MICROPROPAGATION OF TERRESTRIAL ORCHID *PHAIUS TANCARVILLEAE* (L' Her.) Blume.

A DISSERTATION
SUBMITTED TO CENTRAL DEPARTMENT OF BOTANY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR MASTER'S DEGREE IN BOTANY

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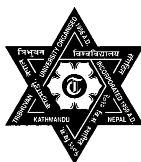
KATHMANDU, NEPAL

2008



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CERTIFICATE

This is to certify that the work entitled **“MICROPROPAGATION OF TERRESTRIAL ORCHID *PHAIUS TANCARVILLEAE* (L’ Her.) Blume.”** has been carried out by Sumitra Shrestha under my supervision. Her findings are based on the experiments carried out by her in our laboratory.

I have a pleasure in recommending the present study as a dissertation work for the partial fulfillment of the requirement for degree in Botany. The results of the present work have not been previously submitted for any other degree.

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LETTER OF APPROVAL

This dissertation paper submitted by Sumitra Shrestha entitled **"MICROPROPAGATION OF TERRESTRIAL ORCHID *PHAIUS TANCARVILLEAE* (L' Her.) Blume."** has been accepted as a partial fulfillment of Masters of Science in Botany.

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ACKNOWLEDGEMENTS

I would like to express my hearty gratitude and appreciation to Dr. Bijaya Pant, Asst. Prof.,CDB, T.U. for her generous guidance, excellent suggestions and continuous encouragement throughout the period of this investigation.

I wish to express my sincere gratitude to Dr. Krishna K. Shrestha, the Head of the Department , CDB, T.U., and Dr. Pramod Kumar Jha the former Head of the Department for providing me lab facilities during this research work.

I gratefully acknowledge the constant encourage and help received from Prof. Dr. Sanu Devi Joshi, the former Head of the Department , CDB, TU and Prof. Dr. Sushila Bhattarai, CDB, TU.

I am grateful to Mrs. Banu Ojha, the Director of Cornell-Nepal Study Programme (CNSP), Kirtipur, for providing me partial financial support to conduct this study.

I am thankful to Ms. Anu Shrestha for her help and suggestions. I am equally thankful to Ms. Srijana Shrestha and Mrs. Nutan Prasai for accompanying me throughout this research work.

I want to express my thanks to my family members for all the support that they gave me during the research work.

Finally my sincere thanks to all the teachers and staffs of CDB, T.U.

Date:- Sumitra Shrestha

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ABBREVIATIONS

BA	Benzyl Adenine
BAP	Benzyl Amino-Purine
BM	Basal Medium
CDB	Central Department of Botany
EDTA	Ethylene Diamino Tetra Acetate
IAA	Indole-3- Acetic Acid
IBA	Indole-3- Butyric Acid
MS	Murashige and Skooge
NAA	Napthalene Acetic Acid
PLBs	Protocorm like bodies
TU	Tribhuvan University
WWF	World Wide Fund for Nature

ABSTRACT

Phaius tancarvilleae (L' Her.) Blume. has been listed as endangered under Biodiversity Conservation Act 1999. The aim of the study was to develop protocol for micropropagation of terrestrial orchid *Phaius tancarvilleae* (L' Her.) Blume. In the present investigation, *in vitro* seed germination and multiplication of shoot tip of *Phaius tancarvilleae* (L' Her.) Blume. was carried on MS medium (Murashige and Skoog, 1962) supplemented with or without different concentration and combination of Napthaleneaceticacid and Benzylaminopurine. Among them, MS medium supplemented with 0.5 mg/l of BAP was found to be the most effective medium for seed germination, growth and development of seedlings. Germination started after 7 weeks of culture and complete seedlings were obtained after 24 weeks of culture. Shoot tip obtained from *in vitro* grown plants was cultured on MS medium and MS medium supplemented with different concentration and combination of NAA and BAP for their mass propagation. The maximum number of shoot multiplication was observed on MS medium supplemented with 1 mg/l of BAP (13.3 ± 1.92). The shoot multiplication was started after 4 weeks of culture. Rooting was observed in all media used but less number of roots were observed in MS media supplemented with BAP. The most effective condition for *in vitro* rooting was observed in MS medium supplemented with 0.5 mg/l of NAA. The rooting was initiated after two weeks of culture. Then, the rooted plantlets were transferred for acclimatization.

CHAPTER - ONE

1.1 INTRODUCTION

Orchidaceae is the second largest and most diverse family in the plant kingdom found throughout the globe except the polar region. There are over 30,000 native species estimated around the world. The orchids are notably diversified in the moist tropics of both hemispheres, where the majority is epiphytes in forests. Most of the temperate and all of the alpine genera are terrestrial. Orchids, a beautiful gift of natural are very distinctive plants. They are known for their long lasting and exquisite beautiful flowers which are unusually oriented and are of excellent form. They are of immense source of aesthetic pleasure. Therefore they are becoming popular plants for decoration purposes. Also these groups of plant have been a suitable experimental material for the researchers and plant breeders.

Orchids are perennial herbs of varying habitat. They can be classified as epiphytes, which are found growing on trees; terrestrial, which are ground-dwelling species; lithophytes, growing on rocks and subterranean. Vegetatively the orchids are divided into two basic groups: sympodial and monopodial.

Nepal with its unique geographical position and climate offers excellent growing condition for orchids. Nepal is endowed with rich and an incredible variety of orchid flora due to wide altitudinal variation, diverse habitats and varied climatic conditions. As a result about 377 species under 100 genera has been reported so far (Rajbhandari and Dahal, 2004). In Nepal, orchids are found at the altitudinal range of 100 to 5000m. Larger number of species are epiphytes (47%) and terrestrial (41%). Terrestrial orchids like *Habenaria, Dactylorhiza, Malaxis, Calanthe, Satyrium, Peristylus* and some species of *Cymbidium* are common in Nepal. About eleven species of orchids have been recorded endemic in Nepal.

Orchidaceae comes third in Nepalese flora in terms of species richness. Nepal is rich in its wild orchid species. Wild orchid is one of the integral parts of Nepal's heritage. Orchid diversity is rich in central and eastern Nepal . In western Nepal, orchid species are sparse. The distributions in different bio-climate zones show that subtropical and lower temperate zones are rich in orchid diversity. Most of the Nepalese orchids have ornamental value and few with medicinal as well as edible value.

Besides the ornamental importance and horticultural values, people use orchids in different manner. Traditional as well as Ayurvedic system of medicine recognizes *Orchis hatagirea* (Panch aunle) as very good tonic and medicine. *Cypripedium cordegenum, Orchis hatagirea* are also used as vegetable, whereas *Planthera clangena* and *Satyrium nepalensis* are used as food.

Aerides, *Ascocentrum*, *Arundina*, *Bulbophyllum*, *Calanthe*, *Coelogyne*, *Cymbidium, Dendrobium*, *Epigeneium*, *Eria*, *Phaius*, *Phalaenopsis*, *Plieone*, *Rhyncostylis*, *Thunia*, *Vanda* are some of the beautiful orchid species of Nepal. Species cultivated for ornamental purpose includes, *Aerides multiflora*, *Ascocentrum ampullaceum*, *Bulbophyllum leopardinum*, *Calanthe masuca*, *C. plantaginea*, *C. tricarinata*, *Coelogyne cristata*, *Cymbidium elegans*, *C. iridioides*, *Dendrobium densiflorum*, *D. nobile*, *Phaius tankervilleae*, *Pleione praecox*, *Rhyncostylis retusa*, and *Vanda tesselata* (Rajbhandari and Bhattarai, 2001).

Some of the important medicinal orchids of Nepal are mentioned by Rajbhandari *et al*. (2000). They are *Brachycorythis obcordata*, *Coelogyne flavida*, *Coelogyne stricta*, *Cymbidium aloifolium*, *Dactylorhiza hatagirea*, *Eulophia nuda*, *Flickingeria macraie*, *Pholidota imbricate*, *Luisia zeylanica* and *Vanda tesselata*. Among the highly exploited orchids for medicinal purpose are *Flickingeria macraie* found in the tropical and sub-tropical zones and *Dactylorhiza hatagirea* found in the subalpine and alpine zones (Bailes, 1985).

Due to these properties there is a serious threat to the conservation of orchids in Nepal which is associated with habitat loss, forest destruction, degradation and over exploitation of beautiful and medicinal orchids. Studies to understand the conservation status of orchids of Nepal are still lacking. Many orchid species of Nepal stand now at the threatened stage. Because orchids are characteristically slow growing and difficult to germinate, excessive picking and futile attempt to transplant have depleted native species. They have been exploited from their natural habitat although orchid species are protected for the purpose of international commerce under Convention of International Trade in endangered species of Wild Fauna and Flora (CITES) as potentially threatened or endangered in their natural habitat, with most of the species under Apendix II.

Orchids have been well known for their horticultural values. They are among the most highly prized of ornamental plants. Orchids of high commercial value are becoming rare and endangered due to heavy collection for national and international trade. So, heavy collection of orchids is a major problem of Nepal. Particularly in Mechi zone

and Illam district, orchids have been widely collected for trade. The Government of Nepal has restricted export of wild orchids but the enforcement is awfully weak. So Nepalese orchids need serious attention from the government and also from the public. Orchid reserves should be established at orchid rich areas for preservation and regeneration. The rare and endangered species of orchids should be introduced to Botanic Gardens.

Vegetative propagation is a very slow process to propagate a large quantity of clonal orchid plants. It needs special ecological and climatic condition. In natural condition the majority of orchid flowers are not pollinated, their ovules are not fertilized and capsules are rarely formed. Less than 5% of seeds germinated in their natural environment because of particular fungal requirement (Rao, 1997)

Orchid seeds are very small; 1.0-2.0mm long and 0.5-1.0mm wide. The dust like seeds are produced in great number, often over a million seeds per capsule.The embryo has a round or spherical form. Most orchid seeds are hardly differentiated because it has no cotyledons, no roots and no endosperm. They form a symbiotic association with mycorrhizal fungi for germination and seedling nutrition as they lack their own food reserves i.e. endosperm.

Tissue culture has become the standard method of propagation for the conservation of orchids. Before the advent of tissue culture techniques, orchids were considered difficult to propagate for two reasons. First, natural vegetative propagation of many species is slow and second, the dust like seeds of orchids are difficult to germinate as they lack nutrient reserves. The young embryo requires mycorrhiza for its development to plants. Germinating seeds *in vitro* is a breakthrough in orchid multiplication (Fay, 1996).

As the germination and further development of seedling is dependent on a symbiotic relationship with a fungus in nature. But it is possible *in vitro* to be independent of the fungus by substituting its action with a nutrient medium, this is known as asymbiotic germination. Sowing *in vitro* makes possible to germinate immature orchid embryos; this leading to a shortening of breeding cycle. Germination and development take place much quicker *in vitro*, since there is a conditioned environment, and there is no competition with fungi and bacteria. Therefore, a systematic research on propagation technique especially the tissue culture should be promoted for commercially valuable orchids. Orchids represent the first floricultural crop successfully mass propagated through tissue culture technique (Peirik, 1997).

Micropropagation technique is not even in an infant stage in commercial scale in Nepal. This has been mainly due to the non-availability of suitable planting material for large scale cultivation, lack of technology for commercial multiplication, lack of trained personnel and lack of incentives, appropriate government policies for exporters.

Tissue culture protocols of 37 species of orchids have been developed in National Herbarium and Plant Laboratory (NHPL), Godawari under Department of Plant Resources (DPR). A number of dissertation works on tissue culture have been carried out in Central Department of Botany, Tribhuvan University (Pant and Ghimire, 2006). Some private organizations like Nepal Biotech Nursery also involved in tissue culture activities of orchids.

Micropropagation of *Phaius tancarvilleae* has not been carried out yet. The present study is mainly focused on *in vitro* culture, especially micropropagation of *Phaius tancarvilleae* (L' Her.) Blume.

1.2 *Phaius tancarvilleae* (L' Her.) Blume.

Phaius is the small genus which consists of approximately 30 species of often large and spectacular, principally terrestrial orchids. In Nepal, two species of *Phaius* have been reported i.e. *P. flavus* and *P. tancarvilleae* (Press *et al*., 2000). The range of *Phaius* is largest one, extending from East Africa and Madagascar, throughout tropical Asia and Indonesia to the Himalayas. *Phaius* refers to dusky. The frequently numerous flowers usually open successively, so that the plants remain bloom for a long period of time. The tubular lip is rather characteristics of the blossoms, which occurs in a wide variety of hues, many of them unusual for orchids.It is an impressive ground orchid thrives in poor soil. It produces brilliantly coloured flowers in combination of yellow, white and red.

1.3 Botany of the plant

Stem of *Phaius tancarvilleae* (L' Her.) Blume. is stout, 60-200 cm tall with conical or ovoid. Leaves: 30-100 cm long and up to 20 cm broad, narrowly elliptic or lanceolate, acute or acuminate. Petioles: 15-25 cm long. Inflorescence: stout, 8-20 large progressively blooming flowers, produced from one of the lower nodes of the pseudobulb. Floral bracts spathaceous and caducous. Flowers: fragrant heavy textured, long lived, purplish-brown ventrally and white dorsally, 8-12.5 cm across. Sepals and petals: subequal, linear-lanceolate, acuminate, spreading, powder white outside, the blade dark wine-red inside. Lip: shorter than the sepals and petals, its lower part tubular and ending in a narrow curved spur 1.2-2 cm long; its anterior part deflexed, conchiform, more or less ovate, dilated, acute or acuminate, sometimes apiculate; tube of lip pink to wine-red with a yellow base inside. Column: erect, glabrous.

Flowering time: March-May.

Distribution: This species is found in Nepal, North India, Bangladesh, Sri Lanka, Myanmar, China and South East Asia to New Guinea, Australia and the South, West Pacific Islands. It occurs as a terrestrial herb in the subtropical and temperate zones of east Nepal at 1300-2100 m altitude.

Chromosome number: 2n=42.

Conservation status: *Phaius tancarvilleae* is listed as endangered under Biodiversity Conservation Act 1999. Illegal collection by orchid enthusiasts is one of the threats facing this species.

1.4 Objectives to the study

The plants undertaken for the present investigation have high ornamental values. But their propagation and domestication are not considered as satisfactory. The present investigation is aimed at-

1. To investigate the effect of various concentration of hormones in seed germination of *Phaius tancarvilleae* (L' Her.) Blume.

2. To develop the protocol for mass propagation.

3. To acclimatize the *in vitro* grown plants.

1.5 Justification of the study

Phaius tancarvilleae (L' Her.) Blume. is a robust terrestrial orchid species listed as endangered under the Biodiversity Conservation Act 1999. This species has high commercial value and horticultural importance having potentiality to export. Habitat destruction, illegal and indiscriminate collection by orchid enthusiast are some of the threats facing this species. So from both commercial and conservation point of view it is important to develop quick methods of propagation of this species. *In vitro* techniques of micropropagation is one of the method that can meet both commercial and conservational demand. Since there is no published information regarding the micropropagation of *Phaius tancarvilleae* (L' Her.) Blume. This investigation may provide protocol for micropropagation of such horticulturally important species.

CHAPTER TWO
LITERATURE REVIEW

Bernard (1909), for the first time, discovered that fungi are important for germination of orchid seeds. He successfully isolated the root infecting fungi which was helpful in seed germination. Comparative germination studies were made of infected and noninfected seeds. When sterilized seeds were grown in association with the fungus, the percentage of germination improved considerably. The most important fungi that have symbiotic relationships with orchids were from the genus *Rhizoctonia*. (Pierik, 1997)

Knudson (1922, 1924, 1925) was the first to propose asymbiotic seed germination of orchids. He developed a suitable nutrient media for the germination of the seeds of orchid without fungal infection. The orginal method was modified as 'Knudson C' media. He also clarified many important things regarding formation of seedlings and organogenesis. The first organ culture was achieved by White (1934) with the demonstration of the potentiality unlimited growth of excised tomato root tip.

Ball (1946) was the first person to demonstrate the possibility of regenerating whole plants *in vitro* from isolated explants of the shoot apex of certain angiosperm. Later, Wetmore and Morel regenerated whole plants from the isolated shoot species, measuring 100-250ìm in length and bearing one to two leaf primordia, for several lower plants and angiosperms.

Rotor (1949) aseptically cultured the flower stalk cutting each with only one axillary bud of *Phalaenopsis*.

Morel (1960, 1965)made an important attempt by developing *Cymbidium* shoot tip (Meristem) culture as a means of clonal propagation (Rao, 1997).

Murashige and skoog (1962) found striking four to five fold increase in yield with tobacco tissue when cultured on White's modified medium supplemented with kinetin and indoleacetic acid (IAA) within a three to four week growth period on addition of an aqueous extract of tobacco leaves.

Raghavan and Torrey (1964) grew seeds of the orchid *Cattleya in vitro* in a medium containing NH_4NO_3 as the sole source of nitrogen. Seeds germinated readily and proceeded to form small plantlets. Development of the embryos was accompanied by an increase in their total nitrogen and a decline in dry weight.

Reinert and Hubertc (1967) successfully propagated *Cattleya* orchid plants by plants by tissue culture of lateral bud meristems 1st in a chemically defined liquid medium with constant agitation, then later transfered to an agar medium. Addition of kinetin after the 1st week of culturing favored the development of callus or protocorm like bodies which produced plantlets.

Tanaka *et al*., (1975) obtained PLBs from leaf tissue from seedlings of *Phalaenopsis* and *Vanda*. A practical medium for PLB formation was formulated. The PLB formed on the explant of both *Phalaenopsis* and *Vanda* grew to juvenile plants on a medium suitable for orchid seed germination.

Arditti *et al*., (1981) germinated seeds of native California and related terrestrial orchids *Calypso bulbosa, Platanthera saccata, Epipactis, Goodyera* and *Piperia in vitro* on five basal media and 43 modifications. Germination periods were extended from several weeks to almost 2 yr.The most suitable media for orchid germination are those devised for the culture of barley embryos (Norstog) and a modified Curtis medium containing urea and calcium carbonate instead of ammonium nitrate and calcium nitrate.

Niraula and Rajbhandary (1985) cultured shoot tips of *Dendrobium fimbriatum* Hook. In MS medium supplemented with cytokinins. From the explants, shoots were proliferated and protocorms were also developed. These shoots and protocorms when transferred in Vacin and Went medium grew into complete plantlets.

Krishnamohan and Jorapur (1986) multiplied *Acampe praemorsa* through seed culture. The VW medium (Vacin and Went, 1949) was used as basal medium (BM) and it was supplemented with peptone, casein hydrolysate, coconut milk, thiamine and niacin in different concentrations and combinations. The seed germination was poor in BM. However, the response was comparatively better when the medium was supplemented with coconut milk. Further increase in the percentage of germination was observed with additional peptone, vitamins, and casein hydrolysate in the medium. The seeds germinated within 10 weeks in BM, whereas these germinated within 6 weeks and developed into protocorm like bodies (PLBs) within 3 months when the BM was supplemented with peptone, vitamins and casein hydrolysate. Well-developed plants with 2-3 leaves and 1-2 healthy roots were obtained in 6 months.

Muralidhar and Mehta (1986) germinated seeds of *Cymbidium longifolium* on three basal media, Kn C (Knudson, 1946), VW (Vacin and Went, 1949) and RT (Raghavan and Torrey, 1964) with or without various levels and combinations of vitamins, hormones, amino acids and micronutrients. The seed germination was assessed at up to 30% on KnC, 60% on VW and 35% on RT. The sequential steps of histomorphological changes from embryo to PLBs were traced out.

Shrestha and Rajbhandary (1988, 1993 and 1994) regenerated plant through meristem culture of *Cymbidium giganteum, C. grandiflorum* and *C. longiflorum.* Shoots of *C. giganteum* were initiated on MS (1962), MS supplemented with BAP (5mg/l), NAA (1mg/l) and 10% coconut milk.The culture of meristem of *C. grandiflorum* was carried out on MS medium supplemented with BAP (2.2mg/l), NAA (1.8 mg/l) and 10% coconut milk.Meristem culture of *C. longiflorum* initiated PLBs within six weeks after culturing in MS medium supplemented with BAP (2 mg/l), NAA (1 mg/l), 10% coconut milk and 3% sucrose. They also propagated *Dendrobium densiflorum* Lindl. using shoot tip. Protocorms were initiated from shoot tip explant in MS medium supplemented with 15% coconut milk, BAP 2.5 mg/l, NAA 1 mg/l and caseinhydrolysate 1g/l.

Anderson and Allan (1991) germinated seeds of *Spiranthes magnicamporum in vitro* on water agar, modified Knudson's medium, and oat medium with the fungal symbiont *Epulorhiza repens* isolated from a naturally occurring plant of the same species.

Yamamoto *et al*., (1991) observed that shoot primordia of *Calanthe sieboldii* were induced from the meristems in the modified B5 medium supplemented with 2.0 mg/l BA. Then PLBs were obtained from shoot primordia after transplanting onto agar medium with the same composition. These PLBs were massively and rapidly propagated by culturing in the same liquid medium. PLBs were regenerated into plantlets. The plantlets were then acclimatized easily.

Fay (1992, 1994 and 1996) concluded that micropopagation has greatly helped botanic gardens to multiply rare and endangered plants in cultivation. *In vitro* laboratory techniques are widely used for vegetative propagation, seed germination and long term storage of germplasm of rare plants, especially those unable to reproduce naturally or which reproduce poorly. This facilitates distribution of material of these species to other institutions around the world because the cultures do not require quarantine due to their sterile nature. *In vitro* propagation has also allowed material to be stored in *in vitro* gene banks. This will increase with further developments in cryopreservation techniques.

Niraula and Rajbhandary (1992) cultured explants of *Vanda teres* Lindl. excised from aseptically grown seedlings on MS medium supplemented with auxin and cytokinin. PLBs were developed from the explants. These PLBs were transferred to a VW medium and grew into complete plantlets.

Reddy *et al*., (1992) studied *in vitro* seed germination and seedling development in four species of south Indian orchids and showed a significant interaction between the media and the orchids. The frequency of germination and protocorm development and the extent of seedling growth varied with the species and the culture medium. MS and RL (Rosa and Laneri, 1977) media yielded better results than KC and VW medium. The ground growing *Spalthoglottis plicata* responded better to MS medium and the epiphytic *Epidendrum radicans*, *Dendrobium crepidatum* and *Cymbidium aloifolium* to RL medium.

Hoshi *et al*., (1994) sowed immature seeds from unripe capsules of four Asiatic *Cypripedium* taxa, *C. debile, C. henryi, C. japonicum var. formosanum,* and *C. tibeticum*, on sterilized MS agar (0.7%) medium in culture flasks. Germination and protocorm development occurred three to five months after sowing. Protocorms *of C. debile* and *C. japonicum var. formosanum* grew to seedlings six months after seed-sowing, while those of *C. tibeticum* and *C. henryi* did not. Nodes of axenically cultured *C. montanum* transplanted onto MS agar medium supplemented with 0.2 mg/l NAA and 2.0mg/l BAP grew readily forming thick, well-branched rhizomes and 20 or more shoots in flasks within a month.

Rajkarnikar (1994) initiated protocorms of *Dendrobium fimbriatum* Hook. from shoot explant in the MS medium fortified with 5 mg/l BAP, 1 mg/l NAA and 10% coconut milk. Protocorms were sub cultured in 1 mg/l BAP, 1 mg/l NAA and 10% coconut milk for multiple shoots and protocorms production. Micro shoots transferred on MS medium with 0.5 mg/l NAA to produce roots.

Shrestha and Rajbhandary (1994) propagated 36 species of native and exotic orchids *in vitro* from seeds. They germinated in different culture media namely, Burgeff, Knudson's C, Vacin and Went, Thomson, Murashige and Skoog and N3F.

Yasugi and Shinto (1994) observed the maximum number of plantlets per single segment of pseudobulb in the treatment of 1 mg/l NAA + 1 mg/l BA of MS (1962) medium in *Dendrobium* (Nobile type). The axillary buds did not develop into a PLB but directly developed into shoots.

Banerjee and Mandal (1999) germinated immature *Cymbidium* seeds obtained from green capsules on defined orchid culture medium supplemented with folic acid, NAA and organic adjuvants (casein hydrolysate, peptone, coconut water and tryptophan) at varying rates. 2-5 mg/l of folic acid in VW medium induced maximum 85% germination while NAA 0.1mg/l could induce 78%. 2, 4-D, GA_3 , BAP and kinetin bore either null relationship or inhibitory effects on seed germination. NAA 2mg/l in VW was most appropriate to induce 3-4 roots in 2 months.

De Pauw *et al*., (1995) investigated that BA and 6-(á,á- dimethyl allylamino) - purine (2 ip) in concentration up to 0.8mg/l enhanced germination of *Cypripedium candidum* significantly in comparison to controls without cytokinin. Protocorms developed faster and the production of multiple protocorms increased with all three cytokinins in comparison to control.

Vij *et al*., (1995) germinated immature seeds from unripe capsule of *Dactylorhiza hatagirea*, collected 16 weeks after pollination on agar modified Knudson 'C' medium supplemented with selective growth adjuncts. A combination containing yeast extract (YE; 1 mg/l) and 6-purfurylamino purine (KN; 1 mg/l) proved very useful during germination and seedling complete with leaf and tuberous root were obtained in 38 weeks.

Pant *et al*., (1996) studied that multiple shoots were induced from the apical domes of shoot tips of *Cnidium officinale* Makino (Apiaceae) by culturing them on Murashige and Skoog (MS)1 static media solidified with 0.2% gelrite and supplemented with 6-benzylaminopurine (BAP) 10^{-6} M. An average of 5.3 shoots per segment were obtained within 6 weeks and this ability did not decline even after two weeks of subculture. Cytological observation in root tip cells of cultivated, as well as *in vitro* propagated plantlets revealed that in both cases the cells had 2n = 22 chromosomes indicating the homogeneity of the clonally propagated plants.

Tanaka *et al*., (1997) concluded that *Spiranthes sinensis* can be micropropagated and conserved for a long time by the "tissue-cultured shoot-primordium method." Numerous protocorms were successfully germinated from 10 to 13 day old immature seeds on the Hyponex medium at concentration of 2.5 g/l supplemented with 35 g/l sucrose, 2 g/l peptone, and 0.9% agar at 22°C under 500 lx full illumination. The PLBs were differentiated and multiplied and later regenerated plantlets in growth substance free B5 liquid medium.

Vij and Kaur (1999) observed rapid clonal multiplication of *Ascocenda* '50th State Beauty' through *in vitro* culture of leaf explant. The success of this system depended upon the juvenility of tissues, use of BAP/ KN/ IAA in the nutrient medium and optimization of surface sterilization procedure for the explants of *in vivo* grown plants. An individual treatment of either of BAP or KN and IAA favored somatic embryogenesis. BAP, however also promoted precocious differentiation of shoot meristem in the embryoids (PLBs). The regenerated shoots developed multiple PLBs at their bases. Nearly 60 plantlets were obtained within 20 weeks from each of the BAP treated explants.

Gangaprasad *et al*., (2000) micropropagated two horticulturally important jewel orchids of the genus *Anoectochilus*. Isolated nodes of *A. sikkimensis* and *A. regalis* were cultured for 12 weeks on Woody Plant Medium (WPM) to produce a maximum of 4.8 and 5.6 callus free axillary shoots respectively at 95 and 98% efficiency. During the subculture of the explants from *in vitro* raised shoots under the same conditions, the total number of shoots obtained from the nodes (21.4) and shoot tips (8.2) of *A. regalis* were significantly higher than those hardy and slow growing shoots of *A. sikkimensis* (12.3 and 4.3) respectively. Shoots (4-6 cm) were rooted in medium containing NAA (2.70 ìm) and activated charcoal (0.2%). The rooted plants were established at 95-98% rate in community pots.

Geeta and Shetty (2000) developed micropropagation protocol for *Vanilla planifolia* using shoot tip and nodal segments as explants. The explants were cultured in MS basal medium with 1 mg/l BAP for 10 weeks and subcultured onto fresh medium every four weeks. The proliferating clusters were cultured in N69 basal medium with BAP (0.5mg/l) +d-biotin (0.05mg/l) + folic acid (0.5mg/l) and 2% sucrose for elongation of shoots, formation of root initials and further proliferation of axillary shoots. Separation and culturing of elongated shoots in fresh medium for 2 to 3 weeks yielded 7 to 8 cm long plantlets, which were acclimatized on polytunnels.

Sheelavanthmath *et al*., (2000) cultured rhizome sections of *Geodorum densiflorum* (Lam) Scnltr on MS (1962) on Knudson C medium supplemented with various growth regulators and 0.1% activated charcoal NAA at 2µM stimulated rhizome growth, BA at 5 µM induced multiple shoots within four weeks of culture and inhibited rhizome growth. The regenerated shoots rooted on MS only or with NAA at 1.0 µM. Shoots were acclimatized and transferred to the green house.

Kabita and Sharma (2001) studied *in vitro* germination and protocorm formation of *Acampe longifolium* Lindl.by using MS, Kn C, VW and B5 media supplemented with different concentration of NAA, IBA and Kinetin. Cent percent germination was obtained on MS medium supplemented with NAA (0.1µg/ml) and Kn (1.0 µg/ml). Formation of spherales and development of distinct green PLBs and leaves also took less time on MS medium than the other three media tried.

Murthy and Pyali (2001) studied micropagation of *Aerides maulosum* Lindl. Young leaf segments from plants growing both *in vivo* and *in vitro* were cultured on MS medium supplemented with auxins (NAA, 2,4 D), cytokinin (kinetin and IBA) and coconut liquid endosperm (cw). The explants from mature leaves did not show any growth and turned necrotic. While those obtained from juvenile leaves growing *in vitro* developed PLBs at their cut surfaces within 4-8 weeks. An optimum of 18 PLBs developed from leaf explants on MS medium supplemented with 2.0 mg/l BA. Upon subculture in basal medium, the PLBs differentiate into plantlets within 6-8 weeks.

Talukdar (2001) developed an *in vitro* method to regenerate multiple shoots of *Dendrobium aphyllum* Roxb. A maximum of 7 shoots were regenerated in Knudson C medium supplemented with kinetin (10 mg/l), coconut water (15% v/V) and banana extract (6% w/v) within 13 weeks of culture. Well-developed shoots were also developed in IAA and NAA treated cultures. Protocorms proliferated and callused in medium containing 2, 4 D.

Nagarju and Upadhyaya (2001) studied the morphogenetic response of *Cymbidium* Lunavian Atlas PLBs on three different basal media and their combinations with activated charcoal (AC). Among the media, Nitsch was the best for growth of plantlets. The addition of 0.3% AC to the media had a marked effect on growth of shoots and roots. The plantlets grown in the presence of AC were healthy and phenolic compounds were not observed. These plantlets established better in pots.

Sheelavanthmath and Murthy (2001) reported an efficient multiplication system based on *in vitro* culture of embryo for *Habenaria marginata* Coleb. Cultures were initiated in NAA (0.5 µM) supplemented Knudson C medium and their multiplication, using protocorm segments, was optimized in BA (0.5 µM) treated cultures. Upto 22 PLBs could be regenerated from each segment; the PLBs developed into plantlets upon their subculture on the fresh medium.

Karanjit (2002) cultured the seeds of *Coelogyne cristata* Lindl. and *Cymbidium iridioides* D.Don. in MS medium and Gamborg B_5(G-B_5) medium. The germination rate of seeds cultured in MS medium was found to be more responsive than G-B_5 medium.

Bhadra and Hossain (2003) subcultured two to three months old *in vitro* raised *Geodorum densiflorum* seedlings bearing 3-4 leaves on agar (0.8% w/v) and sucrose (3% w/v) supplement MS medium and its four different combinations with IAA (1.0mg/l), NAA (1.0mg/l), BAP (0.5-2.5mg/l), 2,4 D(1.0mg/l) and activated charcoal (1.0g/l). Seedlings grown on medium containing 3% (w/v) sucrose, 2.0/2.5mg/l BAP, 1.0mg/l IAA/ 1% (w/v) activated charcoal started flowering within 3-4 months of culture.

Nagarju *et al*.,(2003) showed the effect of media and BAP on protocorms of *Cymbidium* and *Cattleya*. They cultured protocorm sections from *in vitro* grown cultures of *Cymbidium* Lunavias Atlas and *Cattleya maxima* in MS, Nitsch, Street, White, Kn C and Gamborg media supplemented with BAP. Growth rate and multiplication with respect to culture weight, protocorm number and shoot number were significantly superior in *Cattleya* in comparison to *Cymbidium*. Among the media, Nitsch produced the best results for all the morphological characters followed by MS. Supplementation of 0.5 mg/l BAP improved the number of protocorms and shoots. However, further increase in concentration of BAP proportionately reduced the number of shoots and leaf size. Root differentiation in *Cymbidium* was completely inhibited in KC, Street and White's media. About 90% of plantlets of 4-5 leaf stage developed new leaves and roots and established in the community pots, when they were transferred after partial hardening *in vitro* by reducing the concentration of salts.

Ogura *et al*., (2003) studied *in vitro* shoot formation of *Cymbidium ensifolium* from rhizome apices. Through rhizome apices shoot formation was promoted in MS medium supplemented with 10mg/l NAA with or without NAA.

Swar and Pant (2003) cultured the seeds of *Cymbidium iridioides* D.Don. Growth and development of seedlings were favored in MS medium supplemented with BAP (1mg/ l) and NAA (1mg/l). The multiple shoot were obtained through shoot tip culture. The maximum number of multiple shoots were obtained on MS medium supplemented with BAP (0.5mg/l). They also cultured shoot tip of *Coelogyne cristata* Lindl. The maximum number of multiple shoots were obtained on MS medium supplemented with BAP (1mg/l) and NAA (1mg/l).

Bejoy *et al.,* (2004) cultured seeds obtained from 50-55 days old green capsules of *Peristeria elata* Hook. In liquid Mitra *et al*. (1996) medium supplemented with 1 gm/l peptone. Protocorms differentiated in 5-6 wks in this medium. Rooting of young seedlings was achieved when these were subcultured on modified KC medium without any additives. *In vitro* raised *P. elata* seedlings recorded 96% survival in greenhouse condition.

Bhadra *et al.,* (2004) germinated immature seeds of *Bulbophyllum lilacinum* on 0.8% (w/v) agar solidified MS media. The germinated seedlings were transferred to plant growth regulators. The highest rate of elongation was achieved on MS media fortified with 2.0 mg/l BAP and 1.0 mg/l IAA. Strong and stout root system was developed when the seedling of 3-4 cm height were transferred to 0.8% (w/v) agar solidified half strength MS +1.5% (w/v) sucrose. The *in vitro* seedlings were then transferred to outside natural environment through sequential phases of acclimatization.

Ket *et al.,* (2004) developed a rapid and efficient protocol for *in vitro* clonal propagation of an elite cultivar of jewel orchid *Anoectochilus formosanus*. Multiple shoot proliferation was induced in shoot tip explants on Hyponex (H3) media supplemented with 1 mg dm^{-3} benzyladenin or 1-2 mg dm^{-3} thidiazuron (TDZ). Addition of activated charcoal (1 g dm^{-3}) to the TDZ containing medium promoted multiple shoot formation (11.1 shoots per explants). Rooting was induced in 100% of the regenerated shoots in the same media. The plantlets were acclimatized and established in green house.

Nagarju, *et al.,* (2004) studied *in vitro* germination of seeds from unripened green capsules of two *Dendrobium* crosses (*Dendrobium nobile* × *D. nobile* var. *alba* and *D. nobile* × *D. heterocarpum*) which were tested on MS media and those from selfed capsules of *D. chrysotoxum* was tested on Kn C media supplemented with or without 3g/l activated charcoal and 0.5 mg/l of BAP. The study showed that addition of growth regulators and activated charcoal in the medium delayed the onset of germination. Incorporation of BAP in the medium favoured rapid multiplication of protocorms. Activated charcoal proved beneficial for the seedling growth. The growth of seedling was better in MS, while presence of AC in the medium further improved the seedling growth.

Vij, *et al*., (2004) studied that root segments (0.5-1.0 cm long) from *in vivo* sourced segments failed to respond whereas those from *in vitro* grown plantlets of *Cymbidium* (24 wks old) regenerated PLBs on VW medium and its various combinations with plant growth hormones. The frequency, with which the PLBs produced, was markedly influenced by the quality and combination of growth hormones in the nutrient regime. A combination containing BAP (1.5 mg/l) and NAA (1.0 mg/l) proved best for accelerated development of PLBs and proliferations.

Vij, *et al*., (2004) reported the rapid mass multiplication of *Aerides multiflora* Roxb.using leaf segments. Mitra *et al*. (1996: M) medium supplemented with BA (2 mg/l) with NAA (0.5 mg/l) proved the best for initiating regeneration of the explants. It favored high regeneration frequency, early response and high proliferation; nearly 20 plantlets could be produced within 30 weeks.

Chung *et al.,* (2005) showed the effect of four auxins viz. 2,4-D, IAA, IBA and NAA and five cytokinins viz. (N-6-[2-isopentinyl] adenine [2iP], BA, kinetin, TDZ, and zeatin on direct embryo induction of leaf explants of *Dendrobium* cv. Chiengmai Pink cultured on 1/2 MS medium. Whether in light or darkness, explants easily became necrotic and no embryos were obtained on growth regulator free or auxin-containing media after 60 days of culture. By contrast, five cytokinins tested induced direct embryo formation from explants, and explants cultured in light had a higher embryogenic response compared with those cultured in darkness. Regenerated plantlets were successfully transplanted and grown in a greenhouse environment.

Esitken *et al*., (2005) studied the effect of mycorrhiza isolates on symbiotic germination of terrestrial orchids (*Orchis palustris* Jacq. and *Serapias vomeracea*). The objective of this study was to determine the effects of a number of *Rhizoctonias*, isolated from wild orchids. On seed germination and protocorm development of *Orchis* and *Serapias*, when inoculated with these isolates, they germinated within 21 days. While uninoculated control seeds of both species showed lowest germination rate (<1%).

Pant and Gurung (2005) studied *in vitro* germination and seedling development of *Aerides odorata* Lour. on MS static media solidified with agar (0.8%) and supplemented with or without different concentration and combination of NAA and BAP. Hormone free MS medium was found to be the best for early germination, higher number of protocorm formation and their further differentiation. However all the hormone treatments favored seed germination and the further differentiation of 2-3 leaved seedlings. Use of combination of BAP (2.0 mg/l) and NAA (1.0 mg/l) proved to be the best for seed germination among all the hormone treatments.

Karki *et al.,* (2005) cultured seeds of *Vanilla planifolia* Andrews in MS medium without growth hormones. The seed germinated after 6-8 months. The seedlings were subcultured in MS medium supplemented with 1.0 mg/l BAP and 1.5 mg/l Kinetin with 10 % coconut milk. The mature shoots were transferred in cocopit for rooting.

Malabadi *et al.,* (2005) developed a new method of propagation of *Dendrobium nobile* from shoot tip section. Successful shoot regeneration was achieved using thin shoot tip sections and triacontanol (TRIA). PLBs were observed when thin shoot tip sections were cultured on the basal medium of Mitra *et al* supplemented with 4.0 µg/l TRIA. All the newly formed PLBs were survived and ultimately produced healthy shoots with 2-3 leaves. Shoots produced roots when cultured on basal medium supplemented with 2.0 µg/l TRIA. The well-rooted shoots were transferred to pots containing charcoal chips, coconut husk and broken tiles (2:2:1), and a 92% survival rate was achieved.

Nagarju and Manl (2005) studied the influence of plant growth substances medium and potting mixture on protocorm development, differentiation, growth and establishment of *Zygopetalum intermedium.* Embryo from mature green but unripe capsule cultured in half strength MS medium containing 1.5 g/l AC, with 0.25 mg/l PBZ and 0.1 mg/l NAA. Protocorms was developed in 70.7 days after formation of globular bodies. Plantlets with 4-5 well developed leaves with roots are pre hardened in medium supplemented with 0.25 mg/l each PBZ and triacontanol transferred to community pots filled with potting mixture of cocopit and tree fern (1:1) resulted in 72.3% survival *ex vitro.*

Shrestha (2005) studied *ex situ* conservation of *Coelogyne ovalis* through micropropagation. In this experiment, asymbiotic seed germination was observed in MS medium and MS medium supplemented with different growth regulator. The best medium for seed germination, growth and development of seedling was MS + NAA (1 mg/l). The explant, shoot tip, obtained from *in vitro* grown plants was cultured in MS and MS medium supplemented with various combinations of BAP and NAA. The maximum shoot multiplication was observed in MS + 1 mg/l of BAP. Green mass of callus was also observed at the base of some shoots in MS + BAP (1 mg/l) and MS + BAP (0.5 mg/l) + NAA (0.5 mg/l). The best *in vitro* rooting was observed in MS + IBA (2 mg/l). The rooted plants were transferred for acclimatization.

Swamy *et al.,* (2005) studied on *in vitro* germination of orchid seeds. MS and PM media were used to germinate orchid seeds *in vitro*. Seeds of *Aerides maculosum* were germinated on PM medium supplemented with NAA and 2,4D in 0.5mg/l concentration. The seeds of *Coelogyne nervosa* and *Cottonia peduncularis* were cultured on PM medium with the addition of NAA, 2,4D and kinetin (0.5mg/l). Seeds did not germinate on MS medium. Seeds of *Cymbidium bicolor* also germinated on PM medium with supplement of NAA, IAA, 2,4D (0.5mg/ml) and adenine sulphate (5 mg/l). However the seeds of *Dendrobium macrostachyum* were germinated well on both MS and PM media supplemented with NAA and 2,4D in 5.0 mg/l. Addition of coconut water and Casein hydrolysate to the media were found to enhance seed germination and protocorm production.

Vennel and Basavaraju (2005) studied the morphogenetic responses of nodal explants of *Vanilla*. MS medium supplemented with auxin, cytokinin individually and in combination were investigated to find effect of the above on the initiation of axillary bud and organogenesis of shoots. MS medium supplemented with BAP revealed good initiation and formation of shoots when compared to others. The combination of BAP+IBA and BAP+NAA revealed good organogenesis in MS medium. In all multiplication medium the formation of multiple shoots were observed. Of these experiments the multiplication MS medium with BAP + IBA produced good numberof shoots/propagules by continuous subculturing up to 40-50 numbers, which is an advantageous feature for the micropropagation of *Vanilla*.

Yang *et al*., (2005) studied mass propagation of PLBs of ornamental *Dendrobium*. The PLBs of ornamental *Dendrobium* were transversely cut and adopted as explants. They were cultured on MS basal salt media containing 30g/l of sucrose, 80g/l of potato homogenate, 9g/l agar and in combination with 2mg/l of different kinds of plant growth regulators such as NAA, IBA, BA, kinetin, TDZ and zeatin for PLB proliferation. After 60 days, PLBs from the zeatin treatment subcultured on the medium containing yam bean had the higher shoot formation rate. The ¼ diluted basal salt medium had the best root induction and growth rate.

Cheng, J.T. and Chang, W.C. (2006) cultured leaf explants of *Phaleonopsis amabilis* Var. formosa on 1/2- strength modified MS medium supplemented with 0.1, 1and 3 mg dm(-3) TDZ formed clusters of somatic embryos directly from epidermal cells without an intervening callus within 20-30 d. Repetitive production of embryos involved secondary embryogenesis could be obtained by culturing segments of embryogenic masses on TDZ-containing media. Plantlet conversion from embryos was successfully achieved on regulator- free growth medium.

Yamazaki and Miyoshi (2006) cultured immature seeds obtained from young capsules of *Cephalanthera falcata* which were collected every 10d from 50d after pollination until 120 d after pollination on modified Kano medium and ND medium. Of the capsules, 74.5% survived to full maturity. The highest frequency (39.8%) of seed germination was obtained with seeds harvested 70 d after pollination. The frequency declined with progress of seed maturity on the mother plant. Minimal germination was observed with seeds harvested 100 d or later after pollination.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Materials:

3.1.1 Source of Seeds:

The materials used for the present experiment were the young pods of *Phaius tancarvilleae* (L'Her.) Blume. The materials were obtained from the cultivated home garden.

3.1.2 Laboratory Requirements:

All the necessary equipments, glasswares, chemicals & other laboratory facilities were provided by Central Department of Botany, T.U., Kathmandu.

3.2 Methods :

The methods used for micropropagation are described under following headings:

3.2.1 Preparation of stock solution:

The MS medium (Murashige and Skoog,1962) was used as the basal medium (BM) supplemented with different growth regulators in different concentrations for this experiment. The chemical composition of the MS medium is as follows:

A. (1) Macro - nutrients

Components	Composition of MS (Final Conc.) mg/l	(10X) gm/lit Stock Concentration	Volume to be taken for 1 litre medium
Macro - nutrients			100ml
Potassium nitrate (KNO_3)	1900	19.0	
Ammonium nitrate (NH_4NO_3)	1650	16.5	
Calcium chloride ($CaCl_2 2H2O$)	440	4.4	
Magnesium sulphate ($MgSO_4.7H_2O$)	370	3.7	
Potassium dihydrogen phosphate (KH_2PO_4)	170	1.7	

B. (2)Micro - nutrients

Components	Composition of MS(Final Conc.) mg/l	(100X) mg/100ml Stock Concentration	Volume to be taken for 1 litre medium
Micro - nutrients			
Manganese sulphate ($MnSO_4.4H_2O$)	22.3	2230	1ml
Boric acid (H_3BO_3)	6.2	620	
Zinc sulphate ($ZnSO_{4.}7H_2O$)	8.6	860	
Potassium iodite (KI)	0.83	83	
Sodium molybdate ($Na_2MoO4.2H_2O$)	0.25	25	
Cobalt chloride ($COCl_2.6H_2O$)	0.025	2.5	
Copper sulphate ($CuSO_4.5H_2O$)	0.025	2.5	

C. (3)Iron source (Fe, EDTA)

Components	Composition of MS(Final Conc.) mg/l	(10X) mg/100ml Stock Concentration	Volume to be taken for 1 litre medium
Iron Source			
Sodium ethylene diamine tetra acetate (Na_2EDTA)	37.3	373	10ml
Ferrous sulphate ($FeSO_4.7H_2O$)	27.8	278	

C. (3) Vitamins

Components	Composition of MS(Final Conc.) mg/l	(100X) mg/100ml Stock Concentration	Volume to be taken for 1 litre medium
Vitamins			
Glycine	2.0	200	1ml
Nicotinic acid	0.5	50	
Pyridoxin HCl	0.5	50	
Thiamin HCl	0.1	10	
Myo - inositol	100.0	10,000	

(5) Carbon source

Chemicals	g/l
Sucrose	30g

(6) Gelling agent

Chemicals	g/l
Agar	8g

The stock solutions A, B, C and D were prepared which consisted of macronutrients or major salts, micronutrients or minor salts, iron source (Fe,EDTA) and vitamins as Murashige and Skoog (1962) media.

The above chemicals were weighed accurately using digital balance and dissolved completely in distilled water during the preparation of each stock solution. The chemicals were completely dissolved one at each time by using magnetic stirrer. The final volume was mesh upto a litre each for stock A, 100 ml for stock B, C and D. All of these stock solutions were stored in dry, sterile and labelled amber bottle then allowed to preserve in freeze.

3.2.2 Hormones used for the experiments

Cytokinins

1. 6 - Benzylaminopurine (BAP)

Auxins

2. Naphthalene Acetic acid (NAA)
3. Indol-3-Acetic acid (IAA)
4. Indol-3-Butyric acid (IBA)

3.2.3 Preparation of hormone stock solutions

10 mg BAP was first dissolved in few drops of 0.5N NaOH and distilled water was added to make final volume of 10ml. This was considered as the stock of 1000 mg/l. 1 ml of this was used for 1000 ml of medium to make 1mg/l of hormone concentrations in the medium (1mg/l).

Similarly, 10mg of NAA, IAA and IBA each were dissolved in few drops of absolute ethyl alcohol and final volume was made 10ml by adding distilled water. This was considered as the stock of 1000 mg/l. 1ml of this was used for 1000 ml of medium for making 1mg/l of hormone concentration in the medium (1mg/l).

3.2.4. Sterilization of glasswares and metal instruments

During the experiment sterilized glasswares were used. All the glasswares (Petridishes, culture tubes, pipettes, beaker, conical flask, measuring cylinder etc.) were dipped in detergent water solution for 24 hours and washed with the help of bottle brush, cleaned with tap water. Finally the glasswares were rinsed with distilled water. Then the glasswares were sterilized in hot air oven at 155° C for 2-3 hrs.

Glasswares and metal instruments were subjected to dry heat sterilization before their use in the process. Metal instruments like forceps, scalpels and surgical blades were wrapped with aluminium foil before keeping inside the hot air oven for sterilization.

3.2.5. Preparation of medium

For the preparation of 1 litre MS medium following protocol was applied:

a. 400ml. distilled water was taken in 1 litre conical flask and placed on magnetic stirrer.

b. Different stock solution were added in the conical flask as follows:
   - i. Stock A (Macronutrients)-100ml
   - ii. Stock B (Micronutrients)-1ml
   - iii. Stock C (Fe,EDTA)-10ml
   - iv. Stock D (Vitamins)-1ml

c. 30 gm of sucrose was weighed and added to the mixture. It was dissolved completely by magnetic stirrer.

d. Final volume was made to 1000ml by adding distilled water.

e. For the preparation of hormonal medium, hormone stocks were added according to the media requirement

f. The pH of the medium was adjusted at 5.8 with 0.1N NaCl or HCl.

g. The medium was solidified with 8 gm (0.8%) Difco Bacto Agar and it was heated upto boiling to melt the agar. When the solution became clear, the medium was dispensed into sterile culture tubes at the rate of about 20 ml per culture tube. Then, each tube were sealed with aluminium foil.

h. The culture tubes containing medium were autoclaved at 121^{0}C and pressure of 15 lb/sq inch for 20 minutes. After cooling down of autoclave, the tubes were taken out and kept in slanting position.

3.2.6. Surface Sterilization of capsule

Before inoculation, the laminar air flow chamber was thoroughly cleaned by cotton soaked with absolute alcohol. The culture tubes and vessels with media and all required materials for inoculation like spirit, lamp, matchbox , NaOHCl solutions, double distilled water, 70% ethyl alcohol, sterilized aluminium foils, sterilized petridishes, handle with surgical blade, spatula, beaker, forceps were exposed to UV light for 45 minutes to remove the possible contaminants present in and around the transfer area. After UV exposure, the blower was switched on to make the laminar air-flow chamber ready for inoculation.

For the culture of seeds, the immature healthy capsule of the orchid was dipped in water containing few drops of teepol solution. Then they were kept in running tap water for 30-40 minutes. Then the capsule was rinsed with distilled water. The orchid pod was then wrapped with cotton and soaked in 70% ethyl alchohol for 1 minute. and then surface sterilized in 1% NaOHCl solution for 10-15 minutes. Then finally washed with double distilled water for 3-5 times.

3.2.7. Inoculation of seeds:

The surface sterilized orchid pod was then transferred into the petridish and dissected longitudinally by using scalpel. Then immature orchid seeds in cluster were inoculated on the surface of MS medium alone and combination with different hormones. Sterile forceps were used to spread seed on agar medium. The cultures were incubated at 25^0C ($\pm 2^0$C) temperature under the photoperiod of 16 hours.

3.2.8. Histomorphological study

For squash preparation, PLBs obtained after 16 weeks of seed culture in MS medium alone and MS medium supplemented with different hormonal concentrations were fixed in 1% acetocarmine for 24 hours. Small pieces of the PLBs were mounted on slide with few drops of acetocarmine solution. The slides were observed under microscope.

3.2.9. Inoculation of explant

Shoot tip were taken as explant for this experiment. Shoot tip were obtained from the *in vitro* grown seedling by excising them with the help of surgical blade. The explants were inoculated on the surface of the media supplemented with various concentrations and combinations of hormones. All the cultures were maintained at 25^0C ($\pm 2^0$C) temperature under the photoperiod of 16 hours.

3.2.10. Rooting of shoots

For *in vitro* rooting, 2-3cm long microshoots were excised and transferred asseptically to the MS solid media supplemented with rooting hormones. Different concentrations of IAA, IBA and NAA were used as rooting hormones.

3.2.11. Acclimatization

The fully grown well rooted plants were transferred for acclimatization. The plants were removed from the culture tubes and the agar was washed with distilled water and treated with 1 % Bavistin, an antifungal agent. The plants were finally transferred in pots containing cocopit medium. The pots were covered with polythene for increasing humidity. Watering was done by spraying for two times a day. After a week, plastic was removed for one hr/day, the time was increased per day by one hour for 10 days. Then the plastic was removed.

CHAPTER FOUR

RESULTS

The results of *in vitro* seed germination, multiplication of shoot tips, rooting and acclimatization has been described below in different headings.

4.1 Culture of seeds and germination

The immature seeds of *Phaius tancarvilleae* (L' Her.) Blume. were cultured in MS medium and MS medium supplemented with various concentrations of auxin (NAA) and cytokinin (BAP). Germination rate was vigorous in MS medium supplemented with different concentrations of NAA and BAP. An average of 85% of the seeds germinated and began to form protocorms. The fastest germination rate was observed in MS medium supplemented with 0.5 mg/l of BAP. In this medium, germination was started after 7 weeks of culture. It was followed by 1 mg/l BAP+0.5 mg/l NAA. In this medium, germination was started after 8 weeks of culture.

In vitro germination of immature seeds in MS medium supplemented with growth regulators undergoes further differentiation after formation of protocorms. After 24 weeks of culture, the seeds cultured in 0.5 mg/l of BAP developed into complete plantlets followed by 1mg/l BAP+0.5mg/l NAA, 1mg/l BAP and 0.5 mg/l of NAA. While in other combination and basal medium, the seeds could not undergo further differentiation apart from formation of hairy protocorms and development of first leaf primordia even after 30 weeks of culture. The protocorms formed in different media were chlorophyllous and globular. Seedlings were changed to fusiform and/or cylindrical in appearance.

In vitro germination of immature seeds of *Phaius tancarvilleae* (L'Her.) Blume. in MS medium and MS medium supplemented with different hormonal concentrations is given in Table 1.

Table 1: Effect of growth regulators in MS medium on seed germination and seedling growth of *Phaius tancarvilleae* (L'Her.) Blume.

Medium	Growth Hormones	Concentration	Time taken in weeks for: Initiation of Germination	Development of protocorm	Differentiation of: 1st leaf primodia	1st root primodia	Seedling	Remarks
MS	–	–	15	19	–	–	–	Germination favored
MS	BAP	0.5mg/l	7	9	12	18	24	Germination and seedling development favored
MS	BAP	1mg/l	12	15	20	28	35	Germination and seedling development favored
MS	NAA	0.5mg/l	13	18	23	32	38	Germination and seedling development favored
MS	BAP+NAA	0.5mgl+0.5mg/l	13	17	24	39	–	Germination favored
MS	BAP+NAA	1mg/l+0.5mg/l	8	11	14	19	29	Germination and seedling development favored

Culture condition: MS medium, 25 ± 2°C, 6 replicates
Source: Lab Work, 2006.

In vitro germination of seed

The inoculation of seeds of *Phaius tancarvilleae* (L'Her.) Blume. in MS medium and MS medium with different hormonal concentration ie; BAP and NAA is described below :-

1. MS Basal Medium :
Seed cultured in MS Basal media required more time for germination than in the hormone supplemented MS media. Germination was started only after 15 weeks of seed inoculation. The development of PLB was observed after 4 weeks of seed germination. No leaf primordia and root primordia were observed till 35 weeks of seed inoculation (Fig:6).

2. BAP 0.5 mg/l :
Seed cultured in MS medium supplemented with BAP 0.5 mg/l required 7 weeks for seed germination. The development of PLB was observed after 2 weeks of seed germination. First leaf primordia was observed after 3 weeks of PLB formation and first root primordia was observed after 6 weeks of leaf primordia formation. It took 24 weeks to develop into seedling (Fig:14).

3. BAP 1 mg/l :
Seed cultured in MS medium supplemented with BAP 1mg/l required 12 weeks for germination. The development of PLB was observed after 3 weeks of seed germination. First leaf primordia was observed after 5 weeks of PLB formation. Root primordia was observed after 28 weeks of seed inoculation. It took 35 weeks to develop into seedling.

4. NAA 0.5 mg/l :
Seed cultured in MS media supplemented with NAA 0.5 mg/l required 13 weeks for germination. The development of PLB was observed after 5 weeks of germination. Also first leaf primordia was observed after 5 weeks of PLB formation. Root primordia was observed after 32 weeks of seed inoculation. It took 38 weeks to develop into seedling (Fig:10).

5. NAA 0.5 mg/l + BAP 0.5 mg/l :
Seed cultured in MS media supplemented with NAA 0.5 mg/l +BAP 0.5 mg/l required 13 weeks for germination. The development of PLB was observed after 4 weeks of germination. Also first leaf primordia was observed after 7 weeks of PLB formation. Root primordia was observed after 39 weeks of seed inoculation.

6. BAP 1 mg/l +NAA 0.5 mg/l :
Seed cultured in MS media supplemented with BAP 1 mg/l + NAA 0.5 mg/l required less time for germination ie.8 weeks of seed inoculation. The development of PLB was observed after 3 weeks of germination. Also first leaf primordia was observed after 3 weeks of PLB formation. Root primordia was observed after 19 weeks of seed inoculation. It took 29 weeks to develop into seedling (Fig:9).

PHOTO PLATES 1-4: General Morphology of *Phaius tancarvilleae* (L'Her.)Blume.

Plate 1. Cultivated *Phaius tancarvilleae.*

Plate 2. Bud of *Phaius tancarvilleae.*

Plate 3. Pod of *Phaius tancarvilleae.*

Plate 4. Blooming of *Phaius tancarvilleae.*

PHOTO PLATES 5-15: Seed germination of *Phaius tancarvilleae* (L' Her.) Blume. in MS basal medium and in different hormonal media.

Plate 5. Seed cultured in MS basal medium after 10 weeks of culture.

Plate 6. Seed cultured in MS basal medium after 35 weeks of culture.

Plate 7. Seed cultured in MS + BAP (1mg/l) medium after 12 weeks of culture showing protocorm like bodies and shoot primordia.

Plate 8. Seed cultured in MS + BAP (0.5 mg/l) medium after 12 weeks of culture showing protocorm like bodies and hairy shoot primordia.

Plate 9. Seed cultured in MS+BAP (1 mg/l) + NAA (0.5 mg/l) medium after 29 weeks of culture showing hairy shoot primordia.

Plate 10. Seed cultured in MS+NAA (0.5 mg/l) medium after 12 weeks of culture showing shoot primordia and hairy roots.

Plate 11. Seed cultured in MS + BAP (0.5 mg/l) medium after 10 weeks of culture showing protocorm like bodies.

Plate 12. Seed cultured in MS + BAP (0.5mg/l) + NAA (0.5 mg/l) medium after 30 weeks of culture showing protocorm like bodies and shoot primordia.

Plate 13. Seed cultured in MS basal medium after 25 weeks of culture showing protocorm like bodies.

Plate14. Seed cultured in MS + BAP (0.5 mg/l) medium after 24 weeks of culture showing protocorm like bodies and shoots.

Plate15. Seed cultured in MS + BAP (1 mg/l) medium after 25 weeks of culture showing protocorm like bodies and shoots.

4.2 Histomorphological study

The production of orchid seedlings from seeds involves three sequential stages i.e. seed germination, protocorms formation and seedling development. In the present investigation, the histomorphological study from seed germination to protocorms formation was observed.

PHOTO PLATES 16-21: Hisomorphological study

Plate 16. Squash preparation of 12 weeks old mass obtained from seed culture on MS medium supplemented with BAP (1mg/l) + NAA (0.5mg/l) showing globular embryo and embryonic leaves.

Plate 17. Squash preparation of 12 weeks old mass obtained from seed culture on MS medium supplemented with BAP (1mg/l) + NAA (0.5mg/l) showing shoot primordia.

Plate 18-19. Squash preparation of 12 weeks old mass obtained from seed culture on MS medium supplemented with BAP (1mg/l) + NAA (0.5mg/l) showing seeds with oblong embryos located in centre and testa cells longitudinally oriented.

Plate 20-21. Squash preparation of 12 weeks old mass obtained from seed culture on MS medium supplemented with BAP (1mg/l) + NAA (0.5mg/l) showing bunch of seeds with oblong embryos located in centre and testa cells longitudinally oriented.

4.3 Culture of shoot tip

The shoot tips of length 8 -10mm with a very small portion of stem were cultured on MS media supplemented with various concentrations and combinations of auxins and cytokinins for inducing multiple shoots. The shoot tips cultured on MS basal and MS media supplemented with different hormones showed different responses. The effect of BAP and NAA on shoot tip culture has been given in Table 2.

Table 2: Effect of BAP and NAA on shoot tip culture of *Phaius tancarvilleae* (L' Her.) Blume.

BAP → **NAA ↓ (mg/l)**	**0**	**0.5**	**1**	**2**
0	4 S 2RS	2MS, PLB 4RS	4RMS, PLB 2RS	4MS 2RS
0.5	4RMS, HPLB 2RS	2 S 4RS, PLB	2MS 4RS	2MS 4S

Culture condition- MS medium, 25 ± 2^{0}C, 28 weeks. 6 replicates were used in each combination.
Source: Lab work, 2006

S = Shoot, MS = Multiple shoot, RMS = Rooted multiple shoot, RS = Rooted shoot, PLB = Protocorm like body, HPLB = Hairy protocorm like body.

The shoot tip cultured in MS medium supplemented with various concentrations of BAP and NAA treated alone showed following results:-

1. MS basal medium

The shoot tip cultured in MS basal medium showed no shoot multiplication. Only elongation of shoots ranged from 0.4 to 3 cm occurred. 1-3 roots were observed (Fig: 41).

2. BAP 0.5 mg/l

The shoot tip cultured in MS+BAP (0.5 mg/l) resulted in multiplication after 5 weeks of primary culture. 8 to 10 multiple shoots were obtained after 13 weeks. Many PLBs were found at the base of the multiple shoots. The maximum height of the shoot was 6cm. and the smallest was 1cm (Fig: 24 and 38).

3. BAP 1 mg/l

With the increase of the concentration of BAP to 1 mg/l, resulted in multiplication after 4 weeks of primary culture. 10-15 rooted multiple shoots were observed after 12 weeks. PLBs were found at the base of the rooted multiple shoots. The maximum height of the shoots was 7 cm and smallest was 1 cm (Fig: 25 and 34).

4. BAP 2 mg/l

Further increase in concentration of BAP to 2 mg/l, resulted in multiplication after 5 weeks of primary culture. 4-8 multiple shoots of length 2 cm to 3 cm were observed after 17 weeks. The maximum height of shoot was 4cm and the smallest was 5cm (Fig: 37).

5. NAA 0.5 mg/l

The shoot tip cultured in MS+NAA (0.5 mg/l) multiplied after 4 weeks of culture. 10-15 rooted multiple shoots were observed after 13 weeks. Hairy PLBs were found at the base of the rooted multiple shoots. The length of the shoots ranged from 2 cm to 8 cm (Fig:31a and 31b).

The shoot tip cultured in MS medium supplemented with various concentrations of BAP and NAA treated in combination showed following results:-

6. BAP 0.5 mg/l+ NAA 0.5 mg/l

The shoot tip cultured in MS medium supplemented with 0.5 mg/l BAP + 0.5 mg/l NAA resulted in multiplication after 7 weeks of primary culture. 2-3 multiple shoots were observed. The length of the shoots ranged from 2cm to 5cm (Fig: 39).

7. BAP 1 mg/l + NAA 0.5 mg/l

The shoot tip cultured in MS medium supplemented with 1 mg/l BAP + 0.5 mg/l NAA resulted in multiplication after 5 weeks of primary culture. 2-3 stout and big multiple shoots with abnormal roots were observed. The maximum height of the shoot was 8cm and the smallest 3cm (Fig: 29 and 30).

8. BAP 2 mg/l + NAA 0.5 mg/l

The shoot tip cultured in MS medium supplemented with 2 mg/l BAP + 0.5 mg/l NAA resulted in multiplication after 7 weeks of primary culture. 1-2 rooted multiple shoots were observed. The length of the shoots ranged from 1.5cm to 3cm.

4.3.1 Shoot multiplication

After shoot tip culture, regular observation was carried out. Maximum multiplication of shoot was observed after 20 weeks culture in all the combinations. Multiplication of shoot together with shoot growth and root induction was observed.

Table 3: Effect of BAP and NAA on multiple shoot formation, growth and root induction of *Phaius tancarvilleae* (L' Her.) Blume.

Parameters	Hormones			
	BAP in mg/l			NAA in mg/l
	0.5	1	2	0.5
	Mean ± S.E	Mean ± S.E	Mean ± S.E	Mean ± S.E
Shoot Proliferation	7.8 ± 2.4	13.3 ± 1.92	1.80 ± 0.68	7 ± 2.34
Shoot growth (Cm)	4.9 ± 0.38	4 ± 0.66	2.26 ± 0.24	5.16 ± 0.54
No. of roots	0.5 ± 0.31	1.33 ± 0.30	0.33 ± 0.19	4.25 ± 0.45

*S.E = Standard Error.

Culture condition: MS medium, 25°C±2 °C, 20 weeks, 6 replicates were used in each combination.

Source: Lab work, 2006

Table 4: Effect of BAP and NAA on multiple shoot formation, growth and root induction of *P. tancarvilleae* (L' Her.) Blume.

Parameters	Hormone combination BAP (mg/l)+NAA (mg/l)			
	0+0	0.5+0.5	1+0.5	2+0.5
Shoot Proliferation	Mean ± S.E	Mean ± S.E	Mean ± S.E	Mean ± S.E
	1 ± 0.16	1.83 ± 0.28	5.5 ± 1.77	1.45 ± 0.42
Shoot growth (Cm)	1.45 ± 0.17	3.66 ± 0.30	6.33 ± 0.56	1.41 ± 0.17
No. of roots	1.16 ± 0.15	1 ± 0.23	2.83 ± 0.36	0.66 ± 0.28

* S.E = Standard Error.

Culture condition: MS medium, 25°C±2 °C, 20 weeks, 6 replicates were used in each combination.
Source: Lab work, 2006

The mean number of the shoots formed, mean length of shoots, mean number of roots and standard deviation formed in different combinations of hormones used are presented in Table 3 and 4 above .The multiple shoot formation rate was found to be increased more in hormone supplemented MS media than in hormone free MS medium. Maximum multiplication of shoot was observed in MS + 1 mg/l BAP which was 13.33 shoots per shoot tip in 20 weeks (Fig: 34). Increase in concentration of BAP to 2 mg/l decreased the multiplication rate. Less number of roots was developed in media containing various concentrations of BAP. But in the media which was supplemented with NAA maximum roots were induced. Maximum multiplication in MS + 1 mg/l BAP was followed by MS + 0.5 mg/l BAP which was 7.8 shoots per shoot tip (Fig: 38). It was followed by MS + 0.5 mg/l NAA and MS + 1 mg/l BAP + 0.5 mg/l NAA. Least multiplication was observed in MS + 2mg/l BAP +0.5 mg/l NAA.Similarly maximum length of shoot was observed in MS + 1 mg/l BAP +0.5 mg/l NAA (Fig: 30) which was 6.33cm. It was followed by MS + 0.5 mg/l NAA, MS + 0.5 mg/l BAP, MS + 1 mg/l BAP and MS + 0.5 mg/l BAP + 0.5mg/lNAA .

PHOTO PLATES 22-41: Culture of shoot tip.

Plate 22. 5 week old explant (shoot tip) culture on MS basal medium.

Plate 23. 5 week old explant (shoot tip) culture on MS + BAP (1mg/l) + NAA (0.5 mg/l) medium.

Plate 24. 10 week old shoot tip culture on MS + BAP (0.5 mg/l) medium showing protocorm like bodies and multiple shoot with hairy roots.

Plate 25. 10 week old shoot tip culture on MS + BAP (1mg/l) medium showing protocorm like bodies and multiple shoot with hairy roots.

Plate 26. 16 week old shoot tip culture on MS + BAP (1mg/l) + NAA (0.5mg/l) medium showing two rooted multiple shoot.

Plate 27. 16week old shoot tip culture on MS + BAP (1mg/l) medium showing multiple shoot.

Plate 28. 16 week old shoot tip culture on MS + BAP (1mg/l) + NAA (0.5mg/l) medium showing two multiple shoot with giant roots.

Plate 29 and 30. 20 week old shoot tip culture on MS + BAP (1mg/l) + NAA (0.5mg/l) medium showing multiple shoot and giant hairy roots.

Plate 31a and 31b. 20 week old shoot tip culture on MS + NAA (0.5mg/l) medium showing multiple shoot and hairy roots.

Plate 32. 17 week old shoot tip culture on MS + BAP (0.5mg/l) + NAA (0.5mg/l) medium showing two multiple shoot with hairy roots.

Plate 33. 17 week old shoot tip culture on MS + BAP (2mg/l) + NAA (0.5mg/l) medium showing rooted multiple shoot.

Plate 34. 20 week old shoot tip culture on MS + BAP (1mg/l) medium showing multiple shoot.

Plate35. 20 week old shoot tip culture on MS + NAA (0.5mg/l) medium showing multiple shoot with hairy roots.

Plate 36. 20 week old shoot tip culture on MS + BAP (1mg/l)+NAA (0.5mg/l) medium showing multiple shoot.

Plate 37. 17 week old shoot tip culture on MS + BAP (2mg/l) medium showing multiple shoot with roots.

Plate 38. 20 week old shoot tip culture on MS + BAP (0.5mg/l) medium showing multiple shoot.

Plate 39 and 40. 20 week old shoot tip culture on MS + BAP (0.5mg/l) +NAA (0.5 mg/l) medium showing multiple shoot.

Plate 41. 20 week old shoot tip culture on MS basal medium showing shoot with roots.

4.4 Rooting of shoots

The microshoots which were not rooted in the multiplication medium were used for rooting. These microshoots were cultured in MS medium supplemented with different concentrations of auxins i.e. IBA, NAA, IAA which were used for the rooting purpose. MS medium supplemented with 0.5 mg/l NAA was found most effective condition for rooting in this experiment. The rooting was initiated after two weeks of culture in this medium. The roots were thick, fleshy and hairy. The mean number of roots was found 4.33 (Fig: 48 and 49). The length of the root was in the range of 1cm to 5cm after 5 weeks of culture.

In MS medium supplemented with 1 mg/l NAA, less number of roots was observed, in which, mean number of roots was 3 and the length of the root was in the range of 1cm to 3cm. which was less than in MS medium supplemented with 0.5 mg/l NAA. Thus the increase in the concentration of NAA had negative effect on rooting.

In basal medium, rooting was observed after 4 weeks of culture. In MS medium supplemented with 0.5 mg/l IBA, rooting was observed after 2 weeks of culture (Fig: 51). Here, the mean number of roots was found 2.33 and the length of the roots was found in the range 1cm to 5cm. In MS medium supplemented with 1 mg/l IBA, rooting was observed after 2 weeks of culture (Fig: 52). The mean number of root was 2.16 and the length of root was in the range of 0.5cm to 3cm. Thus increase in the concentration of IBA had negative effect on rooting. Table 6 shows the effect of different auxins on rooting of shoot tips.

Table 5: Effect of different auxins on rooting of shoot tip of *P. tancarvilleae* (L' Her.) Blume.

Parameters	Concentrations of Hormones						
Number of roots	MS	IBA (0.5 mg/l)	IBA (1 mg/l)	NAA (0.5 mg/l)	NAA (1 mg/l)	IAA (0.5 mg/l)	IAA (1 mg/l)
	Mean±S.E	Mean±S.E	Mean±S.E	Mean±S.E	Mean±S.E	Mean±S.E	Mean±S.E
	1.51±0.42	2.33±0.31	2.16±0.59	4.33±0.19	3±0.57	2.33±0.73	2±0.33
Lenght of roots	1.82±0.35	2.83±0.62	2.26±0.91	3±0.77	2.25±0.34	1.91±0.43	2.41±0.53

*S.E = Standard Error.

Culture condition: MS medium, 25°C±2 °C, 10 weeks, 6 replicates were used in each combination.

Source: Lab work, 2006

In MS medium supplemented with 0.5 mg/l IAA, rooting was observed after 2 weeks of culture (Fig: 50). The mean number of root was found 2.33 and the length of root was in the range of 0.5cm to 4cm. Increase in the concentration of 0.5 mg/l IAA to 1 mg/l IAA had negative effect for rooting as far as number of roots was concerned but length of root was bigger in 1 mg/l IAA. In 1 mg/l IAA, the mean number of root was 2 and the length of root was in the range of 1cm to5cm. The length of shoots was also increased in MS + IAA (1mg/l) medium (Fig: 53).

4.5 Acclimatization

The rooted plantlets were transferred for acclimatization. Cocopit was used in acclimatization. 60 % survival rate was achieved.

PHOTO PLATES 42-53: Rooting of the shoot

Plate 42. 5 week old culture on MS + NAA (0.5 mg/l) medium showing thick hairy roots.

Plate 43. 5 week old culture on MS + IBA (0.5 mg/l) medium showing two roots.

Plate 44. 5 week old culture on MS + NAA (1 mg/l) medium showing hairy roots.

Plate 45. 5 week old culture on MS + IAA (0.5 mg/l) medium showing roots.

Plate 46 and 47. 8 week old culture on MS + NAA (1 mg/l) medium showing thick hairy roots.

Plate 48 and 49. 10 week old culture on MS + NAA (0.5 mg/l) medium showing thick hairy roots.

Plate 50. 10 week old culture on MS + IAA (0.5 mg/l) medium showing roots.

Plate 51. 10 week old culture on MS + IBA (0.5 mg/l) medium showing thick hairy root.

Plate 52. 10 week old culture on MS + IBA (1 mg/l) medium showing hairy roots.

Plate 53. 10 week old culture on MS + IAA (1 mg/l) medium.

PHOTO PLATES 54-57: Acclimatization of rooted plants

Plate 54. Plants transferred for acclimatization of *Phaius tancarvilleae*.

Plate 55-57. Few plants transferred for acclimatization of *Phaius tancarvilleae*.

CHAPTER FIVE
DISCUSSION

The result obtained from present study of *Phaius tancarvilleae* (L' Her.) Blume. have been discussed on the basis of relevant literature available.

5.1 *In vitro* germination of seeds

Orchid seed germination differs from that of other seeds because of the absence of an endosperm, radicle, and leaf rudiments. Swelling of the embryo is followed by the formation of a round, top-shaped body called as protocorms. Other organ subsequently appear (Arditti 1967, 1979).

Seeds of terrestrial orchids are relatively more difficult to germinate *in vitro* than epiphytic orchids. Since they are much more dependent on their micorrhizal fungi for germination than epiphytic orchids (Stoutamire 1974; Warcup 1975).

In the present study, MS medium alone and MS medium supplemented with different concentrations of hormones were found to be efficient for the germination of immature seeds up to the development of protocorm like body stage. MS medium supplemented with 0.5 mg/l of BAP was found to be the most suitable culture condition for immature seed germination of *Phaius tancarvilleae* (L' Her.) Blume. where germination was observed after 7 weeks of culture, protocorms were developed after 9 weeks of culture and complete seedlings were obtained after 24 weeks of culture. This was found on the basis of time taken for germination, high amount of protocorms formation and further differentiation into seedlings.

In MS basal medium germination was observed after 15 weeks of culture and protocorms were seen after 19 weeks of culture. Further differentiation into seedlings was ceased. This result showed that hormone supplemented media was found to be more effective for seed germination than hormone free medium.

Arditti *et al.,* (1984) reported that the improvement in the nutritional status of the basal medium with additives like vitamins, amino acids and hormones promote seed germination in many orchids (especially terrestrial species).

In the present study, MS medium supplemented with 0.5 mg/l of BAP showed promising result for immature seed germination. Further increase in the concentration of BAP to 1mg/l, took more time for seed germination and protocorm formation. This result was supported by an experiment by Nagarju *et al.,* (2003) in *Cattleya* and *Cymbidium* where supplementation of 0.5 mg/l BAP proliferated protocorms were developed. While increase in the concentration BAP proportionately reduced the amount of protocorms. Similar result has been obtained by De Pauw *et al.,* (1995) on seed germination of *Cypripedium candidum* where 0.8 mg/l BA enhanced faster germination, multiple protocorm formation increased with cytokinin.

The use of BAP and NAA in combination also favored germination and further differentiation into seedling. After 0.5 mg/l BAP, 1 mg/l BAP + 0.5 mg/l NAA was suitable culture condition where germination was observed after 8 weeks of culture and complete seedling was obtained after 29 weeks of culture. This is in conformity with the findings of Vij *et al.,* (2004) who reported that a combination containing BAP (1.5 mg/l) and NAA (1.0 mg/l) proved best for accelerated development of PLBs in *Cymbidium.*

Pant and Swar (2003) also reported that MS media supplemented with 1 mg/l of BAP and 1 mg/l NAA as the best medium for the germination of the immature seeds of *Cymbidium iridioides* D.Don. Pant and Gurung (2005) reported that use of combination of BAP (2.0 mg^{-1}) and NAA (1.0 mg^{-1}) favored seed germination and further differentiation into seedlings. Kabita and Sharma (2001) observed that cent percent germination was obtained on MS medium supplemented with NAA (0.1µg/ml) and Kn (1.0 µg/ml) in *Acampe longifolium* Lindl.

In the present investigation, MS medium was used for asymbiotic germination. This is in agreement with previous reports of germination in orchid. Kim (1982) also found that MS medium was superior over other media. Nagarju and Parthasarathy (1995) recorded similar observation in *Phaius.*

A total of 36 species of native and exotic species of orchids have been germinated *in vitro* in various media including MS medium by Shrestha and Rajbhandary (1994).

Reddy *et al.,* (1992) obtained that MS and RL (Rosa and Laneri, 1977) media were better for germination, protocorms formation and seedling growth in 4 species of south India tropical orchids namely *Cymbidium aloifolium* (L), *Dendrobium crepidatum* Lindl., *Epidendrum radicans* pavon and *Spathoglottis plicata* BL.

Arditti *et al.,* (1981) studied seed germination of North American orchids in modified Curtis medium (Curtis 1936, 1943).

Krishnamohan and Jorapur (1986) reported 86 % seed germination in VW medium (Vacin and Went, 1949) used as basal medium.

This experiment differed with the findings of Hoshi *et al.,* (1994) on seed germination of four species of *Cypripedium* where MS medium lacking growth substances was used for seed germination and early seedling development. It was also differed from experiment by Bannerjee *et al.,* (1999) who concluded that 0.1 mg/l of NAA could induce 78 % seed germination and BAP had either no relationship or inhibitory effects on seed germination of immature seeds of *Cymbidium*. But in the present study, BAP showed stimulatory response rather than inhibitory effect.

5.2 Culture of shoot tip

In the present study, MS medium supplemented with different concentration of growth hormones found to be effective for the initiation of multiple shoots. MS medium alone was not effective for multiple shoot induction. Similar result has been obtained by Yasugi *et al.,* (1994) in *Dendrobium* sp.

The maximum number of multiple shoots was obtained in MS medium supplemented with 1 mg/l of BAP. When the concentration of BAP was increased to 2 mg/l, multiple shoots forming ability was retarded. Hence the increase in the concentration of BAP has inhibitory effect on multiple shoot formation. This result was supported by experiment of Shrestha (2005) in *Coelogyne ovalis* Lindl. where highest number of multiple shoots were obtained in MS + 1 mg/l BAP.

MS medium supplemented with 1 mg/l of BAP was the most suitable media for shoot multiplication; it was followed by MS + 0.5 mg/l of BAP. This result was supported by experiment of Nagarju *et al.*,(2003) in *Cymbidium* and *Cattleya* where highest number of multiple shoots were obtained in MS + 0.5 mg/l of BAP.

The differentiation of root was observed in all media used but less number of roots were observed in MS media supplemented with BAP. Maximum rooting was observed MS medium supplemented with NAA where 8-10 hairy roots were obtained. While in MS media supplemented with 1 mg/l BAP + 0.5 mg/l NAA, 3-4 enlarged hairy roots were obtained.

Least multiplication was observed in MS medium supplemented with 2 mg/l BAP + 0.5 mg/l NAA.
In the present study, the shoot tip explant cultured on MS media supplemented with BAP gave PLBs at the base of the newly formed shoots. These PLBs differentiated into plantlets. Thus maximum amount of shoots were obtained in MS media supplemented with BAP. While the shoot tip cultured in MS media supplemented with NAA gave hairy PLBs which differentiated into multiple shoots and hairy roots.
Tahara (1977) reported that relatively big size (1.5-2.0mm) of shoot tips of *Calanthe discolor* and *C. sieboldii* was required in addition to NAA and BA to MS medium for the survival and the formation of PLBs and callus like tissues.
Yamamoto *et al.,* (1991) obtained multiple shoots and friable callus like tissues in the modified B_5 medium in *Calanthe sieboldii.*

Pant and Swar (2003) obtained maximum number of shoots in MS medium supplemented with 1mg/l of BAP and 0.5 mg/l of NAA in *Coelogyne cristata* Lindl. They also obtained maximum number of multiple shoots in MS medium supplemented with 0.5 mg/l of BAP in *Cymbidium iridioides* D.Don.

Shrestha and Rajbhandary (1994) observed clonal multiplication of *Cymbidium longiflorum* D.Don with shoot apical meristem cultured *in vitro*. Rapid multiplication of shoots was found in MS media supplemented with 1 mg/l BAP and 1.5 mg/l kinetin and 10 mg/l Adeninesulphate.

Rajkarnikar and Niraula (1994) obtained multiple shoots in MS media supplemented with 1 mg/l of BAP, 1 mg/l NAA and 10 % coconut milk in *Dendrobium fimbriatum.*

5.3 *In vitro* rooting of shoots

In the present experiment, the multiple shoots obtained from the culture of shoot tip in MS medium supplemented with BAP were excised and subcultured in MS basal medium and on the root initiating media.

In hormone free MS medium root induction was observed in 4 weeks of culture. This result was supported by experiments by Shrestha and Rajbhandary (1988) who successfully rooted *Cymbidium giganteum* in MS control media in 2 months. Similar result was obtained in *Cymbidium grandiflorum* and *Cymbidium longiflorum* by Shrestha and Rajbhandary (1993, 1994).

In the present study, MS medium supplemented with 0.5 mg/l of NAA was found to be most effective for rooting. It was followed by MS + NAA (1mg/l),MS + IBA (0.5 mg/l),MS + IAA (0.5 mg/l) and MS + IBA (1 mg/l). This result was in conformity with the findings of Rajkarnikar and Niraula (1994) who reported that roots were produced when the shoots were transferred on MS medium supplemented with 0.5 mg/l of NAA in *Dendrobium fimbriatum.* Sheelavanthmath *et al*.,(2000) rooted the shoots of *Geodorum densiflorum* (Lam) Schlta on MS only or with 1.0µM of NAA. Bannerjee *et al*., (1999) reported that 2 mg/l of NAA was most appropriate in producing 3-4 roots in a month in *Cymbidium.*

The rooted plantlets were transferred to the community pot after acclimatization at room temperature for 2-3 weeks.

CHAPTER SIX
SUMMARY

Micropropagation of *Phaius tancarvilleae* (L' Her.) Blume. , a terrestrial orchid was studied in the present investigation.

Asymbiotic germination of *Phaius tancarvilleae* (L' Her.) Blume. was obtained in the all media used for germination. Among them, MS medium supplemented with 0.5 mg/l of BAP was found to be the most effective medium on the basis of time taken for germination, growth and development of seedlings. Germination started after 7 weeks of culture and complete seedlings was obtained after 24 weeks of culture. Seedlings obtained also had developed roots.

The use of BAP and NAA in combination also favored germination and further differentiation into seedling. MS + BAP (1mg/l) + NAA (0.5 mg/l) was suitable culture condition after MS + BAP (0.5 mg/l) where germination was observed after 8 weeks of culture and complete seedlings was obtained after 29 weeks of culture. Hormone supplemented medium was more efficient for seed germination than hormone free MS medium.

The shoot tip cultured on MS + BAP (1 mg/l) produced the maximum number of multiple shoots (13 shoots in 20 weeks). It was followed by MS + BAP (0.5 mg/l), MS + NAA (0.5 mg/l) and MS + BAP (1 mg/l) + NAA (0.5 mg/l). Root differentiation was observed in all media used but less number of roots were observed in MS medium supplemented with BAP. Maximum rooting was obtained in MS medium supplemented with NAA.

For *in vitro* rooting, different concentrations of auxins i.e. IAA, NAA and IBA were supplemented with MS medium. Among them, MS + NAA (0.5 mg/l) was found most effective for rooting. The hairy roots were obtained .The rooting was initiated after two weeks of culture. Then the rooted plantlets were transferred for acclimatization.

The result of the present experiment shows that the endangered species of orchid like *Phaius tancarvilleae* (L' Her.) Blume. can be conserved through microprogation.

CHAPTER SEVEN
CONCLUSION AND RECOMMENDATIONS

7.1 Conclusion

A protocol for the mass propagation *Phaius tancarvilleae* (L' Her.) Blume.was studied. This protocol may be useful for mass propagation of other species of *Phaius*. The result obtained from present investigation, following points could be concluded:

* For asymbiotic seed germination *in vitro* MS supplemented with 0.5 mg/l of BAP was the most effective medium.
* The use of NAA and BAP in combination also favored germination.
* Hormone supplemented medium was more efficient for seed germination than hormone free MS medium.
* Shoot tip explants were used for shoot multiplication.
* Maximum multiplication of shoot was observed in MS medium supplemented with 1 mg/l of BAP.
* Increase in BAP to 2 mg/l decreased the multiplication rate.
* Less number of root was developed in media supplemented BAP. But in the media supplemented with NAA maximum roots were induced.
* For *in vitro* rooting MS medium supplemented with 0.5 mg/l of NAA was most effective.

7.2 Recommendation

From the above study following recommendations are made:

* The mass propagation using shoot tip culture can be started in a commercial scale to conserve the orchids so that it lightens the pressure on natural environ ment and also to upgrade the economic status by commercial cultivation of such horticulturally important orchid.

* The local people should be educated by organizing awareness programmes for protection of orchids. They should be encouraged to participate for conservation of orchids.

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