CHAPTER ONE
INTRODUCTION

1.1. Background

Nepal, occupying the central portion of the Himalayan range represents 6,076 taxa of flowering plants under 1,534 genera and 216 families. About 5% of the vascular flora is endemic to the country while 30% to the Himalayan range. About 1,600 species have recorded uses in traditional medicine covering 90% of the rural population. About 100 species of wild plants including medicinal plants endemic to the country and the Himalayas are harvested for commercial purposes. Medicinal plant based industries are few in the country. Over 90% of the commercial collection in Nepal is exported to India and is the major source and steady basis for the Indian pharmaceutical and aroma industries. The trade in medicinal and aromatic plants is an important source of revenue to the government and a major source of cash income to the rural people of Nepal's impoverished highland villages.

Nepal is rich in medicinal plant resource. The increasing commercial demand of medicinal plant has led to depletion of many medicinal plants. Due to rapidly increasing population, unmanageable collections of medicinal plant are increasing. So many medicinal and aromatic plants are in endangered condition. As the result, Nepal is facing challenges to sustain its medicinal plant resource base and safeguard its rich biodiversity. Recently attempts for systematic study and evaluation of the threat status of wild medicinal plants in the country have been done through national and international efforts. Based on field studies, potential and viable recommendations to develop and manage the country's rich medicinal plant diversity have been provided to the government (Bhattarai and Karki, 2004).

In vitro studies are one of the important methods for conservation. The challenges are immense and it is time to think and develop strategies for success (Murch, 2004).

Tissues of plants such as stem, roots and leaves can grow into an independent plant when cultured in different media in different steps. The tissues are cultured in nutrient medium. The media are generally supplemented with growth promoters called phytohormones. The phytohormone in medium control organ development, the process of differentiation such as the growth of roots, shoots or leaves. Tissue culture technique produces and propagates identical plants called clones. Such plants are often necessary in plant breeding **(**Bhattarai, 2004**).**

Micropropagation is the most promising aspect of plant tissue culture for regeneration of large number of plant species. This technique has now been used as a powerful and promising tool for crop improvement, propagation of endangered, rare and vulnerable plant species.

Plant tissue culture has been investigated as an alternative means to reproduce industrially important natural products such as flavors, insecticides and drugs. Successful application of plant tissue culture technology for medicinal plants includes regeneration of similar clones from the cultured cells at high frequencies with natural product in similar or higher quantity. Long term storage and conservation of the rare and endangered species, manipulation of DNA and protoplast culture to form genetically engineered plants also comes under tissue culture technology. We have no doubts that the feasibility of cultivating suspensions of isolated cells of higher plants *in vitro* will widen the scope of this method even more. Methods for growing cell suspensions are being rapidly developed.

Many reports of *in vitro* culture of different medicinal plants have been found. However, there are no relevant reports on the micropropagation of *Neopicrorhiza scrophulariiflora* so far. In the present investigation a protocol for the *in vitro* multiplication was developed.

1.2 *Neopicrorhiza scrophulariiflora* (Pennell) Hong

Neopicrorhiza scrophulariiflora (Pennell) Hong belongs to family "scrophulariaceae". It is one of the important and threatened medicinal plant resources and is known as Kutki in Nepali. It is a low, hairy herb with a perennial woody rhizome, found at an altitude of 3500 – 4800 m in Nepal and endemic to Himalayan region (Kashmir – Sikkim), North Myanmar, China (Sichuari, Xizang, Yunnan).

1.2.1. Botany of the plant

The plant is a creeper with stout rootstock covered with leaf bases above; leaves 2-6 cm oblanceolate and narrowed below to a winged leaf stalk (Fig. 1) ; flowers dark blue to purple in a dense cylindrical head, borne in a stout stem arising from a rosette of conspicuously toothed ; calyx hairy, nearly as long as the corollary tube, calyx lobes 5, lanceolate , blunt , corolla 1.5 cm with a long 3-lobed upper and a short lower lip; stamen and style exserted capsule 6-10mm.(Joshi & Joshi 2001) (Fig. 2). Seeds minute and netty: many (Fig. 4).This plant comes under endangered state according to IUCN (Shrestha & Joshi, 1996).

2. Medicinal use

Rhizome of this plant is used to control fever and gastralgia. Root powder is used as laxative and also administered for the relief from abdominal pain. It is also prescribed in liver complaints, anemia and jaundice (Joshi & Joshi 2001).

It is also said to stimulate blood formation. Root decoction of this plant is made by boiling 2-3 roots in 1 liter of water which should be consumed in the morning and evening. The dried of ground flowers and leaves can also be used. (Pohle, 1990).

Propagation and domestication of *Neopicrorhiza scrophulariiflora* has not been seriously undertaken. Development of the plant by micropropagation technique will contribute a lot for the domestication and convenient cultivation. The present research has been carried out to propagate plantlets of *Neopicrorhiza scrophulariiflora in-vitro*. Protocols for micropropagation from various parts of the plant will be established. This work will provide information on morphogenetic response of root, internode, node and shoot tip explants on medium supplemented with various concentrations of hormones.

1.3 OBJECTIVES

The present study has the following objectives:

1. To determine whether it is possible to establish culture of high altitude plant.
2. To determine what explants of *Neopicrorhiza scrophulariiflora* (Pennell) Hong constitute the best explants capable of rapid proliferation.
3. To investigate the effects of different phytohormones on *in vitro* morphogenesis of vegetative parts.
4. To acclimatize the *in vitro* grown plants.

CHAPTER TWO
LITERATURE REVIEW

A large number of works has been carried out regarding the plant tissue culture. Some research works carried out *in vitro* culture of medicinal herbs are mentioned over here.

Haberlandt (1902) was the first to propose the cultivating plant cells *in vitro* and introduce the idea of totipotency of plant cell. Lamprecht (1918), Knudson (1919), Nemec (1924) made many attempts to find suitable media and optimal conditions for growing organs, tissue and cells excised from whole plants.

Earle and Langhans (1974) working on *Chrysanthemum sp*. observed the multiple shoots and green leafy callus on MS medium supplemented with various hormones. The callus was suitable for subsequent reorganization into plantlets.

Lowe *et al.*(1985) working on *Zea mays* found that the immature embryos when cultured on MS media containing (0.5mg/l) 2,4-D and 12% W/V sucrose produced a compact white callus. The callus could differentiate into green tissue, which was capable of regenerating into new plant.

Niraula and Rajbhandari (1988) induced shoot of *Poncirus trifoliata* in the MS medium in the presence of BAP (0.1mg/l) and NAA (0.1mg/l) when cotyledonary node was used as explants. On subculture these shoots continued to proliferate in the medium supplemented with BAP 0.1mg/l and NAA (0.1 mg/l). Roots were produced when transferred on medium supplemented with NAA (0.1 mg/l).

Saiju and Rajbhandary (1992) cultured shoot tips of *Lilium longiforum* in MS medium supplemented with NAA for the embryoid initiation. These embryoids were recultured in the medium supplemented with NAA and BAP

for the formation of micro shoots. The tips of micro shoots were cut down and these shoot were sub-cultured in the shoot formation medium. The micro shoots were transferred in the box containing sand for rooting. The established plants were grown in the field.

Asai *et al*. (1994) established the axenic shoot culture of *Mentha arvensis*. The shoots cultured on hormone free MS solid media under 16h/day light grew plantlets with emerged roots after 3 weeks of culture

Kharel and Karki (1994) cultured the shoot tips of *Chrysanthemum morifolium* varieties "Giant fishtail violet" on MS + BAP (1.0mg/l) and NAA (0.01 mg/l). Micro shoots were developed successfully rooted in sand and grown into normal plants.

Koga *et al*. (1994) produced protocol for *in vitro* propagation of *Epimedium sps*. Shoot tips of the plant were cultured on medium supplemented with 4.4µM BAP and 2.9µM GA_3 on MS medium without growth regulator to form shoots. After 4 subcultures on growth regulator free MS medium, these regenerated shoots formed plantlet.

Purohit *et al*. (1994) achieved *in vitro* clonal multiplication of "safed musli" (*Chlorophytem borivilianum*) a medicinal herb on MS + 22.2 µM BAP, using young shoots. All shoots produced roots on ¾ strength MS + 9.8 µM IBA. 60% can survive on pots and soil. Multiplication was also achieved through somatic embryogenesis.

Hazarika *et al*. (1995) developed a protocol for micropropagation of *Murraya koenigii* Spreng. Shoot tips from in vitro grown plantlets were cultured on MS medium supplemented with five levels of BAP viz. 0.25, 0.5, 0.75 and 1.25 mg/l. Elongated shoots from proliferating cultures were harvested and cultured in different carries namely soil rite, sand, FYM, soilrite + FYM, and soilrite + sand. Of the carries used soilrite induced faster root initiation (14.8 days) while soilrite with FYM induced long roots (1.28 cm).

Kannan & Jasrai (1995) obtained multiple shoots from axillary region of the excised nodal segment of *Vitex negundo* on MS + 4. 4 µM of 6 BAP & 2.6 µM of X-NAA acid .*In vitro* generated shoots demonstrated good root induction after 5 days of incubation on half strength MS media supplemented with 9-8 µM of IBA . The generated plantlets were transferred to pots containing vermiculite and perlite (2:1) for acclimatization and then to the field.

Sarkar *et al*. (1996) induced multiple shoots from nodal and shoot apices of *Rauwolfia serpentina* in MS + (1.0 mg/l) BAP and (0.1 mg/l) NAA was found to give the best shoot proliferation rate. Callus formed at cut bases of the explants, which produced shoots when sub cultured on media containing low concentration of BAP (0.5 or 0.1 mg/l). The *in vitro* proliferated shoots were rooted and later transferred to the soil.

Verma *et al*. (1996) obtained shoot proliferation from node stem explant of *Emblica officinalis* on modified MS + BAP (3-5 mg/l) in combination with NAA (0.5mg/l). These shoots elongated on hormone free MS medium were subsequently rooted on ½ MS medium containing IBA (2.0 - 3.0) mg/l.

Hettiarachchi *et al*. (1997) described a tissue culture method for mass production of *Acorus calamus* L. Apical shoot meristems were cultured on MS + BAP or kin (0.1 – 2.0 mg/l) along with IAA, IBA and NAA (0.01-1.0 mg/l) for culture initiation. Well developed shoots were transferred to solid or liquid medium with BAP or kin. (0.5-5.0 mg/l) for shoot multiplication. For culture initiation the medium containing BAP (0.5mg/l) and NAA (0.2mg/l) was the best liquid media contaning BAP (1.0mg/l) and 2.0 (mg/l) both gave the highest number of shoots (26 shoots/explant).The results show that BAP produced significantly more shoots than kinetin. Liquid media were more promising than solid media.

Ramashree *et al*.(1997) developed a protocol for organogenesis in *Arishtolochia indica* (L).Callus was grown from stem explant on MS + BA

(0.1-10 mg/l). Best callusing was obtained in the medium containing (1.5 mg/l) BA. Bud and leaf initiation from the callus was also observed within 40 days of culture on MS + 2mg/l BA and 0.5-2mg/l NAA.

Tailang and Kharya(1997) studied the plantlet production for medicinally important *Glycyrhiza glabra* plant for their commercial propagation resulted in the development of an average 27 axillary buds with 10-36 cm average shoot length from field derived nodal explant on MS + 2mg/l each of BAP and NAA in five weeks. Subculturing of excised *in vitro* propagated shoots on MS + 0.2 mg/l NAA gave maximum root length of about 8 cm in next five weeks.

Lal *et al*. (1998) developed a procedure for a clonal propagation of *Picrorhiza kurroa* Royle ex. Benth through shoot tip culture. MS + kinetin (3.0 to 5.0 mg/l) supported rapid proliferation of multiple shoots from the explant. Addition of IAA (1mg/l) to the kinetin containing medium showed marked improvement in the growth of regenerated shoots. Rooting was readily achieved upon transferring shoots onto MS +NAA (1.0 mg/l). Plants were successfully transferred to soil.

Nayak & Sen (1998) developed a protocol for rapid propagation of *Asparagus robustus* from callus. Callusing and regeneration were maximum using segments of shoot tissue. Shoot and internode tissue retained the ability to form callus with high regenerability in culture for 34 months. BM + 3 mg 2, 4–D and 1 mg Kinetin were more effective in formation of callus. Shoot formation was optimum on medium containing (0.1 mg/l) NAA, (1 mg) BAP & (4 mg) adenine sulfate. Root induction was maximized in ½ strength MS + 0.5 mg IBA. The number of length of roots was greater under an 8 hr. photoperiod as compared with a 16 hr. photoperiod. Regenerated plants could be maintained in ½ strength MS liquid media for 30 days prior to transfer to potted soil.

Reddy *et al*. (1998) regenerated the multiple shoots from mature nodal explants of *Gymnea sylvestre* R.Br. a useful antidibetic medicinal plant when cultured in MS medium containing different combinations of BAP or kinetin with NAA. Maximum number of shoots (7 per explants) was observed on the medium containing 5ppm BAP and 0.2 ppm NAA. Regenerated shoots were rooted on MS ½ medium without supplementing any growth regulator.

Sahoo & Debata (1998) developed a rapid and highly effective method for plant micropropagation from vegetative shoot buds for *Plumbago zeylanica.* Multiple shoots were proliferated from nodal explants cultured on MS medium supplemented with kinetin or N6 – benzyladenine. Excised shootlets cultured on hormone free basal medium rooted within 4 to 7 days. Successful transfer of the rooted shoots to potting soil has been accomplished with 100% survival.

Sardana *et al* (1998) developed a standard protocol for regeneration of *Trachyspermum ammi* from shoot tip explant using liquid and solid media. Complete plantlet regeneration via shoot tip explant excised from germinated seedling on MS + NAA 1.0mg/l and BAP 5.0 mg/l. On solidified medium multiple shoots were produced on BAP (8.0 mg/l) & IAA (3.0mg/l) and rooted individually on IBA (5.0mg/l). However shoots were produced on liquid media with BAP (0.5 mg/l) & IAA (7.0 mg/l), separated at the base and rooted individually on solidified medium with IAA (3.0 mg/l).

Singh *et al*. (1999) obtained multiple shoots tips (1-2cm) of *Bacopa mannieri* in MS + 0.5 mg/l BAP within 6 days of culture. In the case of *Paederia foetida* and *Centella asiatica* multiple shoots were obtained in MS medium supplemented with 1.0mg/l BAP within 7 days of culture. Root induction in *Bacopa sp* was observed within 12 days of culture. In *Paederia sp* and *Centella sp* rooting was observed within 12 and 21 days of culture when single shoot of both the plants was cultured on to MS media containing (0.25 mg /l) IBA and (0.5 mg/l), BAP + (1.5 mg/l) NAA respectively. About 70% of these plantlets were transferred to soil.

Deka *et al*. (1999) achieved *in vitro* micropropagation from axillary buds and shoot tips of *Withania somnifera* on MS + BAP (0.1-1.0) mg and kin (0.1-0.4)mg. From various concentrations of hormones treated the combined effect of BAP (0.3mg/l) and Kin. (0.2mg/l) had promising response. Lower concentration of hormones also induced growth of shoots. Rooting of excised shoots was initiated on growth regulators free MS medium. Pantlets were successfully transferred to the green house.

Niraula (1999) regenerated the shoots from the aseptically germinated seedling of *Rheum emodi* wall. As an explant when cultured in MS + BAP (1mg/l) and NAA (0.1mg/l). The roots were initiated when the microshoots were transferred in non-sterile sand.

Rajkarnikar and Saiju (1999) regenerated the multiple shoots of *Rauwolfia serpentina* from excised shoot tips on MS medium supplemented with (3mg/l) BAP and (0.1mg/l) of NAA. Subcultures of these microshoots were carried out on the same medium with lower concentration of BAP (1mg/l) and NAA (0.1 mg/l) for shoot proliferation. The microshoots were rooted in non-sterile sand and rooted plants successfully established in the field.

Shrestha and Joshi (1999) investigated the root, leaf, nodal part, shoot tips of *Guizotia* ab*yssinica* produced calli in MS medium. However, proliferation of the explants into callus was more effective in the presence of hormones in the medium. Culture of shoot tips in BM + BAP (3ppm) + NAA (0.1ppm) showed the maximum number of shoot multiplication. Excised shoots differentiated roots on half strength MS liquid medium contaning IBA (1ppm). The plantlets raised were acclimatized.

Sinha *et al*. (1999) regenerated multiple shoots through cotyledons with shoot tip of *Scutellaria discolor* on MS + (1 mg/l) BAP + (0.1 mg/l) NAA was the best medium.

Niraula and Saiju (2000) obtained multiple shoots from young shoot tips of Valeriana *jatamansii* in MS medium supplemented with BAP (1mg/l) and NAA (0.1mg/l). 90% microshoots rooted in non sterile sand and these plants were successfully established in the field

Rajkarnikar *et al*. (2000) showed the microshoots sprouting from the base of shoot tip explant in the MS medium with BAP (1mg/l, 2mg/l) along with (0.1mg/l) NAA in *Rauwolfia serpentina.*

Catapan *et al*. (2002) developed efficient micropropagation, callus culture and root culture protocols for the medicinal plant *Phyllanthus urinaria* (Euphorbiaceae) using single node explants. Maximum multiplication (16-20 shoots per explant) was achieved on MS + 5.0 µM kinetin. MS and Anderson Rhododendron media promoted significant shoot culture growth in terms of number of shoots and nodes produced per explant. Rooting was achieved with 93-100% of the microshoots on MS medium without growth regulators, although 1.25-5.0 µM NAA acid significantly increased the number of roots per explant. Regenerated plantlets were successfully acclimatized and 91% of plantlets survived under *ex vitro* conditions.

Ranjit *et al* (2000) induced shoots from nodal explants of *Foeniculum vulgare* on MS + BAP 1ppm and NAA 0.5 ppm after four weeks of culture. An average of six shoots was developed from single shoot after four weeks of culture. The multiple shoot formation has not been declined after one year of sub culture. Nodal explants were taken from in vitro germinated plantlets as hormones free MS medium. Roots were developed as shoots an MS medium supplemented with NAA 2ppm.

Shah (2002) obtained the plantlet from stem callus in Asparagus racemosus Wild. in similar concentration of NAA and BAP viz. 0.5

Poudel (2003) obtained maximum number of multiple shoots of *Mentha spicata* L.on MS + BAP (1ppm) from both node and shoot tip explants.

Singh and Sudarshana (2003) observed efficient method of direct plantlet regeneration using nodal explants of mature plants of *Baliospermum axillare* for the first time. Nodes were cultured in MS medium containing IBA at 1 and 2 mg/l in combination with BAP at 1, 2 and 5mg/l. A maximum number of 15 multiple shoots per explant were obtained in MS + 2mg/l BAP and 1mg/l IBA. The regenerated shoots rooted in MS basal media.

Bhatt *et al*. (2004) showed the development of plantlets with in 5to 6 weeks from the nodal explants of hybrid of Asiatic Lily ultured in the MS media supplemented with (0.5mg/l) BAP and 0.1 (mg/l) NAA. The established shoot tips were subcultured in the same medium for four weeks. 8 to10 microshoots developed in the 3rd subculture. These microshoots rooted in non-sterile sand within 3 to 4 weeks.

Rajkarnikar *et al* (2004) showed the multiplication of micro-shoots from the explants (1-2 mm shoot-tips and leaves) of *Swertia ciliata* cultured in MS medium supplemented with 1mg/L BAP and 0.01mg/l NAA The developed micro shoots were transferred in non sterile sands for rooting.The roots were initiated within 2 to 3 weeks of transplantation. The rooted plants were established successfully in field.

Bhadel (2005) obtained callus from *in vitro* culture of *Ephedra girardiana* on MS + 2,4- D and BAP (2ppm). In the same medium multiple shoot were produced. Root initiation was observed after subculture of callus on the medium with NAA (1ppm).

Nguyen et al (2005) developed a protocol for micropropagation of *Curcuma roscoe* by rhizome sprout culture. Maximum shoot induce from rhizomes on MS + BAP (0.5-5.0 mg/l), well developed shoots were on MS + (2mg/l) NAA.

Chand (2006) developed a protocol of *in vitro* study of *Clinopodium umbrosum*. Shoot multiplication induced from nodal explant cultured on MS media supplemented with BAP (2ppm).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

The materials used for the present study were seeds of *Neopicrorhiza scrophulariiflora* (Pennell) Hong commonly known as kutki. The seeds were collected from Manang district situated at 3200m altitude from the sea level. Experiments were done in Central Department of Botany, Tribhuvan University, Kathmandu.

3.2 Methods

The nutrient medium used for the culture was Murashaige and Skoog(1962) medium (MS) as the basal medium (BM). The chemical composition of the medium is as follows:

Components	**Composition of MS Final conc.(mg/l)**	**(10X) gm/l Stock concentration**	**Volume to be Taken for 1 Litre medium**
MACRONUTRIENTS			
Potassium nitrate (KNO_3)	1900.00	19.0	
Ammonium nitrate (NH_4NO_3)	1650.00	16.5	
Calcium Chloride($CaCl_2.H_2O$)	440.00	4.4	
Magnesium sulphate($MgSO_4.7H_2O$)	370.00	3.7	100ml
Potassium biphosphate(KH_2PO_4)	170.00	1.7	
Components	**Composition of MS Final conc.(mg/l)**	**(10X) gm/l Stock concentration**	**Volume to be Taken for 1 Litre medium**
MICRONUTRIENTS			
Manganese sulphate ($MnSO_4.4H_2O$)	22.300	4.46	

Zinc sulphate($ZnSO_4.4H_2O$) Boric acid(H_3BO_3) Potassium iodide(Kl) Sodium molybdate($Na_2MO_4.2H_2O$) Copper sulphate($CuSO_4.5H_2O$) Cobalt chloride($CoCl_2.6H_2O$)	8.600 6.200 0.830 0.250 0.025 0.025	1.72 1.24 0.166 0.05 0.005 0.005	5ml
IRON SOURCE Sodium EDTA (Na_2-EDTA) Ferrous sulphate($FeSO_4.7H_2O$)	 37.30 27.80	 7.46 5.56	5ml
VITAMINS Glycine Nictotinic Acid Pyridoxine Hydrochloride Thiamine Hydrochloride Myo-Inositol	 2.0 0.5 0.1 0.5 100.0	 0.4 0.1 0.02 0.1 20	5ml
CARBON SOURCE Sucrose	 30.0		
GELLING AGENT Agar	8.0		

3.2.1 Preparation of Stock Solution

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.

During the preparation of each stock solutions A,B,C and D the above chemicals were weighed accurately and dissolved in distilled water. To dissolve the chemicals completely, solution was stirred by magnetic stirrer . Final

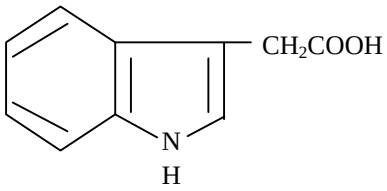
volume was made to a litre each for stock A, B, and C and to 500ml for stock D.

All these stock solutions were stored in a dry, sterile and labeled amber bottle and kept in freeze at 4^{o}C.

3.3 Hormones used for investigation

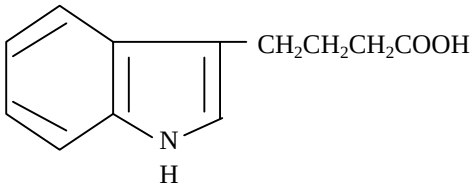
Auxins:

(i) IAA



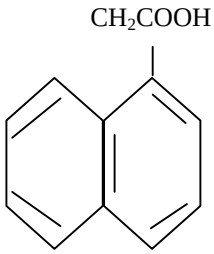
Indole 3-Acetic Acid

(ii) IBA



Indole 3-Butyric Acid

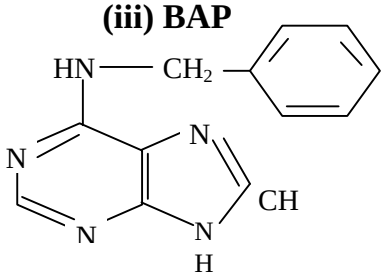
(iii) NAA



Naphthalene Acetic Acid

Cytokinins:

(iii) BAP



6-Benzylaminopurine

3.3.1 Preparation of Hormone Stock Solution

All the auxins and cytokinins were dissolved in few drops of absolute alcohol and then required quantity of distilled water was added.

10mg auxin was first dissolved in few drops of absolute alcohol and made up to a final volume of 10ml by adding distilled water. That was the stock of 1000 ppm. One ml of that stock was used for 1000ml of medium to make 1ppm of hormone concentration in the medium. All these solutions were stored in refrigerator.

3.4 Sterilization of Glasswares and Metal Instruments

All the sterilized glasswares and metal instruments were used for the experiments. All the glasswares were dipped in detergent water solution for 24 hours and washed with the help of bottle brush and cleaned with tap water. Finally the glasswares were rinsed with distilled water and sterilized in hot air oven at 120°C for 3-4 hours. Metal instruments were also washed with tap water and wrapped with aluminium foil and sterilized in autoclave at 121°C for 15 minutes.

3.4.1 Preparation of Medium

For the preparation of one litre of media, about 400ml deionozed or distilled water was taken in a 1l conical flask. Required amounts of stock solutions were added to it in order of macronutrients, micronutrients, Fe-EDTA, vitamins and hormones.100ml of stock A, 1ml of stock B, 10ml of stock C and 1ml of stock D were added respectively. Then 30 gm of sugar was added and dissolved. Required amount of auxin and cytokinin was added. Final volume was maintained to 900ml by adding distilled water. The pH of the medium was adjusted at 5.8±0.1 with the help of 0.5N HCL and 0.5 N NaOH. Then the volume of the medium was made 1000ml by adding distilled water.

The medium was solidified by adding 0.8% Difco Bacto Agar gelling agent. Then the medium was heated for few minutes to dissolve the gelling agent quickly. About 20ml of medium was dispensed into culture tubes. These tubes were sealed with aluminium foil and rubber band. The medium was autoclaved at the temperature of 121^{o}C and pressure of 15 lb/sq. Inch for 15 to 20 minutes. After autoclaving , the culture tubes with the medium were slanted slightly.

3.5 Preparation for inoculation

Before inoculation the laminar airflow chamber was thoroughly cleaned by spirit. The culture tube and vessels with media, all required materials for inoculation like spirit, lamp matches, sodium hypochlorite solution, double distilled water, sterile water, alcohol etc were exposed to ultra violet (UV)light for 45 minutes. Forceps, watch glasses, petridishes, beakers, knives, blades those required for culture were autoclaved for sterilization before culture.

3.6 Seed Inoculation

The viable seeds were first washed in running tap water with few drops of teepol solution for 30-40 minutes and finally washed with distilled water. Soaked seeds were then dipped in 70% ethyl alchohol for 1 minutes and sterilized with 1% sodium hypochloride for 10 minutes and washed 4-5 times with sterile distilled water inside laminar air flow cabinet.Surface sterilized seeds were carefully inoculated in sterilized MS basal medium. In each tube 6 seeds were inoculated. The cultures were maintained in controlled room at 25 ± 2°C temperature.

3.7 Culture of Explants and Subculture of Callus

For explants root, leaf, internodes, nodal part and shoot tip were aseptically excised in 3-5mm pieces from about 5 week old plantlet. The explants were grown in MS media with or without hormones. All the cultures were maintained at 23±2°C. In the process of callus sub culture, the callus grown *in vitro* was taken out aseptically and transferred to medium with or without various combinations of growth hormones. The cultures were maintained in controlled room at 23±2°C temperature. Healthy and non infected plantlets were grown for 9 weeks in culture room under 8-16 hrs light and dark conditions. Each subculture was done after 3 weeks on fresh media.

3.8 *In vitro* Rooting of Shoot

For *in vitro* rooting, 2-3 cm long shoot were excised and transferred aseptically to half and full strength of MS solid media with different concentration of different rooting hormones (IAA, IBA and NAA).

3.9 Acclimatization

The plantlets grown in vitro were transferred to the pots containing sterilized sand, soil and vermiculite in 1:1:1 ratio covered with polythene bags. The plantlets were watered two times daily.

3.10 Cytological Study

The callus produced from different explants on different media were fixed on 1% acetocarmine solution for 24 hour and squash preparation was made and observed under microscope.

CHAPTER FOUR

RESULT

4.1 Culture of seeds

The seeds were cultured on MS basal medium. The cultures were kept out in controlled condition at temperature 25 ± 2°C. 25% of seeds started germinating within 3 weeks of culture. After 4 weeks of culture 40% of seeds were germinated. All plantlets have normal root and shoot (Fig. 5). The shoot and root growth was vigorous for 9 weeks of culture (Fig. 6).

4.2 Culture of Root Explants:

The explant from *in vitro* grown plantlets was inoculated in different combinations of MS basal media**.** The explant about 0.75-1 cm inoculated on MS+NAA (0.5ppm) swelled on cut ends after 8 days and callus was initiated after two weeks of culture (Fig. 7). In 6 weeks callus growth was so vigorous that the identity of the explant was lost. The explant cultured on MS media + NAA (1ppm) also showed response as in NAA (0.5ppm) but did not show any response in MS + BAP and in combination with NAA and BAP both. The callus developed on media supplemented with NAA (0.5ppm) and NAA (1ppm) was yellow greenish and friable. The calli could differentiate small hairy roots after 3 weeks (Fig. 8).

4.2.1 Subculture of Root Callus:

The root callus was subcultured on MS with or without various growth hormones. The calli subcultured on MS media remained unchanged. The calli subcultured on MS + NAA (0.5ppm) and MS + NAA (1ppm) media showed growth of the callus with hairy roots. The nature of callus mass was light green and soft with hairy roots (Fig. 9).

4.3 Culture of Internodal explants:

The explants about 4-6 mm in length were cultured on MS + BAP (0.5ppm) + NAA (0.5ppm) swelled after 2 weeks of culture and produced callus after 3 weeks of culture. The callus was yellowish green and soft. (Fig. 10 & 11).

The explant cultured on MS + BAP (1ppm) + NAA (0.5ppm) produced globular callus after 3 weeks of culture (Fig. 12).

4.3.1 Subculture of Internodal Callus

Subculture of callus on same media exhibit vigorous growth of the callus. (Fig.13). Further differentiation did not occurr.

4.4. Culture of Nodal Explants:

The explant from 9 weeks old *in vitro* grown plantlet for inducing multiple shoots were inoculated on MS basal media supplemented with or without various growth hormones of various concentrations. The explant on MS media showed swelling and increased in length (Fig. 14).

The explant inoculated on MS + BAP (0.5 ppm) showed swelling of explant with creamy and greenish colour of callus after 3 weeks of culture (Fig. 15) and on MS + BAP (1ppm) obtained greenish compact form of callus after 3 weeks (Fig. 16).The explant inoculated on MS + NAA (0.5ppm) and MS + NAA (1ppm) slightly increased in length and the cut ends produced the callus after 3 weeks of culture (Fig. 17 & 18). The callus increased vigorously with formation of hairy roots after 5 weeks of culture (Fig. 19). The emerged shoots thus obtained were thicker than the normal plant (Fig. 20).

The explants were also inoculated on MS + BAP (0.5ppm) + NAA (0.5ppm) could differentiate shoot bud. The inoculated explants were swollon after two weeks of culture (Fig. 21).The callus could differentiate into leaf bud proliferation after 4 weeks of culture (Fig. 22 & 23).

The explant inoculated on MS + BAP (1ppm) + NAA (0.5ppm), formed callus after 2 weeks of culture. The callus was greenish and friable (Fig. 24).

On MS + BAP (1ppm) + NAA (1ppm), the nodal part slightly increased and produced callus (Fig. 25). The natures of the calli were friable and green. Response of node explants in MS medium with different concentration of BAP and NAA was tabulated as shown in Table No. 4.1.

Table No. 4.1

Response of node explants in MS medium with different concentration of BAP and NAA in ppm.

Hormone concentration in ppm		Nature of explant	Response by nodal (explant)	
BAP	**NAA**		**Callus initiation**	**Length (cm)**
0	0	E	****	4
0	0.5	E	+ + +	3-5
0	1	E	+ +	3- 4
0.5	0.5	SB	+ +	- - -
1	0.5	SW	+ +	- - -
1	1	E	+	2-3
0.5	0	SW	+	- - -
1	0	SW	+ +	- - -

Culture condition: MS, 23±2°C, 4 Replicates, 5 weeks of culture, ****= no response, + + + =Response of callus, E = Elongation, SW = Swelling & SB = Shoot buds

4.4.1 Subculture of Nodal Callus

The callus obtained from MS + BAP (0.5ppm) + NAA (0.5ppm) were subcultured on same media and after 4 weeks bulk of shoot bud proliferation were differentiated (Fig. 26).The nodal part emerged out shoots after 5 weeks of culture, which were thicker than normal one (Fig. 27).

Subculture of the calli obtained from MS + BAP (0.5ppm) + NAA (0.5ppm) did not show multiplication of shoots except callus formation.

Subculture of the calli obtained from MS + NAA (0.5ppm) on MS + NAA (1ppm) showed elongation of shoot with vigorous growth of callus with hairy roots (Fig. 28). The callus growth was vigorous developing hairy roots after 8 weeks (Fig. 29 & 30).

4.5 Culture of Shoot-tip Explants:

For inducing multiple shoots, shoot-tip with node measuring about 3-5mm were inoculated on MS basal media supplemented with or without various

growth hormones. On MS media the explants remained unchanged, but after 12 days shoot-tip increased in length (Fig. 31).

The explants inoculated on MS + BAP (0.5ppm, 1ppm, 1.5ppm & 2ppm) alone remained unchanged for 2 weeks of culture. After 4 weeks shows slight elongation of explants.

The explants on MS + NAA (0.5ppm & 1ppm) increased in length and produced callus from cut end after 2 weeks. The explants cultured on MS + NAA 0.5ppm showed best response than in NAA (1ppm).The callus was greenish and friable (Fig. 32). The primary shoot-tip that was inoculated elongated in length and number of shoot were differentiated from callus mass within 4-5 weeks with number of hairy roots (Fig. 33, 34 & 35).

The shoot tip inoculated on MS media with BAP and NAA showed best response. The explants cultured on MS +BAP (0.5ppm) + NAA (0.5ppm) swelled cut end in the first week of culture. After 3 weeks of culture, the explants could differentiate into callus from the cut portion, the shoot tip elongated. The callus mass was greenish in colour and friable (Fig. 36). After 6 weeks of culture shoot buds were proliferated (Fig.37, 38 & 39).The globular calli was observed on MS + BAP(1ppm) + NAA(1ppm) after 6 weeks (Fig. 40).

The shoot-tip explant on MS media supplemented with BAP (1ppm) + NAA (0.5ppm) produced compact and greenish callus from the cut end. The shoot tip swelled than normal size at other end after 2 weeks of culture (Fig. 41).The greenish callus growth was vigorous which could differentiate micro-shoots after 5 weeks of culture (Fig. 42 & 43)

When the concentration of BAP was increased from 0.5ppm to 1ppm compact and greenish callus were formed at the ends. The growth of the callus growth was vigorous which could differentiate microshoots after 5 weeks of culture (Fig. 44). Response of shoot-tip explants in MS medium with different concentration of BAP and NAA was tabulated as shown in Table No. 4.2.

Table No. 4.2

Response of shoot-tip explants in MS medium with different concentration of BAP and NAA (ppm).

Hormone concentration in ppm		Number of Shoots (Mean ± SE)	Response by shoot tip (explant)	
BAP	NAA		Callus initiation	Length (cm) (Mean ± SE)
0	0	1 ± 0	***	3 ± 0.4
0	0.5	6 ± 0.4	+ + +	2.5 ± 0.28
0	1	5.75 ± 0.25	+ +	1.7 ± 0.25
0.5	0.5	**SB**	+ +	***
1	0.5	13.8 ± 0.4	+ + +	1.5 ± 0.2
1	1	**C**	+ +	***
0.5	0	**E**	***	2.2 ± 0.25
1	0	**E**	***	2.5 ± 0.2

Culture condition: MS, 23±2°C, 4 Replicates, 5 weeks of culture, ****= no response, SB = Shoot buds, C = Callus and E = Elongation, + = Formation of callus and SE = Standard Error.

4.5.1 Subculture of Shoot-tip Callus

The microshoots with callus obtained from MS + BAP(1ppm) +NAA (0.5ppm) subcultured on MS hormone free media obtained microshoots increased in length (Fig. 45&46)whereas in same concentration MS + BAP (1 ppm) + NAA (0.5 ppm) of hormones it produced vigorous growth of callus with short length microshoots (Fig. 47)

The microshoots obtained from MS + NAA (0.5ppm) subcultured on MS + NAA (1ppm) obtained thicker shoots with callus and hairy roots (Fig. 48).

4.6 Shoot Multiplication

For inducing multiple shoots the microshoots with callus were sub-cultured in MS basal media with and without different combinations of BAP and NAA. Shoot multiplication was observed after 2 weeks of sub-culture in MS media without hormones and MS + NAA (0.5ppm).The different combination of hormones also affect the length and thickness of shoots. On MS + BAP (1ppm) + NAA (0.5ppm) the shoots differentiated were thicker than the plantlets obtained from seed germination (Fig. 49). The microshoots excised with callus when transferred to MS basal media the shoot length increased (Fig. 50). The shoot multiplication in MS + BAP (1ppm) + NAA (0.5ppm) showed thicker and short length shoots. On MS + NAA (0.5ppm) and MS + NAA (1ppm) hairy roots were differentiated from the microshoots along with multiple shoots (Fig. 51, 52 & 53).

The shoots were also obtained from 9 week old callus (Fig. 54). Mean number and length of shoots formed in different combinations of hormones used are tabulated as shown in Table No. 4.3

Table No. 4.3

Effect of BAP and NAA combination on shoot multiplication comparing number and length of shoots.

Combination of hormones (in ppm) **BAP →** **and** **NAA ↓**	**0**		**0.5**		**1**	
	Number of shoots (Mean±SE)	**Length (cm) (Mean±SE)**	**Numberof shoots (Mean±SE)**	**Length (cm) (Mean±SE)**	**Number of shoots (Mean±SE)**	**Length (cm) (Mean±SE)**

0	12.7 ± 0.62	7.5 ± 0.2	**C**	- - -	**C**	- - -
0.5	6.5 ± 0.28	5.2 ± 0.25	**SB**	0.6 ± 0.1	14.7 ± 0.4	1.5 ± 0.47
1	6.25 ± 0.25	4.5 ± 0.2	**C**	- - -	**C**	- - -

Culture condition: MS, 23±2°C, 4 Replicates, 4 weeks of culture, SB = Shoot buds, C = Callus and SE= Standard Error.

4.7 *In vitro* rooting of shoots

For root inducing, the *in vitro* grown multiple shoot with 1-2 nodes were used. The basal cut end of the shoot produced roots as well as callus depending on the hormone and its concentration supplemented in the medium. The excised micro shoots were inoculated in different combinations of IAA, NAA and IBA.

The shoots cultured in different concentration of IAA, IBA and NAA with full and ½ strength of MS media with 0.8% agar produced callus as well as roots. The shoots inoculated on ½ strength of MS media with hormone did not showed better response in rooting.

The shoots regenerated were cultured in MS basal media differentiated roots after 3 weeks of culture (Fig. 55 & 56). The normal roots produced in MS basal media without hormones were developed into hairy roots after 6 weeks of culture (Fig. 57). The excised shoots in other combination of rooting hormones developed hairy roots with callus formation after 4 weeks of culture. On full and ½ strength MS + NAA (0.5ppm) and MS +NAA (1ppm) the microshoots produces hairy roots along with callus (Fig. 58 & 59).

The microshoots cultured on ½ strength of MS + IAA (0.5ppm) could produced only callus with short length root (Fig. 60) whereas long hairy brownish root with callus on full strength of MS + IAA (0.5ppm) (Fig. 61 & 62).

On MS + IBA (0.5 ppm) the inoculated shoots produced roots from basal callus on full strength and ½ strength MS media (Fig. 63, 64 & 65).The inoculated shoots laterally increased in length on full strength of MS + IBA (0.5ppm) (Fig. 64).

In this investigation, some regenerating shoots from the callus developed roots after 9 weeks in MS + BAP (1ppm) and NAA (0.5ppm) (Fig. 66 & 67).Rhizogenesis was also obtained on 9 weeks old callus cultured on MS + BAP (1ppm) +NAA (0.5ppm) (Fig. 68) and MS + NAA (0.5 ppm) (Fig. 69).

Maximum numbers of roots were obtained in hormone free MS media without formation of callus. Effects of NAA, IAA and IBA on number and length of rooting was tabulated as shown in Table No. 4.4.

The plants were acclimatized in pots containing composition of soil: sand: vermiculite in the ratio of 1:1:1. (Fig. 70, 71 & 72).

Table 4.4

Effects of NAA, IAA and IBA on number and length of rooting

Hormone	Concentration (in ppm) (Mean ± SE)	Number of Roots (Mean ± SE)	Length of Roots (cm) (Mean ± SE)	Formationof Callus (Mean ± SE)
Without Hormone	0	10.5 ± 0.28	7 ± 0.67	***

NAA	0.5	6.7 ± 0.25	4.7 ± 0.47	+++
	1	5 ± 0.4	4.2 ± 0.25	+++
IAA	0.5	3.5 ± 0.2	3 ± 0.4	+
	1	2.5 ± 0.28	1.5 ± 0.2	++
IBA	0.5	2 ± 0.4	1.2 ± 0.25	+
	1	1.5 ± 0.28	0.8 ± 0.23	+

Culture condition: MS, 23±2°C, 4 Replicates, 4 weeks of culture, SE= Standard Error & + = Formation of callus

From the above table rooting percent was found better in MS hormone free media and in other combination roots were proliferated along with callus. The length and number of roots was found maximum on MS hormone free media without formation of callus.

The plants were acclimatized in pots containing composition of soil: sand: vermiculite in the ratio of 1:1:1. (Fig. 70, 71 & 72).

CHAPTER FIVE
DISCUSSION

In the present investigation the germination behaviour of *Neopicrorhiza scrphulariiflora* was found to be normal on MS basal medium which is similar to the works on seed germination of *Fagopyrum esculentum* by Joshi (1989), on Linseed by Lane (1979), *Rauwolfia serpentina* by Rajkarnikar and Saiju (1999).

Most of the works reveal that auxins like IAA, IBA and NAA induce root while cytokinins like BAP and kinetin induces embryoids, shoots, buds and

plantlets. Shoot regeneration in cultured tissues can be induced in many systems by an appropriate balance of an exogenous supply of auxins and cytokinin. Xu and Liu (1980) working on *Nicotiana tobaccum* reported that shoot bud proliferation is affected by the presence of BAP in the medium. Fane (1986) achieved rapid micropropagation of *Rubus sp*. by culturing shoot tip on MS + BAP (3ppm). Shrivastava *et al*. (1989) working on *Citrulus vulgaris* observed the requirement of BAP in shoot formation. But, in present investigation it was found that BAP not only induces callus, shoot buds but also have prohibitory effect in shoot elongation.

In the present study MS basal medium was not effective for callus induction but found to be effective in shoot elongation. Similar result was obtained by Wawrosch and Maskay (1999) on *Swertia chirata*.

In the present study, the root segments cultured on MS medium with different concentration of NAA proliferated calli from which hairy roots were differentiated but did not produce shoot and plantlet. Davoyan and Smetanin (1980) induced callus from root tip explants of rice plant on the media with 2, 4-D. In the present work, the callus on MS + NAA (0.5ppm) and MS + NAA (1ppm) showed

devlopement of greenish friable callus. Similarly Amatya and Joshi (1986) obtained the callus from root segment of *Brassica rapa* L. in synthetic nutrient media with or without supplement of auxins and cytokinins. The callus grown were subcultured on same medium grew fast giving soft and friable mass of callus. The growth was followed by differentiation of roots only. Similar result was obtained by Hahi and Akram (1986, 1987) in *Rauwolfia serpentine* and by Subash and Christopher (1988) in *Capsicum fructescens*.

In *Neopicrorhiza scrophulariiflora* rhizogenesis was recorded from root callus subcultured on MS + NAA (0.5ppm) after 4 week of culture. Similar results were observed by Joshi and Dhawa (1984) on *Brassica campestris*.

Lal et al (1988) reported successful establishment of plantlets raised through shoot tip culture of *Picrorhiza kurroa* for rapid clonal propagation. Shoot tip explant cultured on MS + kinetin (3.0 to 5.0 mg/l) supported rapid proliferation of multiple shoots from the explants. Addition of IAA (1.0 mg/l) to the kinetin containing medium showed marked improvement in the growth of regenerated shoots.

In the other hand, in present investigation shoot tip cultured on MS + BAP (1ppm) +NAA (0.5ppm) exhibit vigorous callus formation after 3 weeks of culture. The callus could differentiate shoot after 4 weeks of culture. Similarly, Nayak *et al* (1998) initiated optimum shoot formation from shoot tip explants of *Asparagus robustus* on MS+ 0.1 mg/l NAA + 1mg/l BAP. Niroula and Saiju (2000) obtained multiple shoot from young shoot tip of *Valeriana jatamansii* in MS + BAP 1mg/l and NAA 1mg/l. According to Bender and Neuman (1978), auxin and cytokinin were found to be vital factors for the organ differentiation. Saiju and Rajbhandary (1992) cultured shoot tips of *Lilium longiforum* in MS medium supplemented with NAA for the embryoid initiation. These embryoids were recultured in the medium supplemented with NAA and BAP for the formation of micro shoots. The tips of micro shoots were cut and these shoot were sub-cultured in the shoot formation medium. Shrestha and Joshi (1999) obtained maximum number of shoot multiplication from shoot tip explant of *Guizotia abyssinica* in BM + BAP (3ppm) + NAA (0.1ppm). Sinha *et al* (1999) regenerated multiple shoots through cotyledons with shoot tip of *Scutellaria discolor* on MS + BAP (1 mg/l) + NAA (0.1mg/l). Niraula and Saiju (2000) obtained multiple shoots from young shoot tips of *Valeriana jatamansii* in MS + BAP (1mg/l) + NAA (0.1mg/l).

Rajkarnikar and Saiju (1999) obtained microshoots on BAP (3mg/l) + NAA (0.1 mg/l) by shoot tips of *Rauwolfia serpentina* and subculture of these shoot tips on lower concentration of BAP (1mg/l) + NAA (0.1mg/l) resulted in shoot proliferation. In present study also shoot tip cultured on MS + BAP

(1ppm) + NAA (0.5ppm) obtained callus formation at base of shoot with formation of shoots and roots.

Sarkar *et al* (1996) induced multiple shoot from shoot apices of *Rauwolfia serpentina* in MS + BAP (1mg/l) and NAA (1mg/l) and callus formed at cut bases of the explants produced shoots when subcultured on media containing lower concentration of BAP (0.5 or 0.1mg/l). Sardana *et al* (1998) obtained complete plant regeneration from shoot tip explant of *Trachyspermum ammi* excised from seedling on MS + BAP (5.0mg/l) + NAA (1 mg/l). Deka (1999) investigated that lower concentration of hormones induce growth of shoots on culture of shoot tip explant of *Withania somnifera*.

In present investigation, the callus of shoot tip explant obtained from MS + BAP (1ppm) + NAA (0.5ppm) subcultured on same media resulted in proliferation of microshoots. These shoots elongated on hormone free MS medium. Similarly, Verma and Kant (1996) obtained elongation of shoot proliferated from nodal explant of *Emblica officinalis* Gaertn. on hormone free MS medium.

Wawrosch *et al* (1999) working on *Swertia chirata* Buch. – Ham. ex. Wall. found that shoot tip was proliferated into multiple shoots on hormone free media.

In present investigation also the microshoots obtained from shoot tip explant on MS medium supplemented with BAP (1ppm) + NAA (0.5 ppm) increased in length of shoots on MS hormone free medium. The regeneration capacity of shoots was found increased with repeated subculture of the adventitious shoots with callus at the cut end. The increase in multiplication rate per subculture might be a result of the fact that juvenile tissues tend to have a greater capacity for restoration (Murashige and Skoog, 1962).

Newburry (1986) studied *in vitro* multiplication in nine varieties of *Antirrhinum majus* L. by using shoot tip explants. Kharel and Karki (1994)

developed microshoots from shoot tips of *Chrysanthemum morifolium* varieties "Giant Fishtail Violet" on MS + BAP and NAA. Pradhan (1999) observed the regenerated plantlets from excised shoot tips of 5 years old plant of *Santalum album* supplemented with BAP (0.1mg/l). In this present study, shoot tips on BM supplemented with different combinations of BAP and NAA produced callus and microshoots. The callus obtained on MS+BAP (1ppm) + NAA (0.5ppm) induced roots with shoots after 9 weeks.

In present investigation stem explant induces callus on MS in presence of BAP with NAA. The explants on MS + BAP (0.5ppm) + NAA (0.5ppm) and MS + BAP (1ppm) + NAA (0.5ppm) produced calli at the cut ends of the explants. These calli differentiate into shoot bud proliferation and multiplication followed by formation of roots after 9 weeks of culture on MS + BAP (1ppm) + NAA(0.5ppm). Similarly Mudgal et al (1981) observed roots in the aseptic culture of stem explants of *Iberis amara* on MS medium with cytokinin and auxin hormones.

In this experiment nodal explant did not produce callus in MS media without hormone. The MS + BAP (0.5ppm) and NAA (0.5ppm) was found to be best for formation of callus. The callus subculture in same medium and MS + BAP (1ppm) + NAA (0.5ppm) resulted shoot bud proliferation. Similarly Ramashree *et al*, (1997) obtained bud leaf proliferation from the explants cultured on MS + BA (2mg/l) + NAA (0.5-2 mg/l). Verma and Kant (1996) also obtained shoot bud proliferation from nodal explant of *Emblica officinalis* on modified MS medium supplemented with BAP (3.0-5.0mg/l) in combination with NAA (0.5mg/l).

In present work different concentration of MS medium with or without hormone were used for *in vitro* rooting of shoots. Rooting hormones like IAA, IBA and NAA were used. Shoots cultured on hormone free medium could produce normal root after 3 weeks. On MS + IAA 0.5ppm yellowish hairy roots were produced after 4 weeks. On MS medium supplemented with IBA and

NAA the shoots could produce root with callus. On MS + NAA (0.5ppm) and MS + NAA (1ppm) showed vigorous growth of callus and hairy root after 3 weeks of culture.

In present investigation, MS medium without hormone induces better result than MS medium without hormone for rooting. Similarly Sahoo and Debata (1998) obtained rooting of excised shootlets of *Plumbago zeylanica* on hormone free basal medium within 4-7 days. Reddy *et al* (1998) also formed rooting of regenerated shoots of *Gymnema sylvestre* on MS ½ strength medium without hormone. Sharma and Chandel (1992) obtained 40% rooting on medium without an auxin in culture of *Tylophora indica.* Herrera *et al* (1990) investigated direct rooting of the new shoots obtained from shoot multiplication of shoot tip explant of *Digitalis thapsi* after 4 weeks of culture in MS hormone free media.

The study made by Lee *et al* (1985) showed that presence of NAA in the BM induced callus at the base of explant. Frequently roots formed on the surface of the callus and these cultures failed to become established in soil because callus separated roots from the base of shoots.

Rooting was obtained with all auxins at all concentration along with callus, but in the auxin free medium roots obtained without callus. The developed roots were hairy after 5 weeks. Similarly, Singh and Seghal (1999) obtained about 92% of the *in vitro* regenerated shoots rooted on MS hormone free medium within 2-3 weeks utilizing young inflorescence explants of *Ocimum sanctum*. Purohit *et al* (1994) obtained 100% rooting on the auxin free media in culture of *Chlorophytum borivilianum.*

The result of this experiment shows that the best hormone for shoot multiplication is MS + BAP (1ppm) + NAA (0.5ppm) and the best explant for micropropagation is shoot tip.

CHAPTER SIX

SUMMARY

In vitro micropropagation of *Neopicrorhiza scrophulariiflora* was conducted. The morphogenetic response and totipotency of various parts like root node and shoot tip were studied. The multiple shoots were obtained through shoot tip.

For *in vitro* studies seeds were germinated on MS basal medium. All the plantlets had normal root and shoot, which show 30-40% of germination. Various parts of the plant *in vitro* were inoculated on various concentrations of hormone and hormone free media. Root explants proliferated into callus and produced small hairy roots after 3 weeks on MS media with NAA 0.5ppm. Root explant on other combinations didn't show any satisfactory response. The nodal

explant on different concentration of BAP and NAA like MS+ BAP 0.5ppm + NAA 0.5ppm produced friable green callus with shoot bud proliferation. The callus on subculture into different concentration of BAP and NAA produced embryoids which gave roots after 9 weeks of culture.

The internodal explants produced callus on MS + BAP (1ppm) and NAA. (0.5ppm). The calli could not be differentiated into root and shoot even after subculture on different concentration.

The shoot tip explant cultured on MS basal media showed best response in formation of micro shoot on MS basal media with BAP 1ppm and NAA 0.5 ppm. The inoculated shoot tip increased in length with formation of callus at the base. The callus could differentiate into microshoots. These micro shoots on subculture into same media did not show any multiplication but on MS media without hormone the shoots multiplied with increase in length.

The shoot tip and node cultured on MS + NAA (0.5ppm) showed vigorous growth of callus at base with formation of hairy roots on multiplication of shoots.

In present experiment the shoot tip explant used for micropropagation was found to be best in obtaining microshoots than nodal explant and hormone free media was found to be best for shoot multiplication in length.

For *in vitro* rooting of shoots, auxins IAA, IBA and NAA were used. The microshoots inoculated in auxin free media obtained best result and on MS + IAA (0.5ppm) produced yellowish hairy root without formation of callus. The best rooting was observed in hormone free media. Then the regenerated plantlets were transplanted to the small pots containing composition of soil: sand: vermiculite in the ratio of 1:1:1.

CHAPTER SEVEN
CONCLUSION

The result of present investigation reveals that the endangered species of high altitude plants like *Neopicrorhiza scrophulariiflora* (Pennell) Hong can be conserved through micropropagation.

From the study on *Neopicrorhiza schrophulariiflora* following conclusions have been made.

1. Hormone free MS medium was found sufficient for the seed germination and seedling formation.
2. The better medium for callus proliferaration with hairy roots from root, node and shoot tip explant was found to be MS + NAA (0.5ppm).
3. The better medium for callus proliferation from shoot tip explant was found to be MS + BAP (1ppm) + NAA (0.5ppm) whereas for node and

internode explant MS + BAP (0.5ppm) + NAA (0.5ppm) was found to be better medium.

4. The shoot tip was best explant for shoot multiplication.
5. MS medium supplemented with BAP (1ppm) and NAA (0.5 ppm) was found effective in multiplication of high number of healthy shoots and MS medium alone was found to be effective for elongation of shoots.
6. NAA induced vigorous growth of callus in shoot explants with hairy roots.
7. IBA induced callusing of shoot explants and caused brown discoloration of shoot tissues with lateral growth of plantlets.
8. IAA showed rooting of this medicinal plant with formation of callus after 4 weeks.
9. MS medium without hormone was found to be effective for rooting of medicinal plant without formation of callus after 3 weeks.
10. The survival rate of *in vitro* raised rooted shoots in natural condition was only 45%.

Basically *in vitro* grown tissues have more percentage of valuable phytochemicals than *in vivo* since *in vitro* grown cells have controlled environmental conditions. Moreover, extraction of such chemicals is easier and more economic than *in vivo* plants (Kutney, 1990).

Bastola (2006) has conducted a research on comparative phytochemical study of *Neopicrorhiza scrophulariiflora* (Pennell) Hong from different localities i.e. sample A from Gyokcho, B from Gosaikunda, Rasuwa C from Chonkar Goth, Manang, D from Samarlek, Mustang and E (*in vitro* callus) from Tissue culture lab, callus obtained from present investigation and concluded following results.

The highest percentage of petroleum extract was found in sample collected from Gosaikunda Rasuwa and lowest in sample 'E' ie *in vitro* callus. Similarly, the highest percentage of ethanolic extract was found in sample

'E' i.e. from *in vitro* callus and lowest in 'C' i.e. from Chankar Goth, Manang. Phytochemical screening of petroleum extract showed the presence of sterol, triterpenes, coumarin, fatty acid and carotenoid. Similarly polyphenols, reducing compounds, glycosides, quinones, flavonic glycoside, coumarin derivative were found to be present in ethanolic extracts.

Phytochemical screening showed that plant is rich in sterol, triterpenes, reducing compounds, glycosides, polyphenols, carotenoids, flavonic glycoside. Iridoid glycoside, picroside I was found highest in *in vitro* callus augumented with BAP (1ppm) and NAA (0.5ppm) on Murasige- Skoog (MS) media.

So it has been expected that the medicinally important new components of *Neopicrorhiza scrophulariiflora* can be detected and extracted in future.

CHAPTER EIGHT
RECOMMENDATION

From the above study the following recommendations are made:

The techniques of *in vitro* cultures are much expensive and time consuming process. So, the protocols developed once must be well documented and definite national policy should be formed to conduct such research activities. *Neopicrorhiza scrophulariiflora* is a highly valuable medicinal alpine herb, cited in IUCN endangered category. People of Himalayan region are exploiting this plant indiscriminately for income generation and herbal remedies. So, it should be conserved by *in situ* and *ex situ* measures. For sustainable use of this plant cultivation procedure is to be followed.

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