was comparatively faster in *C. flaccida* than *C. stricta*. It might be due to different genetic constitution of the plant materials and different endogenous growth regulating substances present in their seeds (Basker *et al.,* 2012; Kaur and Bhutani, 2013). According to Yam *et al.* (1989), the nutritional requirements of germinating orchid seeds vary with their physiological state and this may be species specific. The nutrient requirement of orchid seeds in terms of quantity as well as form may vary at different stages of development for various species (Ernst, 1974; Arditti and Ernst, 1984; Shobhana and Rajeevan, 1993; Nagaraju and Parthasarathi, 1995).

The first root initials were obtained after 22 weeks and 23 weeks of culture in *C. flaccida* and *C. stricta* respectively. Pant *et al*. (2011) in the seed germination of *Phaius tancarvilleae* found 18 weeks needed for the first root formation. Similarly, Pradhan and Pant (2009) on *Dendrobium densiflorum* took 19 weeks and *Cymbidium elegans* 31 weeks for the root initiation.

The seed germination of orchid involves sequential phases of germination, protocorm formation and seedling development. In the present investigation also same sequence of seedling development was observed when grown on the medium *in vitro* in both species. Several researchers have suggested various growth regulators under different concentrations to promote seed germination and seedling growth (Arditti, 1979).

Tissue culture techniques have been extensively used for the mass propagation of rare, endangered, and economical plants including orchids. Medium formulation determines the efficiency of this technique. Sometimes culture explant exudes phenolic compounds which upon oxidation to quinines turn the media brown. Browning is often found to be toxic to *in vitro* growing plants (Onuoha *et al*., 2011; Ahmad *et al*., 2013). Light browning was observed in few cases of *C. flaccida* after 22 weeks of culture while it was not observed in *C. stricta*. Seed germination of orchid differs from that of

other seeds because it contains undifferentiated embryo and lacks endosperm. On medium seeds swell and later develop into round shaped structure called protocorm. Other organs like shoot and root appear subsequently (Arditti, 1967).

Complete plantlets of *C. flaccida* and *C. stricta* were obtained after 22 weeks and 23 weeks of culture respectively. This was supported by the findings of Pant *et al*. (2011) on *Phaius tancarvilleae* which took 24 weeks to develop into complete plantlets and Paudel *et al*. (2012) on *Esmeralda clarkei* which took 25 weeks to develop into complete plantlets.

A total of 36 species of native and exotic species of orchids have been germinated *in vitro* in various media including MS (1962) medium by Shrestha and Rajbhandary (1994). MS medium supplemented with BAP (1 mg/l) and NAA (1 mg/l) was found to be best for seed germination of *C. stricta*. This was supported by the findings of Pant and Swar (2011) in the study of seed germination of *Cymbidium iridioides* and Rajkarnikar (2010) in the study of the seed germination of *Dendrobium amoenum*. The condition was found to be best for the PLBs formation and seedlings growth.

Seed germination of *Coelogyne ovalis* was found to be most effective on MS medium supplemented with 1 mg/l NAA by Shrestha (2005). Pant and Gurung (2005) found hormone-free MS medium as the most effective for seed germination of *Aerides odorata*. Similarly, Pradhan and Pant (2009) found hormone-free MS medium to be most effective for the seed germination of *Dendrobium densiflorum* and MS medium supplemented with 1 mg/l BAP for *Cymbidium elegans*. Pant et al. (2011) found MS medium supplemented with 0.5 mg/l BAP as the most ideal condition for seed germination of *Phaius tancarvilleae*.

10 samples from each 15 combination of medium were taken after 25 weeks of culture to compare the growth rates of both species developed *in vitro*. Samples were taken randomly and sample size was made to 10 to minimize the error. Growth rates with respect to shoot lengths were taken after 25 weeks of culture because by the 25^{th} week complete plantlets having fair shoots and roots were developed in different hormonal combinations of both species. Under similar conditions (light, temperature and medium) growth rates are different for different species, and so for the intra-generic species. The difference in the growth rates of both species might be due to their

different genetic constitution, physiological state and endogenous growth regulators present in them.

In vitro grown well rooted plantlets of *C. stricta* (90%) and *C. flaccida* (70%) were best acclimatized on the potting mixture ratio of 2:1:1 in coco-peat, sphagnum moss and sand followed by coco-peat and sphagnum moss in the ratio of 2:1 suggesting the usefulness of coco-peat and sphagnum moss in the successful acclimatization of *in vitro* grown epiphytic orchids (Franco *et al*., 2007). Coco-peat and sphagnum moss were used because of their high water holding capacity to provide moisture to the plantlets and sand might provide the heat needed for the initiation of new roots that better suit to adapt to the natural environment. This was supported by the findings of Pant and Thapa (2012) in the acclimatization of *Dendrobium primulinum* where 70% of plantlets survived in the potting mixture of coco-peat and sphagnum moss in the ratio of 2:1. Similarly, Paudel and Pant (2012) successfully hardened 85% plantlets of *Esmeralda clarkei* in soil, sand and sawdust in the ratio of 1:1:1 and Pradhan *et al*. (2013) was successful in hardening 85% plantlets of *Dendrobium densiflorum* in 2:1:1 ratio of coco-peat, litter and clay.

CHAPTER VI

CONCLUSION

MS medium supplemented with BAP (1 mg/l) and NAA (1 mg/l) was found to be the best for *in vitro* seedlings growth of *Coelogyne stricta* with respect to other combination of hormones and hormone free media while MS medium supplemented with BAP (0.5 mg/l) and NAA (0.5 mg/l) was found to be the most effective for *in vitro* seedlings growth of *Coelogyne flaccida*. This suggests that the phytohormones BAP and NAA both are necessary for the fast growth and development of *in vitro* growing orchids from seeds to seedlings stage. Furthermore, statistical analysis showed significant difference in the growth rates of *C. stricta* and *C. flaccida* taken with respect to shoot lengths after 25 weeks of culture.

Phytohormone NAA was found to be essential for root initiation in both species as none of the *in vitro* grown species developed roots in media lacking NAA even after 32 weeks of culture.

The best acclimatization of both species on plastic tray containing coco-peat, sphagnum moss and sand in the ratio of 2:1:1 showed the importance of sand besides coco-peat and sphagnum moss for the successful acclimatization of the both epiphytic species.

There was no occurrence of intermediate callus in any tested media suggesting the possible homogeneity of the *in vitro* propagated seedlings.

CHAPTER VII

RECOMMENDATIONS

Following recommendations have been drawn from the present research work:

- Both orchids are rare and endangered so the incoming researchers are highly recommended to preserve their germplasm and conserve them in their natural habitat.
- Activated charcoal should be used for the culture of *Coelogyne flaccida* as it produced phenolic compounds in the media after 22 weeks of culture.
- Different substrates should be tried for the acclimatization of both orchids.
- Central Department of Botany should launch awareness programme or workshop regarding the status, conservation and utilization of orchids from time to time.

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APPENDICES

1. Average shoot length of 10 samples taken after 25 weeks of culture of seeds.

Name of plant	Medium combination	N	Average shoot length (cm)	Standard Deviation	S.D. Error	Minimum shoot length (cm)	Maximum shoot length (cm)
Coelogyne stricta	MS	10	1.0100	.32472	.10269	.50	1.50
	MS+0.5BAP	10	.8100	.30714	.09713	.30	1.30
	MS+1BAP	10	.3600	.08433	.02667	.30	.50
	MS+1.5BAP	10	.4200	.04216	.01333	.40	.50
	MS+2BAP	10	.2400	.05164	.01633	.20	.30
	MS+0.5NAA	10	1.9300	.53344	.16869	.90	2.60
	MS+0.5BAP +0.5NAA	10	1.3800	.38816	.12275	.70	1.80
	MS+1BAP +0.5NAA	10	.8200	.38816	.12275	.40	1.50
	MS+1.5BAP +0.5NAA	10	.9000	.43970	.13904	.30	1.60
	MS+2BAP +0.5NAA	10	1.8800	.40770	.12893	1.20	2.50
	MS+1NAA	10	.9900	.30714	.09713	.60	1.60
	MS+0.5BAP +1NAA	10	1.0700	.35606	.11260	.60	1.80
	MS+1BAP +1NAA	10	1.7300	.47621	.15059	.80	2.40
	MS+1.5BAP +1NAA	10	1.4900	.53841	.17026	.70	2.20
	MS+2BAP +1NAA	10	1.0500	.23688	.07491	.80	1.40
	Total	150	1.0720	.61547	.05025	.20	2.60
Coelogyne flaccida	MS	10	1.3500	.51262	.16210	.60	2.00
	MS+0.5BAP	10	.9900	.19120	.06046	.80	1.40
	MS+1BAP	10	.6300	.12517	.03958	.50	.80
	MS+1.5BAP	10	.7100	.17920	.05667	.40	1.00
	MS+2BAP	10	.6100	.09944	.03145	.50	.80
	MS+0.5NAA	10	2.7000	.87050	.27528	1.40	4.00
	MS+0.5BAP +0.5NAA	10	2.3200	.82030	.25940	1.20	3.80
	MS+1BAP +0.5NAA	10	1.6000	.63944	.20221	.70	2.40
	MS+1.5BAP +0.5NAA	10	1.7800	.66299	.20966	.60	2.80
	MS+2BAP +0.5NAA	10	2.4700	.60194	.19035	1.40	3.40
	MS+1NAA	10	3.2700	.94522	.29891	1.80	4.70
	MS+0.5BAP +1NAA	10	2.2800	.67462	.21333	1.20	3.30
	MS+1BAP +1NAA	10	2.3700	.82334	.26036	1.20	3.80
	MS+1.5BAP +1NAA	10	1.5600	.49933	.15790	.70	2.20
	MS+2BAP +1NAA	10	1.3900	.32472	.10269	.80	1.90
	Total	150	1.7353	.97355	.07949	.40	4.70

2. Normality test of the shoot length data.

Name of plant	Medium combination	Shapiro-Wilk		
		Statistic	df	Sig.
C. stricta	MS	.963	10	.821
C. flaccida	MS	.903	10	.234
C. stricta	MS+0.5BAP	.985	10	.985
C. flaccida	MS+0.5BAP	.857	10	.070
C. stricta	MS+1BAP	.717	10	.001
C. flaccida	MS+1BAP	.831	10	.034
C. stricta	MS+1.5BAP	.509	10	.000
C. flaccida	MS+1.5BAP	.976	10	.937
C. stricta	MS+2BAP	.640	10	.000
C. flaccida	MS+2BAP	.886	10	.152
C .stricta	MS+0.5NAA	.947	10	.635
C. flaccida	MS+0.5NAA	.936	10	.513
C. stricta	MS+0.5BAP+0.5NAA	.913	10	.305
C. flaccida	MS+0.5BAP+0.5NAA	.955	10	.727
C. stricta	MS+1BAP+0.5NAA	.908	10	.269
C. flaccida	MS+1BAP+0.5NAA	.909	10	.277
C. stricta	MS+1.5BAP+0.5NAA	.926	10	.414
C. flaccida	MS+1.5BAP+0.5NAA	.958	10	.760
C. stricta	MS+2BAP+0.5NAA	.973	10	.918
C. flaccida	MS+2BAP+0.5NAA	.950	10	.663
C. stricta	MS+1NAA	.913	10	.305
C. flaccida	MS+1NAA	.974	10	.926
C. stricta	MS+0.5BAP+1NAA	.958	10	.759
C. flaccida	MS+0.5BAP+1NAA	.974	10	.926
C. stricta	MS+1BAP+1NAA	.966	10	.851
C. flaccida	MS+1BAP+1NAA	.962	10	.809
C. stricta	MS+1.5BAP+1NAA	.936	10	.511
C. flaccida	MS+1.5BAP+1NAA	.953	10	.706
C. stricta	MS+2BAP+1NAA	.852	10	.061
C. flaccida	MS+2BAP+1NAA	.970	10	.890

3. Paired T- test on normal data of shoot length.

Medium combination	Paired Differences			t	df	Sig. (2-tailed)
	Mean	Std. Deviation	Std. Error Mean			
MS	-.34000	.69154	.21868	-1.555	9	.154
MS+0.5BAP	-.18000	.39384	.12454	-1.445	9	.182
MS+0.5NAA	-.77000	1.01768	.32182	-2.393	9	.040
MS+0.5BAP+0.5NAA	-.94000	1.06687	.33738	-2.786	9	.021
MS+1BAP+0.5NAA	-.78000	.77431	.24486	-3.186	9	.011
MS+1.5BAP+0.5NAA	-.88000	.94728	.29956	-2.938	9	.017
MS+2BAP+0.5NAA	-.59000	.64541	.20410	-2.891	9	.018
MS+1NAA	-2.28000	1.03580	.32755	-6.961	9	.000
MS+0.5BAP+1NAA	-1.21000	.85952	.27180	-4.452	9	.002
MS+1BAP+1NAA	-.64000	.91918	.29067	-2.202	9	.055
MS+1.5BAP+1NAA	-.07000	.49900	.15780	-.444	9	.668
MS+2BAP+1NAA	-.34000	.32042	.10132	-3.356	9	.008

4. Paired sample Wilcoxon test on non-normal data of shoot length.

Medium combination	**Test Statistics[b]**	
	Z	Asymp. Sig. (2-tailed)
MS+1BAP	-2.829[a]	.005
MS+1.5BAP	-2.536[a]	.011
MS+2BAP	-2.829[a]	.005
a. Based on negative ranks, b. Wilcoxon Signed Ranks Test		

5. Kruskal-Wallis test on shoot length growth of *C. stricta* and *C. flaccida.*

Name of plant	**Test Statistics[a,b]**		
	Chi-Square	df	Asymp. Sig.
C. stricta	106.223	14	.000
C. flaccida	104.445	14	.000
a. Kruskal Wallis Test, b. Grouping Variable: Media_code			