CHAPTER ONE

INTRODUCTION

1.1 Background

Nepal, with its land area of 1, 47,181 sq. km in the central part of the main Himalayas has a host of complex topography, variation in the altitudes and hence, diversity in climate. Such a complex topography has provided a variety of habitats, ecological and bio-geographical zones which are further characterized by diverse vegetation types, flora and fauna. Owing to the richness, complexity and diversity of vegetation types in the rich monsoon rainfall, Nepal has been ranked the 10^{th} highest flowering plant diversity in Asia and the 27^{th} position in the world in biodiversity richness.

Medicinal plants that are defined as plants with healing properties (Oxford dictionary), are 30,000 to 70,000 across the world; and 80 percent of the world's rural people meet their needs of primary health care from such plants (Sarkar, 1996). Over 60 percent of all pharmaceuticals are plant based (Evans, 1996). The wealth of medicinal plants in Nepal may be considered as one of the important natural resources for the economic benefit of the nation. Out of 6,500 flowering plants of Nepal, 1,624 are found to have medicinal values of which 1,515 are angiosperms (Shrestha *et al*., 2001). Out of the estimate 2000 drugs which have been used in the Indian sub- continent, more than 1500 are of plant origin, and out of these about 700 spp. are used in the Nepalese traditional system of medical treatment (Singh 1997). Out of the 246 endemic species of flowering plants of Nepal (IUCN), 101 species are under endangered and eight under feared extinct groups (Shrestha & Joshi 2001). Most of these endangered species are the plants of medicinal and aromatic values, which are either confined in small areas and/ or are collected recklessly from their natural habitat.

Medicinal properties of plants are due to the active chemical constituents present in different parts of the plant (Mitsher *et al.*, 1980). Plants have been a rich source of medicines because they produce a host of bioactive molecules, most of which probably evolved for chemical defenses against predation or infections (Cox and Balick, 1994). The main groups or components are alkaloids, glycosides and saponin (Akerele, 1993). Most of the plants possess one or more of the medicinal properties, viz. antibacterial, antifungal, antiviral, anti-helmintic, anticancer, laxative, sedative, cardio-tonic, diuretic and others (Parajuli *et al.*, 1998).

Many modern drugs are derived from natural plants products or are chemical simulation of such substances (Joshi and Evington,1990). Of the two types of metabolites –primary and secondary – produced by plants, the latter includes alkaloids, phenolics, flavonoids, steroids, tannins and terpenes that possess medicinal properties (Barz., 1977).

The quality, quantity and controlled production of desired chemical substances have been possible through tissue culture technology. In a single species, there has been proven evidences of the differences in the contents of chemicals between the *in-vitro* developed plants and the *in-vivo* plants. Similarly, the chemical constituents in callus, hairy roots, leaf primordial, tender stem, rhizome, etc. vary with in the same plants species cultured *in-vitro*. Thus, it is important to investigate the different stages and aspects of an *in-vitro* plant.

With the ever increasing trend of population growth and the rapid revolution in science and technology, there has been tremendous research on chemical composition in plants in order to meet the global demand on medicines and other pharmaceutical products. Naturally occurring plants in the wild have not been able to fulfill these demands both quantitatively and qualitatively. Besides that, the propagation of flowering plants in the nature is relatively slow enough to meet the demand of raw materials placed by the ever flourishing pharmaceutical industries. Tissue culture has been the only viable, sustainable

and commercially profitable alternative to reproduce or regenerate the plants adequately in order to fulfill the demand placed by the pharmaceutical industries for the requisite raw materials especially from flowering plants.

Tissue culture, which in strict sense meant for the growth or maintenance of a plant or mammalian tissue in a nutrient medium (*in-vitro*), is now used as a blanket term for protoplast, cell, tissue, organ or whole plant culture under aseptic conditions. The term 'plant tissue culture' denotes genetically all cell, tissue and organ cultures, and employs the basic dogma: totipotency. The technique essentially involves separation of a cell, tissue and organ from the donor plant under aseptic conditions and growing it on a synthetic medium in a suitable container under controlled environment. Tissue culture systems allow for axenic propagation of plant material with high multiplication rates. Among other applications, tissue culture has been successfully dovetailed to conservation and exchange of germplasm in horticulture, medicinal plant production and crop improvement. Next, it has been proven that tissue cultured plants are superior to normal plants in synthesizing primary and secondary metabolites. The increased quantity is correlated to the increased chlorophyll content which in turn enhanced the photosynthetic rate in tissue cultured plants (Tejavanthi *et al.,* 1994). Plant tissue culture has been investigated as an alternative means to produce industrially important natural products such as flavors, insecticides and drugs. Successful application of plant tissue culture technology may help to conserve rare and endangered plants, including regeneration of similar clones from the culture cells at high frequency with natural product in similar or higher quantities. *In –vitro* propagated species of many important medicinal plants were found to be uniform, showing less variation in their content of secondary metabolites than their wild cultivated counter parts (Ramada *et al.,* 1991). Tissue culture technique is quite useful and the only alternative to increase production to those plants whose propagation is slow by means of the conventional sexual seed reproduction. Shoot primordia propagate vegetatively with very high rates and are genetically

quite stable, and whenever needed, they can readily re-differentiate to new plants having the same properties as the mother plants (Miyagawa *et al.*, 1986). In a nutshell, when plant materials are rare, the desired chemical synthesis is not adequate from the wild plants, and cost of such chemical products are high, tissue culture technology is used to obtain the desired product (Ramawat, 1990). Further, tissue culture helps to improve crop yield through mass production of disease free and disease resistant plants. It also enables to obtain hybrid plant in sexually incompatible species by somatic hybridization, and in the international level for the exchange of plant materials without much subject to quarantine procedures. Thus, plant tissue culture technique has been recognized, developed and implemented as the effective alternative means to propagate, cultivate and conserve medicinally and aromatically valuable plant species which are fairly rare in the wild and are becoming scarcer with time due to several causes including ignorance of the people to the importance of plants, deforestation, uncontrolled grazing, extension of agricultural land, indiscriminate collection of medicinal plants and their parts, pollution, climate change and environmentally hostile infrastructural development, i.e. without adequate Environmental Impact Assessment.

1.2 Description of *Valeriana jatamansii* Jones

Distribution

Valerianaceae is a herb family, more than half of those members are in the genus *Valerian.* They are mostly herbs, but a few have a shrubby habit. While in parts of South America, some are cushion plants. The Valerianaceae is primarily a Northern Hemisphere family, being absent from Australia and much of Africa. *V. jatamansii* Jones is found distributed in the Himalayan region (1200-3000 m). Locally known as nakalli jatamansii or Indian valerian, the species flourishes with its characteristic luxuriant growth in moist, shady and virgin soil.

Description

V. jatamansii Jones is a perennial herb, about 45cm tall with thick, horizontal, nodular, aromatic rootstock with descending fibers and tufted stem. Leaves are both cauline and radical. The stem is often decumbent below, very rarely divided. The radical leaves are often 1-3 inch diameter; deeply cordate, usually acute and toothed. Liliac flowers are small and white. Plants are unisexual and flower during spring i.e. March to April. Rhizome, the main source of valuable chemicals, remains attached to the green stem for a number of years. However, a two-year-old rhizome is suitable to extract the chemicals and then secure luxuriant growth.

Chemical constituents

Valerian, the main and useful chemical compound in *Valerian jatamansii* Jones, is composed of a yellowish green to brownish yellow oil, which is 0.5-2 percent in the dried root with the average yield of 0.8 percent. A dry and stony soil yields a root richer in the oil content than of the moist and fertile soil. The oil is a complex substance composed of valerianic, formic and acetic acids, and an alcohol known as borneol. The valerianic acid is an isobalerianic in nature with its characteristics unpleasant odour of valerian. The oil is readily soluble in alcohol and ether but sparsely soluble in water. Similarly, the root also contains two alkaloids-Chatarine and Valerianine which are under investigation for their composition and utility. Also, the other physically active compounds: glycoside, alkaloid and resin have been identified from the fresh rhizome by Chevaleer (1907).

1.2.3 Economic Importance

Plants within the Valerianaceae family have a long tradition in herbal medicines. *V. jatamansii* Jones (sugandhawal) syn *V. wallichii* DC, is widely

used medicinal plant due to its chemical constituents as essential oil and valepotriate under the trade name valerian. The therapeutic use of its root and rhizome is stimulant, carminative, antispasmodic, and useful in hysteria, epilepsy and neurosis. The plant is used in hypochondriasis, nervous unrest and similar emotional disturbances. Dried rhizomes are used in perfumes and hair preparations (The wealth of India, 2000). The drug valerian alleviates pain and induces sleep. Thus, it is beneficial to the patients suffering from nervous overstrain and nervous anxiety. Oil of valerian is used as cholera drops to cure cholera. The oil is also an ingredient in soap perfumery. Similarly, leaf decoction is used to nourish and improve eyesight by way of strengthening the weak optic nerves. Next, the juice of the fresh root under the brand name of Energetene of valerian has been recommemended as a narcotic in insomnia and as anti-convulsant in epilepsy. The oil too is effective in treating cardiac palpitation and restoring normal cardiac rhythms.

Status of the plant

Nepal Government has enlisted the plant as a commercially threatened species and is banned for export as raw material under the Forest Act 1993.

1.3 Description of *Rauvolfia serpentina* L. Benth. ex Kurz.

Botanical name	:	*Rauvolfia serpentine* L. Benth. ex Kurz.
Common name	:	Rauwalfia
Nepali name	:	Sarpagandha
Family	:	Apocynaceae
Chromosome number	:	n = 10, 11, 12, 22

Rauvolfia, the genus named in honour of Leonhard Rauwolf, a German physician of the sixteenth century, has its five species recorded in the Indian

Sub-Continent. *R. Serpentina* has attained a great reputation as a medicinal plant obviously due to its relatively broader medicinal importance.

R. Serpentina, is an upright, perennial, evergreen, glabrous under shrub with tuberous roots of 0.2 – 2 cm width and 4 – 12 cm length. It has a characteristic slightly wrinkled and course surface. The root bark is grayish yellow to brown and displays irregular longitudinal fissures. The leaves are simple, glabrous, lanceolate or obovate and are generally in whorls of three to four, crowding the upper part of the stem. The inflorescence is generally terminal but sometimes axillary, and usually consists of dense crowded cymes.

1.3.1 Distribution

The plant grows in tropical and subtropical regions, benefiting from the monsoon rains. It may be grown almost anywhere at low or medium elevations where rainfall is not less than 75 cm. It flourishes in hot and humid conditions and can be grown both in open and in partial shade. Soil with plenty of humus and a pH of 4.0 – 6.3 are desirable for luxuriant growth. It is widely distributed in the sub-Himalayan tract (up to an elevation of 1000 to 1150 m). In Nepal, *Rauvolfia serpentina* is distributed in the foothills of the Churiya Range where the forests are natural and the soil is rich in humus. Partial shade with moderate sunshine has been favourable for the healthy growth of the plant. Experimental cultivation has been undertaken in Kankai Mai and Dhulabari areas of Jhapa, east Nepal.

The plants are best raised from root-cuttings, but seeds and stem-cuttings can also be used for propagation. For commercial exploitation, roots are generally gathered two to three years after planting. It has been estimated that the alkaloid contents of the roots harvested after the shedding of leaves is far richer than the roots dug out in the foliate condition of the plants, i.e. during June-August.

Status of the plant in Nepal

R. serpentina is a protected plant species in Nepal under the Forest Act. 1993. Under this Act, any form and part of the plant is banned for export in crude form. The plant is enlisted in CITES II.

1.3.2 Chemical Constituents

The total alkaloid content of the root varies from 1.7 to 3.0 percent, of which, the bark alone accounts for nearly 90 percent. The leaves and the stem contains small amount of alkaloids. A large number of alkaloids (50 or more) have been isolated from various species of *Rauvolfia*. Reserpine ($C_{33}H_{40}N_2O_9$) is pharmacologically the most potent. Other important alkaloids are: reserpinine, rescinnamine, deserpidine, deseerpideine, serpentine, serpentinine, ajmaline, ajmalinine, ajmalicine, isoajmaline, rauwolfinine and yohinbine.

1.3.3 Medicinal Importance

Reserpine is a universally known medicine for hypertension. The utility of this plant in treating mental disease and snakebites was made known to the world by the Indians. Reserpine is also a proven medicine for several menstrual and menopause problems, lunacy, schizophrenia and some heart diseases. Reserpine stimulates uterine contraction and, therefore, is recommended for use in labour pain. An extract of the leaves has also been employed as a cure for the opacity of the cornea. Further, the root extracts is used as hypnotic, sedative, specific for insanity and a remedy in painful affection of the bowels.

1.4 Objectives

1.4.1 *Valeriana jatamansii* Jones

i) To determine the suitable batch of subculture for the optimum shoot formation rate *in-vitro*.

ii) To develop a suitable protocol for the *in-vitro* propagation of hairy roots for future analysis.

iii) To investigate the effects of different hardening media on the survival and propagation of the *in-vitro* developed plants and develop a protocol thereof .

1.4.2 *Rauvolfia serpentine* L. Benth. Ex kurz.

i) To determine the suitable explants for the rapid proliferation of the plants *in-vitro*.

ii) To investigate the effects of different phytohormones on the *in-vitro* multiplication of the plant.

iii) To acclimatize the *in-vitro* grown plants (in future).

1.5 Justification of the Study

Valeriana jatamansii Jones and *Rauvolfia serpentina* L. Benth. ex Kurz. are fairly exploited medicinal plants for traditional medicinal use and for the export of crude raw materials, although illegally. Both the species are enlisted in the commercially threatened species of plants of Nepal under the Forest Act, 1993 (Shrestha and Joshi, 1996). *Rauvolfia serpentina* L. Benth. ex Kurz is enlisted in the CITES II (in the endargered list of medicinal plants) where as *Valeriana jatamansii* Jones is enlisted in the commercially threatened species. Because of the selective nature of these plants in respect of agro-climate, their natural habitat as well as cultivation is very much restricted. Therefore, it is felt essential to investigate on their shoot proliferating capacity *in-vitro* and establish them *in-vivo* for commercial utility. These plants are gradually but

certainly being depleted from their natural habitat due to unscientific harvest, extension of agricultural land, deforestation and most significantly, due to the lack of conservation knowledge and sustainable use of these threatened species. There is an urgent need to conserve these plant species in their natural habitat as far as practicable, and also cultivate them on commercial plantation basis by the production of disease free, cost effective, genetically homogenous and chemically adequate plants through *in-vitro* culture and followed by their effective field trial. Thus, the commercial demand of these species can be met without affecting their wild relatives in their natural habitat. Similarly, the *in-vitro* propagated plant species can be transferred to their natural habitat through afforestation scheme, and enrich their population through their balanced distribution in a time bound manner. Thus, these indispensable medicinal plants can be sustained economically and ecologically. To materialize the aforementioned sustainable harvest of these species, a suitable protocol should be developed which can help the stakeholders: from a researcher to a farmer to cultivate these plants from lab to land and utilize the various useful parts as raw materials to the pharmaceutical industries and for traditional use. Through the research inspired efforts, the germplasm of these species can be donated, loaned or sold to foreign research laboratories and biotechnological enterprises that place their demand for these species. Thus, it is indispensable and urgent to conserve and utilize these threatened species. In the context of Nepal, Department of Plant Resources (DPR) under the Ministry of Forest and Soil Conservation (MoFSC) has enlisted most of the important medicinal plant of Nepal. *Valeriana jatamansii* Jones and *Rauvolfia serpentina* L. Benth. ex Kurz are being cultivated. Yet, the cultivation has not been sufficient to meet the commercial demand. Next, mass propagation of shoots *in-vitro* by using large scale bioreactors are in practice in developed countries in order to obtain tremendous yield of *in-vitro* plantlets and then acclimatize them in soil. Also, *in-vitro* developed plants are sought for obtaining superior quality secondary metabolites that are essential for manufacturing medicines, cosmetics etc. Such practices can be developed in Nepal too in future. Hence, these plant species are selected for basic scientific investigation in the present study.

CHAPTER TWO

LITERATURE REVIEW

Comprehensive work on tissue culture of medicinal plants was done by Staba (1963). He studied various medicinal plants (*Agave, Gingko, Belladona, Datura, Opium, Mentha, Digitalis,* etc.) *in- vitro.*

Murashige (1974) has described a three-stage procedure that normally requires alteration of culture medium or growth conditions between stages. Stage I pertains to the establishment of tissue *in-vitro*. Stage II: production of multiple shoots. Stage III must result in roots formation and conditioning of propagules prior to transfer to the green house. High light intensity is important in stage III.

Karki and Rajbhandary (1984) cultured the shoot tips of *Chrysanthemum cinerariefolium* Vis (Pyrethrum) in MS media supplemented with BAP 5 mg/I and IAA 0.5 mg/l, for shoot bud initiation.

Stapfer *et al.* (1985) observed rapid multiplication of *Veronica spicata* by using shoot tip explants. The greater number of shoots greater than or equal to 5 mm in length was produced on MS media containing 8 μm BA plus 0.01 μm NAA. Similarly, Swamy and Sahijram (1988) successfully regenerated multiple shoots from shot tip culture of *Bougainvellea glabra* on MS media supplemented with BA.

Agrawal *et al.* (1988) propogated multiple shoots from nodal explants of inflorescence axis of Delphinum Linn. in MS medium supplemented with 2-isopentenyladenine (2 ip) 10 mgl^{-1} and myo-inositol 100mg^{-1}. After 8 passages, growth of shoots continued on a medium with reduced concentration of 2 ip and omission of myo-inositol. IAA (0.1-5.0 mgl^{-1}) was found the best Auxin to induce 100 percent rooting *in-vitro* shoots while NAA (0.1-5.0 mgl^{-1}) induced callus and inhibited root formation.

Amatya and Rajbhandari (1988) produced multiple shoots from the aseptic culture of cotyledonary nodes of *Ficus auriculata* Lour. in the MS medium supplemented with BAP 1mgl^{-1} and NAA -.01 mgl^{-1}. Multiple shoots thus formed were sub-cultured every eight weeks to produce more shoots.

Arora and Bhojwani (1988) undertook to conserve a medicinally important and endangered species, *Saussurea lappa* C.B. Clarke by *in-vitro* regeneration and then propagation of shoots, various explants – colyledons, hypocotyle and root segments (from 15-day-old aseptic seedlings) and plumular leaves (from *in-vitro* formed shoots) were excised and cultured on MS medium supplemented with growth regulators. Shoot multiplication was achieved via adventitious bud formation form the compact, nodular callus formed at the base of the shoot explants. Maximum rate of shoot multiplication (3.2 fold in 3 weeks) occurred on MS+BAP ($5x10^{-6}$m) + GA_3 ($3x10^{-6}$m).

Barna and Wakhlu (1988) developed a protocal for micro propagation of *Plantago ovato* Forsk from axillary shoots by using shoot tip as explants. Ms supplemented with kn 1mgl^{-1} and NAA 0.01mgl^{-1} was found optimal for axillary shoot formation. These shoots showed best rooting response on half-strength MS medium supplemented with IBA (1mgl^{-1}). The plants were successfully transplanted into pots and are being grown to maturity under green house conditions.

Dantu and Bhojwani (1988) initiated the shoot cultures of three commercial cultivars of Gladiolus corms. MS medium supplemented with 0.5 mgl^{-1} BAP was found to be most suitable for shoot multiplication. Excised fleshy leaves (4-6 mm long) also formed adventitious shoots buds. Interestingly, in liquid medium good shoot elongation occurred. Even in the presence of 0.05mg/l BAP. These shoots of 10-12 cm in four weeks were readily rooted in MS+NAA (0.1 mgl^{-1}). However, the plantlets did not survive after transplantation to pots.

Joarder *et al. (*1988) enhanced the rapid multiplication of mulberry tree (*Morus alba* L.) through culture of excised tissues and organs. Shoot tips and lateral

buds were used as explants. The MS medium supplemented with 1mgl^{-1} BA or BAP resulted remarkable increase in leaf and internode extension. High concentration of BA or BAP (2 mgl^{-1}) decreased leaf and internode extension but enhanced more shoot production giving bushy appearance.

Sengupta and Sharma (1988) induced multiple shoots formation in *Withania somnifera* L., and undershrub of solanaceae family that contains a narcotic substance known as 'withanolide'. Multiple shoot formation was examined in the plant on MS medium using cytokines alone or in combination with auxins. BAP 0.5, 1.0 and 2-5 mg/l were found equally effective to induce multiple shoot formation.

Niraula and Rajbhandari (1988) induced multiple shoots from the cotyledonary node of *Poncirus trifoliate* that was used as the explants. The shoot induction was caused in the MS medium supplemented with BAP 1mgl^{-1} and NAA 0.1 mgl^{-1}. On subculture, these shoots continued to proliferate in the basal medium supplemented with lower concentration of BAP (0.1mgl^{-1}). Roots were produced in shoots when transferred on basal medium supplemented with NAA 0.1 mgl^{-1}.

Upadhyaya *et al.* (1988) carried out the *in-vitro* studies of *Picrorhiza kurroa* Royle ex Benthl., an endangered species of medicinal importance. Shoot cuttings (1.5-2 cm long) were used to initiate aseptic cultures. MS medium containing 3 percent sucrose and gelled with 0.8 percent agar was used as basal medium (MS). It was supplemented with a range of concentrations of BAP (5 x 10^{-7} m^{-5} x 10^{-6}m) and zip (1 x 10^{-5}m-5 x 10^{-5}m). Within 2-3 weeks, the buds present on the explants exhibited growth and after four weeks, multiple shoots were formed. Shoot proliferation was largely due to forced axillary branching. The maximum number of shoots was obtained with 1×10^{-6}M BAP (90 percent cultures developed 20-30 shoots at the end of fourth week).

Rajbhandari and Bajaj (1989) had successfully carried out rooting of *in-vitro* produced shoots in non-sterile sand – an inexpensive and efficient technique for en-masse micro propagation of *in-vitro* plants in the *in-vivo* condition.

Niraula *et al.* (1992) induced multiple shoots on cotyledonary node culture of *Citrus limon* L. MS medium supplemented with 1mg/l BAP and 0.1 mg/l NAA. These shoots on subculture continued to proliferate in the same medium supplemented with lower concentration of BAP 0.5mg/l and NAA 0.01 mg/l. The micro shoots produced roots when transferred in non-sterile sand and successfully established in the field.

Awal, Shrestha and Rajbhandari (1993) obtained multiple shoots from shoot tip culture of *Dianthus Caryophyllus* on MS medium supplemented with BAP 1mg/l and 0.01 mg./l IAA. Culture resumed growth after six weeks storage.

Duproz *et al.* (1994) obtained tropane alkaloids in transformed roots of *Datura quercifolia*. Hairy root cultures of *D. quercfolia* were established following infection with *Agrobacterium rhizogenes* strain LBA 9402. Eight tropane alkaloids were identified in the hairy roots, hyoscyamine being the major constituent. The growth and the hycoscyamine content of transformed roots were investigated under various conditions. Gamborg B5 medium was identified as the best for growth as well as for hyoscyamine accumulation. The influence of sucrose concentration was examined and a 5% concentration was found to be the most appropriate for growth and for alkaloid production.

Karki (1993) generated the multiple shoots of *Zingiber officinale* by using buds (2 mm) from rhizome, as the explants, on MS medium (1962) supplemented with 1.0 mg/l BAP and 1.5 mg/l kinetin. The subculture of *in- vitro* shoot in fresh MS medium of the same concentration increased the number of shoots.

Kayastha (1993) induced multiple shoots of *Artocarpus heterophyllus* Lam in the MS medium supplemented with 1.0 mg/l BAP and 0.01 mg/l NAA when

cotyledonary node was used as explants. The roots were produced when the micro-shoots were transferred to non-sterile sand.

Tanaka and Matsumoto (1993) had obtained a clone of a Ri-transformed regenerated plant of *Ajuga reptans* Va. a *tropurpurea.* The plant was derived from a hairy root line selected from more than 120 hairy root clones. It produced high growth rate in culture as well as more than four times as much 20-hydrooxyedysone as the original wild-grown Ajuga plant.

Akita *et al.* (1994) had obtained 64.6 kg of *in-vitro* shoots from 460 g of the inoculated shoot primordial of *Stevia rebaudiana* by using a large scale bioreactor. These shoots were easily acclimatized in soil. Aseptic plants of *S. rebaudiana* obtained from apical meristem were used. Shoots were cut to single nodal segments and transferred to 100 ml MS medium supplemented with 0.1 ppm NAA and 1 ppm BA, with suitable ph adjustment to 6.2. Every flask was shaken at 120 rpm under 25°C, 16 hour photoperiod (about 6000 lux.). Clusters of multiple shoot primordia were formed within one month.

Kharel and Karki (1994) cultured shoot tips (2-4 mm) of *Chrysanthemum morifolium* varieties “giant fishtail violet” on MS medium supplemented with BAP 1.0 mg/l and NAA 0.01 mg/l. Microshoots were successfully rooted in sand and grown into normal plants. The plants showed no abnormalities in flower morphology and colour.

Shrestha and Rajbhandari (1994) achieved clonal multiplication of *Cymbidium longiflorum* D. Don form shoot apical meristems cultured *in- vitro*. Rapid multiplication of shoots occurred in MS medium containing $1mgl^{-1}$ BAP, 1.5 mgl^{-1} kinetins and 10 mgl^{-1} Adenine sulphate. The proliferation continued on subculturing on the same medium.

Bansal and Bansal (1995) studied efficient multiple shoots and plantlets formation in *Cicer arietinum* L. from the seedling explants on MS media, enriched with different concentrations of auxin and cytokinin.

Kayastha (1995) obtained shoots *in-vitro* from the cotyledonary nodes of *Artocarpus heterophyllus* lam. when cultured in MS medium supplemented with 5mgl^{-1} BAP. These shoots were subcultured in the same medium supplemented with 1.0mg/l BAP and 0.01 mg/l NAA for multiplication. The roots initiated on non-sterile sand after five weeks but did not survive well on transplantation to the field.

Martin and Hariharan (1995) had obtained multiple shoot regeneration from callus initiated from young rhizome of *Alpinia calcarata* Rosc. On MS medium containing a range of concentrations of 2, 4-D, NAA and BAP. Sixty days old callus on modified MS medium with a combination of BAP (1.5 mg/l) and 2, 4-D (0.25 mg/l), produced a single shoot along with 4-6 shoot primordial and numerous meristematic protuberances.

Pant *et al*. (1995) induced multiple shoots form the apical dome of shoot tips of *Mentha arvensis* L. Var. *piperoscens* by culturing them on MS liquid media supplemented with BAP 10^{-6}M, solidified with 0.2 percent gelnite. Subsequent transfer of the regenerated shoots on MS media supplemented with NAA 10^{-7}M with BAP 10^{-7} M resulted in root formation.

Tanaka *et al*. (1995) had successfully extracted a chemical named vincamine from the multiple shoot culture derived from hairy roots of *Vinca minor* L. A previously established Ri-transformed clone, Vm-101, proliferated rapidly in *in-vitro*, which displayed a high degree of lateral branching and rapid shoot elongation and had a growth index 2.5 times that of an untransformed plant. The addition of 2.2 μM BA to the culture medium increased the shoot number without decreasing the growth index.

Martin and Hariharan (1996) obtained a sustained rate of shoot multiplication, i.e., 15-20 shoots in 3-weekly subcultures after fourth subcultures in *Ullucus tuberosus* and *Oxalis tuberosa*. For *U. tuberosus*, elongated shoots after 3 weeks of growth period were set as individual microcutting in the sand bed under high humidity and polythene cover to obtained rooted plants. Except for

O. tuberosus, the multiplied shoot buds had to be transferred to the basic medium containing 1 mg/l GA to induce elongation. The elongated shoots, however, rooted readily in the nonsterile sand.

Miyagawa *et al.* (1986) carried out shoot multiplication *in-vitro* in *Stevia primorida* by using MS – agar medium containing kinetin (10^{-5} M) at 25°C, 16 hours light a day. The primary shoot tips with one-two leaves were subcultured in BAP 2 ppm and NAA 0.2 ppm and BAP 2 ppm and NAA 2 ppm, with slow rotation of 2 rpm for two months. Subculture was continued by using the shoot premorida (*in-vitro*) for several times. From a small aggregate of shoot primordia, 50 to 60 individual shoot primordial were obtained. Therefore, their propagation rate was about 3n, where 'n' is the number of weeks. When the pieces of shoot primordia in the media (MS + BAP 2 ppm and NAA 0.2 ppm , and BAP 2 ppm and NAA 2 ppm) were transferred on agar medium B5 containing 0.02 ppm BAP and 2% sucrose in static culture in 2 to 3 weeks, they easily developed into a large number of young plantlets with shoots and roots which could be grown to normal plants.

Sarker *et al.* (1996) induced multiple shoots from nodal segments and shoot apices of *Rauvolfia serpentina* L. Benth. ex Kurz. on MS media containing 1.0 mg/l BAP and 0.1 mg/l NAA. Callus formed at the cut bases of the explants which produced shoots when subcultured on MS media containing low concentration of BAP (0.5 or 0.1 mg/l) and NAA (0.1mg/l). the *in-vitro* proliferated plants were transplanted to the soil.

Reddy *et al.* (1998) regenerated the multiple shoots from mature nodal explants of *Gymnea sylvestre*, a useful antidiabetic medicinal plant, when cultured in MS medium containing combinations of BAP and NAA. They observed maximum shoot sprouting on MS medium containing 5 mg/l BAP and 0.2 mg/l NAA. Regenerated shoots were rooted on MS half strength medium without any growth regulator.

Jaya and Ramakrishna (1999) developed hairy roots of *Plumbago indica* by using leaf explants. The sterized leaf explants wre inoculated in MS medium

with different concentrations of BA, IAA and IBA. The regenerated shoots were transferred to MS basal medium, incubated in dark for two days and then inoculated with three – days old cultures of *Agrobacterium rhizogenes* and again kept in dark for 15 days. Hairy roots were developed which were subsequently cultured in liquid MS basal medium with carbencillian, an antibiotic. Plumbagin, a useful chemical, was extracted from the hairy roots.

Karki and Niroula (1999) obtained the multiple shoots of through *in-vitro Acacia auriculiformis* A. Cunn. excised from their 12-16 days old seedlings. Twenty five to thirty multiple shoots were proliferated in MS medium supplemented with BAP 0.5 mg/l and NAA 0.1 mg/l after the fourth sub-culture. The micro shoots 3-4 long were excised and rooted in non-sterile sand. The roots were initiated after 13-21 days. The rooted plants were successfully established in soil.

Kaur *et al*. (1999) studied the *in-vitro* propagation of V*aleriana jatamansii* Jones. The sterilized explants' shoot buds were established on solid media supplemented with Benzyl adenine (BA) alone or in combination with IAA or NAA. The buds cultured on media supplemented with BA and IAA or NAA formed shoots which after 3-4 weeks produced roots on the same medium.

Pant and Basavaraju (1999) successfully obtained multiple shoots from single axillary bud and that of female inflorescence leading to the formation of fruit in single BAP treatment in the migropropagation of *Morus indica* linn.

Rajkarnikar and Saiju (1999) regenerated the multiple shoots of *Rauvolfia serpentina* (L.) Benth. ex. kurz. from excised shoot tips on MS media supplemented with 3mg/l BAP and 0.1 mg/l of NAA. Subcultured of these micro shoots were carried out on the same media but with lower concentration of BAP (1mg/l) and NAA (0.1mg/l) for shoot proliferation.

Ranjit (1999) obtained callus from the different explant of *Rhus parriflora* on different concentrations of growth hormones but in *Bauhinia variegata* L., the

nodal explants gave multiple shoots on MS media supplemented with 1 ppm BAP and 0.5 ppm NAA. However, leaf, root and stem explants could not give any response.

Thakur and Ishii (1999) successfully cultured the motile zygotic embryos of *Pinus gerardiana* wall. on two different media with 30 treatments of different growth regulators. Maximum adventitious bud initiation and growth were obtained on a half strength MS medium supplemented with 0.5 mg/1 BPA. Shoot elongation was achieved on half-strength MS medium lacking growth regulators but containing 2g/1 activated charcoal. Highest rhizogeneis of 40 percent were obtained when shoots were kept in an upright position in medium containing 0.25g/l IBA, 0.25g/l NAA and 15g/l sucrose for two weeks in dark at 4°c, two weeks in light at 25±2°c and then for two months on hormone free medium.

Joshi and Singh (2000) found MS medium supplemented with 1 ppm BAP and 0.5 ppm NAA gave good result in *Bauhinia variegata* and MS medium with 0.5 ppm BAP along with 0.1ppm NAA was found best in *Bauhinia purpurea* for shoot multiplication when nodal explants were taken for culture initiation.

Joshi *et al*. (2000) proliferated the microshoots from the nodal explants of *Elacocarpus sphaericus* (Gaertn) K. Schum. in the MS medium supplemented with BAP 0.5 mg/l and NAA 0.01 mg/l. The proliferated microshoots when subcultured in MS medium with less Ammonium nitrate (300 mg/l) supplemented with BAP 0.25 mg/l at the interval of 3 to 4 months, it showed good result of proliferation with no browning of microshoots. The micro roosts developed roots successfully in non-sterile sand and within 15 to 20 days in green house condition.

Karki and Saiju (2000) developed a protocol for large scale production of palntlets of *Amomum subulatum* Roxb. from the two cultivators viz., Ramsai and Golsai. The shoot tips (1-2 mm) were excised from the mother plants and cultured in MS solid medium (1962) supplemented with 1.0 mg/l BAP and

0.1mg/l NAA multiple shoots with roots were produced by repeated sub-cultures in same quantities of liquid medium. Rooted plants were successfully planted in the field.

Kayastha (2000) developed a protocol for the micro propagation of *Swertia chirata* (wall.) C.B. Clarke. The shoot tips of two months old plants were cultured in MS medium supplemented with 1 mg/l of BAP and 0.01 mg/l of NAA for multiplication. Ten to fifteen microshoots were developed after fourth subculture. These micro shoots were transferred in non-sterile sand for rooting. The roots were developed within two to three weeks.

Niroula and Saiju (2000) obtained multiplied shoots from young shoot tips of V*aleriana jatamansii* Jones in MS medium supplemented with BAP 1 mg/l and NAA 0.1 mg/l. Ninety percent microshoots that were transferred to non-sterile sand, developed roots. These plants were successfully established in the field.

Rajkarnikar *et al*. (2000) showed the micro-shoots sprouting from the base of shoot-tip explant in the MS medium with BAP 1 mg/l, 2 mg/l along with 0.1 mg/l NAA in *Rauvolfia serpentina* L. Benth. ex Kurz.

Yoshikawa *et al*. (2000) studied about the production by transgenic tissue, i.e. hairy root cultures, which were induced by the transfer into plant cell or RI plasmid DNA in *Agrobacterium rhizogenes*. This has been expected as an effective method to produce the useful secondary metabolites, which has not been produced by the usual culture methods using callus or cell cultures. The pure saponin content is produced by hairy root. Culture was almost the same as it of the native root in a fresh weight, and four times higher in dry weight. The effects of saponin extracted from hairy root culture were better than those of the native roots because the cultured class were purer and rich in the saponin content per dry material than those of the field cultivated root-which may contain agricultural chemicals.

Pereira *et al*., (2003), induced multiple shoots of *Anenopaegmg arvense* (Vell.) *Stellfeld exde* Souza, an endangered medicinal plant, by using nodal segments as explants on MS media supplemented with 4.4 mm of kinetin. Acclimatization of un-rooted plants into soil was carried out successfully.

Poudel (2003) obtained maximum number of multiple shoots of *Mentha specata* L. on MS mediums supplemented with 1ppm BAP for both node and shoot tip explants.

Basnet (2004) developed a protocol for the *in-vitro* propagation of *Coffee arabica* L. and *Citrus aurantifolia* swingle. MS medium supplemented with BAP 1.0 ppm and NAA 0.5 ppm was proven effective for micropropagation of shoots for *in-vitro* rooting of *C. arabica* L. Similarly, half-strength of MS medium supplemented with 1.5 ppm IBA was the best rooting media *in-vitro*. He found nodal explant of *C. aurantifolia* effective for micropropagation of multiple shoots. On the other hand, leaf explant was found to be effective for callus formation in *C. arabica* L.

Bhatt (2004) investigated the suitable plant combinations of plant growth regulators for the micropropagation of *Orxylum indicum* (L.) Kurz. Nodal explants were found to be the best for inducing multiple shots in MS medium supplemented with BAP (2.0 ppm). In contrast, maximum root differentiation in the shoots was found in half-strength MS medium containing 2.0 ppm IBA. Further, MS medium with 1.0 ppm IBA was found effective for root elongation.

Devkota (2004) investigated the appropriate combinations of plants growth regulators for the micropropagation of *Valeriana jatamansii* Jones by using node and shoot tip as explant. They had found that MS medium supplemented with BAP 1 ppm was suitable for shoot initiation, and MS media with NAA 0.5 ppm for rooting.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

The present investigation was carried out in the Central Department of Botany (CDB), Tribhuvan University (TU) Kathmandu, Nepal.

The initiating material used for the investigation of *Valeriana jatamansi* Jones was the *in-vitro* shoots established in the *in-vitro* condition in the tissue culure laboratory of Biotechnology Unit of CDB, by the senior investigator, Samjhana Devkota (2004). The material was used with the kind permission of the investigator and that of my supervisor.

For the investigation of *Rauvolfia serpentina* L.Benth. ex Kurz, 25 young plants packed in polythene bags were brought from a private nursery of Domukha village Kankai mai, Jhapa (altitude:610 m), eastern Nepal. The plants were initially nurtured in the green house of the CDB in order to maintain the desired temperature for the plants and enable them to sprout with young shoots. The shoots were used as the explant for the *in-vitro* investigation.

3.2 Methodology

For the investigation, Murashige and Skoog (1962) solid media (MS) supplemented by two phytohormones or plant growth regulators (PGR): BAP and NAA were used. The phytohormones were used either alone or in different combinations of the two along with the MS.

3. 2.1 Sterilization of Glasswares and Metal Instruments

The requisite glasswares were dipped in detergent rich water for 24 hours and then cleaned them with bottlebrush. The glasswares were washed thoroughly with tap water and finally rinsed with distilled water. They were sterilized in Hot-Air Oven at 160^{o}c for two to three hours. Forceps, knife, scalpel and gloves were wrapped in aluminium foil and then autoclaved them at 121^{o}c for 30 minute at 15 lb/sq. inch pressure.

3.2.2 Preparation of Stock Solution for MS Medium

Each stock solution was prepared in the given concentration by first weighing the requisite ingredients in an electronic balance and then dissolving them completely in distilled water in the sequence of the ingredients as established in the protocol. Each stock solution was kept in sterilized brown bottles with label indicating the name of the preparations and the date. The stock solutions were preserved in refrigerator at 4^{o}c.

Macronutrients- stock 'A'

Chemical	**10 X (gm/ litre)**
Potassium Nitrate (KNO_3)	19.00
Ammonium Nitrate (NH_4NO_3)	16.50
Magnesium Sulphate ($MgSO_4.7H_2O$)	3.70
Potassium dihydrogen phosphate (KH_2PO_4)	1.70
Calcium Chloride ($CaCl_2.2H_2O$)	4.40

Micronutrients- stock 'B'

Chemicals	**(100X) mg/ 100 ml**
Boric Acid (H_3BO_3)	620
Manganese Sulphate ($MnSO_4.4H_2O$)	2230

Zinc Sulphate ($ZnSO_4.7H_2O$)	860
Sodium molybdate ($Na_2MoO_4.\ 2H_2O$)	25
Copper Sulphate ($CuSO_4.\ 5H_2O$)	2.5
Cobalt Chloride ($CoCl_2.\ 6H_2O$)	2.5
Potassium Iodide (KI)*	8.3

* KI was prepared and stored separately

Iron EDTA – Stock 'C'

Chemicals	**10X (mg/ 100 ml)**
Sodium Ethylene diamine tetracetate (Na_2EDTA)	620
Ferrous Sulphate ($FeSO_4.7H_2O$)	278

Vitamins– Stock 'D'

Vitamins	**(100X) mg/ 100 ml**
Glycine	200
Nicotinic Acid	50
Pyridoxin HCl	50
Thiamin HCl	10
Myo - inositol	10000

Carbon Source

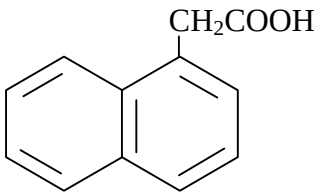
Chemicals	**gm/ litre**
Sucrose	30

Solidifying agent

Chemicals	**gm/ litre**
Difco Bacto Agar	8

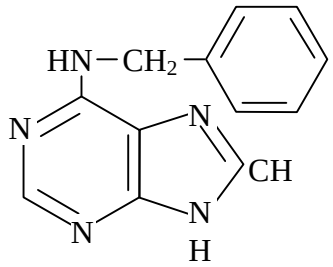
3.3 Hormones Used for Investigation

A) Auxin: Naphthalene acetic acid (NAA)



NAA

B) Cytokinin: 6-Benzylaminopurine(BAP)



6-Benzylaminopurine (BAP)

3.4 Preparation of Hormone Stock Solutions

Preparation of desired NAA (Auxin)

10 mg of NAA was first dissolved in 2 ml of 95 percent ethyl alcohol. Then 98 ml double distilled water was added in the dissolved NAA in 2 ml ethyl alcohol resulting to 100 ml volume.

By formula,

- 1 mg NAA dissolved in 1000 ml water becomes 1 ppm NAA
- 10 mg NAA dissolved in 1000 ml water becomes 10 ppm NAA

Therefore, 10 mg NAA dissolved in 100 ml water became 100 ppm NAA stock solution. This stock solution was labeled and kept in refrigerator for the desired use throughout the initial investigation.

Preparation of desired BAP (Cytokinin)

10 mg of BAP was first dissolved in 2ml of 0.5N NaoH. Next, 98 ml double distilled water was added to the above 2 ml volume of 0.5N NaoH where 10 mg of BAP was dissolved. The total volume became 100 ml.

By formula,

- 1 mg BAP dissolved in 1000 ml water becomes 1 ppm BAP
- 10 mg BAP dissolved in 1000 ml water becomes 10 ppm BAP
- 10 mg of BAP dissolved in 100 ml water makes 100 ppm BAP

Therefore, the 10 mg of BAP dissolved in 100 ml volume (2ml of 0.5 N NaoH + 98 ml distilled water) made 100 ppm stock solution of BAP. It was labeled and kept in refrigerator at 4^{o} C for further use.

However, in both the preparations, the pH values were measured with a pH meter and the pH value of 5.8 was maintained in both the solutions by adding either 0.1N NaOH or 0.1N HCl and then shaking the solutions. The solutions were transferred into freshly sterilized brown bottles, labeled them including the date of preparation and then preserved in refrigerator at 4°c.

3.5 Preparation of MS Media (as the basal media)

To Prepare one litre of MS Medium, 400 ml of distilled water was taken in a clean, dry and sterilized conical flask (2 l). The water-filled flask was kept on the magnetic strirrer which was then set on motion. Then from the stock solution 100 ml of macronutrient (stock A), 1 ml of micronutrient (stock B), 10 ml of Iron EDTA (stock C) and 1ml of vitamins were added into the flask respectively until each of the solutions dissolved completely before adding the next in the order. Next, 30 gm of sucrose (3%) was dissolved little by little with the motion set by the magnetic stirrer. The solution was adjusted to 900 ml with sterile or double distilled water. The solution was maintained at pH 5.8 by

adding 0.1 N NaoH or 0.1 N HCl when required. Finally, additional volume of sterile distilled water was added and made the final volume of one litre. MS medium or the basal medium was made ready. Next, as per the requirement of the investigation procedure, required concentration of phytohormones was prepared by using the following formula and added to the MS medium.

Volume of stock solution required (v_1) =

$$\frac{\text{required concentration } (S_2) \times \text{medium vol.} (V_2)}{\text{concentration of stock solution } (S_1)}$$

Where,

S_1 = Concentration of hormone in the stock solution

V_1 = volume of hormones to be added in the given medium from the stock solution

S_2 = concentration of hormone required in medium

V_2 = volume of medium desired for the investigation

(Source: Media preparation and Handling, P. 25, Laboratory facilities, Operation, and Management by O.L. Gamborg, G.C. Phillips, Eds. 1995)

The prepared hormone(s) was added in the dispensed volume of the MS medium in different beakers as desired by the procedure. Then, agar powder (Difco Bacto Agar), at a rate of 0.8 gm per 100 ml solution was dissolved in the hot medium which was kept on the electric heater to enhance the faster solubility of agar in the solution. The medium was dispensed into the clean and freshly sterilized culture tubes (150 mm × 25 mm). They were capped with aluminium foil and tied with durable rubber bands in order to maintain aseptic environment in the medium. The medium was labeled specifically with the type and the concentration of hormones used. The medium filled culture tubes were autoclaved at 121˚C for 15-20 min at 15 lb/sq-inch (Bhattrai, 2000). The autoclave was allowed to cool to normal atmosphere pressure and the culture tubes with media were taken out. They were kept on the culture tube stands and

were finally placed in the aseptic chamber of tissue culture laboratory at 45˚ inclination for solidification. The media in the tubes were observed for a week. If there is no sign of microbial contamination, the media is perfectly sterilized and thus, it is suitable for inoculating the desired explants or sub-culturing materials.

3.6 Preparation of Inoculation Chamber

To ensure absolute aseptic condition of the inoculation chamber, the Laminar Air Flow chamber or clean bench was thoroughly cleaned with 95 percent ethanol. The culture tubes with media, sterilizing box containing autoclaved glass wares were cleaned by spraying ethanol (95 percent) externally. All the necessary materials with their containers viz. spirit, ethanol, sodium hypochlorite solution, sterile distilled water, rubber bands, aluminum foils, match box, marker pen and gloves were exposed to ultra-violet (UV) light for 45 min. Then, the UV light was put off. The air blower was kept on for 30 min prior to inoculation. In the mean time, hands were washed with liquid detergent, dried and then disinfected with 75 percent ethanol. Gloves were also sterilized with ethanol. Then the inoculation of explants was carried out with the continuous flow of air in the chamber. After the inoculation was completed, the culture tubes were labeled with indelible ink. The culture tubes were kept back in the aseptic growth chamber of tissue culture laboratory.

3.7 Subculture of *In-vitro* Shoots for Optimum Shoot Multiplication Rate

Healthy nodes explants were taken from two years old *in-vitro* raised plants. These nodes of 0.5-1cm each were inoculated in MS medium supplemented with 1ppm BAP and 0.5 ppm NAA. The second passage of nodes was used as the explants for the third batch of subculture. The process was continued until

the sixth batch or passage of subculture. Each subculture was maintained for four weeks, in the laboratory condition of 25 $\pm$ 2^{o}C, 16 hour photo period with four replicates each.

3.8 Subculture of Hairy Roots of *Valeriana jatamansi* Jones on the Different Concentrations of NAA

For the subculture of hairy roots on the different concentrations of NAA, MS supplemented with four different concentration of NAA viz, 0.5 ppm NAA, 1.0 ppm NAA, 1.5 ppm NAA and 2 ppm NAA were established. Nodes of 0.5 cm – 1 cm were obtained from the *in-vitro* developed shoots. These nodes were subcultured on the four different concentrations of NAA + MS.

3.9 Sub-culture of Hairy Roots of *Valeriana jatamansi* Jones on Different Strengths of MS

To determine the suitable strength of MS for the optimum proliferation of hairy roots of *Valeriana jatamansii* Jones, four different strengths of MS medium:: $\frac{1}{4}$MS, $\frac{1}{2}$MS, $\frac{3}{4}$MS, and full strength of MS were supplemented with 0.5 ppm NAA. The healthy *in-vitro* nodes were inoculated in each of the different strengths. The subcultures were maintained in the laboratory condition of 25±2°C, 16 hours photoperiod. The observation was made on weekly basis for six weeks.

3.10 Acclimatization

The six to eight week old *in-vitro* plantlets were selected. Their caps were opened and exposed for five days at laboratory or room temperature. The media was washed from the plantlets with clean and then distilled water. Then Fungicide (Bavistine 0.1 percent) was spread uniformly. The tips of the *in-vitro* roots were cut. The tip of the trimmed roots was dipped in IAA (1 gm/litre) for 10 minutes. However, the leaves and the shoots must not be immersed; hence,

shoots were prevented from the contact of IAA. Nine trays were taken (2.5 x 1.5 square foot each). In a batch of three trays each, three different hardening media were taken respectively: cleaned and sun-dried sand, coco pit and the mixture of non-sterile but sun-dried sand and soil (1:1). The three different media were spread on the surface of the trays. A small volume of water was added in the trays to soften the soil. Eight acclimatized plantlets were transferred to each tray with three trays containing 24 plantlets for each hardening medium. After that, light watering was done to maintain humidity. A miniature green house was constructed around each tray. A small volume of water was kept on the roof of the green house to keep the internal environment cool. The required labels were kept. The plantlets were maintained in the above conditions for 22 days with light watering twice a day. Observation was made in the twenty third day. The entire process was repeated twice. The survived and hardened plants in the above hardening media were transferred to polythene bags filled with the mixture of sand + humus + soft soil (1:2:1).

The plants were kept in the polythene bags for two months. Thereafter, they were transferred to the soil. The growth of the rhizome was noted after every two months up to six months. The entire plant growth and rhizome development was observed up to ten months.

3.11 Sterilization and Culture of Explants of *Rauvolfia serpentina* L. Benth. ex Kurtz.

The explants (shoot tips and tender nodes) were taken from the terminal and axillary parts of the plants. They were washed in running water for one hour. Next, the explants were kept in a beaker with water and added a few drops of detergent viz. tween twenty. The beaker was kept on the magnetic stirrer for proper stir of the explants. The explants were washed repeatedly with distilled water. The explants were taken to the inoculation chamber. The explants were surface sterilized in 70 percent ethanol, prepared in sterile distilled water, for

one minute. Next, the explants were treated with 1 percent sodium hypochlorite (NaOCl) solution for 5 min. However, the loss of chlorophyll was prevented by minimizing the treatment period and by diluting the concentration of the sterilizing solutions. The explants were washed repeatedly with sterile distilled water (for five times) kept on five separate Petri plates. The thoroughly washed explants were kept on the sterile filter paper to get rid of surface water on the explants. Then the explants were cut into small size of 0.5 cm. These pieces were inoculated in the MS medium (free of hormones) as well as in all the fourteen other various combinations of MS + BAP and NAA. Repeatedly sterilized surgical blade, knife and forceps were used for inoculation. All the cultures were maintained at 25±2˚C and 16 hour photoperiod daily. Frequent monitoring was done to minimize possible contamination; and to ensure undisturbed illumination of the inoculation materials in the culture tubes. Observation was made weekly until the end of sixth week.

CHAPTER FOUR

OBSERVATION AND RESULTS

4.1 *In-vitro* Study of *Valeriana jatamasii* Jones

4.1.1 Subculture of Shoots for Optimum Shoot Multiplication

Nodal explants were taken from one year old *in-vitro* raised plants (Fig. 1). These nodes of 0.5-1 cm each were inoculated in MS medium supplemented with 1 ppm BAP and 0.5 ppm NAA. The second passage of nodes was in turn used as the explants for the third batch of subculture. The process was continued until the sixth batch or passage of subculture. Each passage was observed for four weeks in the laboratory condition of 25±2°C, 16 hr photoperiod daily and with four replications (in each passage).

Table No. 4.1.1
Effect of the repeated subculture of the multiple shoot formation rate

No. of replicates per batch	No. of shoot in replicate 1	No. of shoot in replicate 2	No. of shoot in replicate 3	No. of shoot in replicate 4	Average no. of shoots	S.E. (±)
First batch of subculture	2	3	3	2	2.5	.2887
Second batch	4	4	5	3	4	.4082
Third batch	6	5	5	7	5.75	.4787
Fourth batch	8	8	10	10	9	.5774
Fifth batch	11	10	11	12	11	.4082
Sixth batch	10	11	10	11	10.5	.2887

Cultural condition: MS Solid media supplemented with BAP 1 ppm + NAA 0.5 ppm, 25±2°C, 16 hours photoperiod, 4 replicates, 4 weeks of subculture for each passage.

S.E. = Standard Error

After one to two weeks of subculture, the explants showed response and became green. Shoot proliferation was observed after the third week of

subculture. The mode of differentiation is organogenesis, that is, the direct formation of plants from the excised segments–nodes. The new shoots emerged due to adventitious shoot formation at the basal part of the explants. Shoot proliferation appeared due to forced axillary branching at the base. In each of the passage, new shoots emerged as the axillary shoots from the basal part, generally. With increasing passage of the subculture, the number of shoots increased fairly up to the fourth batch and thereafter, the average rate of shoot proliferation remained constant (Photo No. 1). The leaves appeared dark green and healthy with increased passage of subculture. The size of the shoots was generally the same in the given period of observation, i.e. four week. The average number of shoots was 2.5, 4, 5.75, 9, 11 and 10.5 in the first, second, third, fourth, fifth and sixth batches of subculture respectively. The highest average number of shoots was observed in the fifth passage of subculture (11 shoots) (Photo No. 2). There were two to three roots per replicate in all the batches of subculture.

4.1.2 Subculture of Nodes

Nodes of approximately 0.5 cm to 1 cm were selected from the *in-vitro* grown shoots of *V. jatamansii* Jones. They were subcultured in MS medium supplemented with four different concentrations of NAA: MS + 0.5 ppm NAA, MS + 1 ppm NAA, MS + 1.5 ppm NAA and MS + 2 ppm NAA. Four replicates per concentration were established. A weekly observation was made for six weeks.

Table 4.1.2

Effect of different concentrations of NAA on the growth of hairy roots

Observed parameters	MS + 0 ppm NAA	MS + 0.5 ppm NAA	MS + 1 ppm NAA	MS + 1.5 ppm NAA	MS + 2 ppm NAA
Relative density of hairy roots	$\left(\frac{4}{4}\right)$ NA	$\left(\frac{3}{4}\right)$ ⊕⊕⊕⊕⊕	$\left(\frac{3}{4}\right)$ ⊕⊕⊕⊕	$\left(\frac{4}{4}\right)$ ⊕⊕	$\left(\frac{3}{4}\right)$ ⊕
Relative density of callus formed	NA	⊙	⊙⊙	⊙⊙⊙⊙⊙	⊙⊙⊙
Characteristics of hairy roots	NA	• Hairy roots were bristle-like, straight, distinct, soft and denser towards the callus.	• Hairy roots were shorter and did not extend beyond the surface of the embedded callus. • Hairy roots diminished abruptly at the terminal ends.	• Hairy roots were not distinct and they appeared to have been glued uniformly on the surface of brown callus.	• Absolutely insignificant development of hairy roots. • Bristle-like brown hairy roots of sparse nature emerged from the very short and creamy adventitious roots.
Nature of callus formed	NA	• The node swelled within a week. Greenish brown callus formed in the second to third week.	• The node turned into a creamy brown callus with the lower end denser brown and the upper exposed surface: creamy white.	• Distinct brown callus had spread rapidly as a strip along the tilted side of the media on the tube.	• A patch of brown callus formed at the upper end of the media. • The callus was relatively harder, friable and slow growing.

Cultural condition: MS solid media, 25±2°C, 4 replicates, 6 weeks of subculture, 16 hours photoperiod, NA = No any, ă = Relative density of hairy roots, ⊙ = Relative density of callus formed

On MS + 0 ppm NAA, callus was not formed. Hairy roots too did not appear. A shoot developed from the upper end of the node. The shoot became pale green and died by the fourth week (Photo No. 6).

On MS + 0.5 ppm NAA, dense hair roots proliferated from the surface of the white, soft, fleshy and thick adventitious roots which had, in turn, emerged from the surface of greenish brown callus (Photo No. 7).

On MS + 1 ppm NAA, relatively indistinct hairy roots were camouflaged by the creamy white callus. The hairy roots were dispersed in the white and viscous-natured callus. The density of the hairy roots diminished with time (Photo No. 8).

On MS + 1.5 ppm NAA, relatively shorter and indistinct roots emerged from the cracked surface of the callus. The callus formed was partly friable and soft with very short hairy roots spread on the surface of the callus. Three leaves without a shoot emerged from the upper end of the callus (Photo No. 9).

On MS + 2 ppm NAA, the callus formed was relatively harder, dark brown and friable. There was absolutely insignificant development of hairy roots. Bristle-like brown hairy roots emerged on the surface of very short and creamy adventitious roots. Leaves did not appear (Photo No. 10).

4.1.3 Subculture of Nodes for Determining the Suitable Strength of MS Media for Hairy Roots Proliferation

Green and healthy nodes (0.5 cm – 1 cm) were selected from the six weeks – old *in-vitro* grown shoots of *V. jatamansii* Jones. The nodes were subcultured in four different strengths of MS medium supplemented with 0.5 ppm NAA: full strength of MS + 0.5 ppm NAA, $\frac{3}{4}$ strength of MS + 0.5 ppm NAA, $\frac{1}{2}$ strength of MS + 0.5 ppm NAA and $\frac{1}{4}$ strength of MS + 0.5 ppm NAA. Four replicates were established for each different combination. The subculture was maintained at 25±2°C, 16 hours illumination daily, and for six weeks with weekly observation.

Table 4.1.3

Effect of different strengths of MS media on the Proliferation of hairy roots

Strength of MS	**$\frac{1}{4}$ MS + 0.5 ppm NAA**	**$\frac{1}{2}$ MS + 0.5 ppm NAA**	**$\frac{3}{4}$ MS + 0.5 ppm NAA**	**Full strength MS + 0.5 ppm NAA**
Relative density of hairy root proliferation	⊕	⊕⊕⊕⊕	⊕⊕⊕	⊕⊕

Cultural condition: MS solid medium, 25±2°C, 16 hr illumination, 4 replicates, 6 weeks of subculture.

Where, ă = relative density of hairy roots

Different characteristics were observed during the development of the hairy roots in the various strengths of MS solid media. On $\frac{1}{4}$ MS + 0.5 ppm NAA, the subcultured node swelled, became greenish brown and gave rise to dense hairy roots from the surface of greenish brown callus. However, hairy roots progressively disappeared along the terminal ends of the long, white and soft adventitious roots on whose surface, the hairy roots were originated. The hairy roots did not persist beyond the fifth week (Photo No. 14).

On $\frac{1}{2}$ MS + 0.5 ppm NAA, the subcultured node swelled and remained light green in the first week. With in the second week, dense hairy roots emerged from the surface of white, fleshy and tender roots that in turn originated from the entire upper surface of the node. The hairy roots were dense. There was one or two green shoot-like appearance from the center of the swollen node. The hairy roots persisted beyond fifth week. However, no new adventitious roots emerged after the third week. The hairy roots were dense, distinct, relatively longer and persisted for a relatively longer period than in the other three strengths of MS media (Photo No. 13).

On $\frac{3}{4}$ MS + 0.5 ppm NAA, the subcultured node swelled, turned dark green first and then light green. Within the second week, white, fleshy and tender adventitious roots emerged from the center of the callus. The hairy roots that emerged from the surface of the white adventitious roots were sparse at the terminal ends of the adventitious roots. The hairy roots diminished in quantity with the elongation of the adventitious roots (Photo No. 12).

On full strength of MS medium + 0.5 ppm NAA, the subcultured node swelled and turned to brownish green callus. White, fleshy and tender adventitious roots emerged from the callus within the second week. The hairy roots developed from the surface of the adventitious roots. Brownish callus further grew and occupied the central region of the calloid mass. The spread of the calloid mass has interfered with the proliferation of hairy roots. Thus, there was poor persistence as well as proliferation of hairy roots in the full strength of MS. Next, the hairy roots began to clump together and lost their straight and individual structure after the fifth week. The density of the hairy roots diminished beyond fourth week (Photo No. 11).

4.1.4 Acclimatization and Transfer of *in-vitro* Developed Plantlets to the Soil (land)

Table no. 4.2.1

Effect of the different hardening media on the growth of *in-vitro* plantlets

Hardening Media	Coco pit	Sand	Sand and soil mixture (1:1)	Mean	S.E. (±)
Number of plantlets survived	91.67%	83.34%	54.17%	22.0000	.4629
Comparative length of shoot in average (mm)	85	70	55	19.8750	.5154
Density of root (average)	ă ă ă	ă ă ă ă	ă ă	13.6250	.4978
Average number of leaves per plant	3	4	4	22.0000	.4629

Condition: Atmospheric temperature, 24 plantlets per hardening media, 22 days after transplantation

ă = relative density of roots

S.E. = Standard Error

There was 91.6 percent survival of the *in-vitro* plantlets in the coco pit. The average length of the shoot was 85 mm. There were 3-4 leaves per plant. The leaves were relatively healthy and dark green. The internode was shorter. Two leaves have longer petiole that originated from the basal node just above the roots (Photo No. 15, 16 and 18).

The survival rate of *in-vitro* grown plants in the sand was 83.3 percent. The average length of the shoot was 70 mm with an average of four leaves per plant (Photo No. 17). The leaves were dark green. The density of roots was relatively higher.

There was 54.1 percent survival rate of *in-vitro* plants in the hardening media of sand and soil mixture (1:1). The average length of shoot was 55 mm with moderate density of roots. There were four leaves per plant on the average. The leaves were moderately green to yellow, indicating the poor contents of chlorophyll.

The plantlets were transferred to the humus rich soil contained in polythene bags for two months. The plantlets that were hardened in sand adapted to the soil and showed relatively faster growth. Their roots proliferated faster. The plantlets that were hardened in coco pit (Photo No. 23) showed moderate growth and adaptation in the soil. The roots could not go deeper into the soil. The plantlets that were hardened in sand and soil mixture although adapted to the soil faster yet their growth was relatively poorer and stunted (Photo No. 22). The plantlets that were hardened in the sand grew faster; and three to four branch shoots that originated from the basal node (Photo No. 21). The plantlets that were hardened in coco pit became longer and thinner with lesser branching of shoots. All the plants were finally removed from the polythene bags in the third month of hardening process and they were transferred to the soil which is rich in humus, sand and soft soil. The plants have shown better growth in sufficient shade with partial sunlight and adequate soil moisture (Photo No. 24, 25, 26, 27, 28 and 29).

5.1 *In-vitro* Study of *Rauvolfia serpentina L.* Benth. ex Kurz.

Seedlings, raised in a nursery, were brought from Domukha village of Kankaimai, Jhapa. They were further nurtured in the green house of CDB, TU. These seedlings were used as the source of explant for *in-vitro* culture of shoot tips and nodes in the present investigation (Photo No. 30).

5.1.2 Culture of Shoot Tip Explant

Shoot tip explants obtained from *in-vivo* grown seedlings were inoculated on MS medium alone (Photo No. 31), and in different combinations and concentrations of the growth hormones: BAP and NAA.

Table no. 5.1.2

Shoot tip culture on MS + BAP and NAA in various concentrations

BAP (ppm) / NAA (ppm)	0	0.5	1.0	1.5	2.0
0	4Pl	(3/4) MS	4 MS	(3/4) MS	(3/4)C + SS
0.5	(3/4) SS	(3/4) SS	4 C + MS	(3/4) C+ MS	4 C + MS
1.0	(3/4) C + SS	(3/4) C	(3/4) C+ SS	(3/4 C + MS	(3/4) C+MS

Culture condition: MS solid media, 25°C ± 2°C, four replicates, 16 hour light, 8 weeks of culture

Pl = Plantlet
MS = Multiple shoots
SS = Single shoot
C = Callus
C + SS = Callus with single shoot
C + MS = Callus with multiple shoots

The inoculated shoot tips began to show differentiation and growth from about 15 days of culture. The observation was recorded from the third week until the end of eighth week.

Table no. 5.1.3

Effect of different combinations of BAP and NAA on shoot tip explants

S. N.	Composition of media	Response	No. of shoot
1	MS	Single plantlet with a small greenish callus like base. The plantlet had two leaves. The shoot died at the end of fifth week (Photo No. 31).	Single shoot
2	MS + 0.5 ppm BAP	From the sides of small creamy callus, several healthy shoots have sprouted; thick shoots but shorter. The shoots were branched and separated from the thick basal shoot (Photo No. 32).	6 shoots
3	MS + 1.0 ppm BAP	Thin basal callus with several leaves sprouted from it. Four shoots emerged. Shoots were not distinguished into node and internode. Thick leaves at the base.	Four shoots
4	MS + 1.5 ppm BAP	Thick leafy base. Small over whorled leaves. A single, thick but fleshy and tender shoot that arose with two branches. Shoots were very short and indistinct (Photo No. 33).	Single shoot
5	MS + 2 ppm BAP	Several curled, thick, creamy and greenish leaves sprouted from the base of creamy callus. Single, short shoot guarded by whorled leaves.	Single shoot
6	MS + 0.5 ppm NAA	Single shoot emerged. No callus formation. Single and thin shoot with no branches. Leaves, node and internode were distinct.	Single shoot
7	MS + 0.5 ppm BAP + 0.5 ppm NAA	Single healthy shoot with two leaves emerged from a thick and greenish pod-like basal structure. No callus formed (Photo No. 33).	Single shoot
8	MS + 1.0 ppm BAP + 0.5ppm NAA	Callus formed. From the base of the callus, four long and thin shoots with distinct internodes emerged. Four to six small and thin leaves sprouted at the upper part of shoot (Photo No. 45).	Four shoot

9	MS + 1.5 ppm BAP 0.5 ppm NAA	Brownish green callus formed just around the base of the single shoot which in turn gave rise to several branches of shoots. The shoots are comparable to the pods of orchid.	Multiple shoots branched off from a single shoot
10	MS + 2.0 ppm BAP + 0.5ppm NAA	Prominent basal callus: round, brown and appeared like a yam. Thick, green shoots with moderately distinct internodes sprouted from the tip of the callus. Healthy shoots developed (Photo No. 35).	Multiple shoots: four to six
11	MS + 1.0 ppm NAA	Vigorous callus: brown and soft. A single shoot emerged from the callus with two brown leaves at the tip. The shoot turned yellow.	Single shoot
12	MS + 0.5 ppm BAP + 1 ppm NAA	Patches of dark green callus with bright green upper region and brown lower region. No shoot formed.	No shoot
13	MS + 1.0 ppm BAP + 1 ppm NAA	Patches of brown and green callus with soft edges. From the edge, sprouted a thick, fleshy and short shoot which is phylloid-like.	Single shoot
14	MS + 1.5 ppm BAP + 1 ppm NAA	Patches of brown and green callus which were soft. Short, thin and yellowish shoots sprouted from the callus	Multiple shoots (four to five)
15	MS + 2 ppm BAP + 1 ppm NAA	Round, green and brown patches of callus spread on the media. Very short (about 1.5 cm) shoots sprouted from the edge of upper green callus. The shoots were not distinguished into internodes and nodes. They were more leaf like (Photo No. 46).	Short shoots (four to six)

Culture condition: MS solid media, 25°C ± 2°C, 16 hour light, four replicates, 8 weeks of culture.

Shoot tip explants were treated with 15 different combinations of MS medium supplemented with different combinations of BAP and NAA. The observation was made on weekly basis for eight weeks. The specific response of each of the treatments was made (table no 4.3.2). Among the fifteen different combinations, MS medium supplemented with 2 ppm BAP and 0.5 ppm NAA produced relatively the best result, i.e. thick, healthy and multiple shoots with

distinct node and internode. The shoots emerged from the surface of round, brownish callus of soft nature. There were four to six shoots per culture of the explants. All the four replicates gave identical result.

MS medium supplemented with BAP of different concentrations gave rise to shoots but without distinct nodes and internodes. These shoots have more leaves than the prominent structure of shoots which are essential for adaptation *in-vivo*.

On the other hand, MS medium supplemented with BAP and then higher concentration of NAA gave rise to callus with or without shoots and leaves. The shoots formed are more leaf-like: thick and fleshy tender leaves with short and thick shoots emerged from the basal callus.

5.1.4 Culture of Nodes

Nodes of about 0.5 cm were selected from the tender shoots of *in-vivo* grown *Rauvolfia serpentina* L. Benth. ex Kurz. The nodes were cultured on MS media supplemented with BAP and NAA either alone or in different concentrations and combinations.

Table no. 5.1.4

Node culture on MS + BAP and NAA in various concentrations

BAP (ppm) / NAA (ppm)	0	0.5	1.0	1.5	2.0
0	(3/4) SS	4 MS	(3/4) MS	(3/4) MS	4 C+MS
0.5	4 SS	4 MS	4 MS	(3/4) MS	(3/4) MS
1.0	(2/4) SS	(3/4) C + SS	(3/4) C	4 C + SS	(3/4) C + MS

Culture condition: MS solid media, 25ºC ± 2 °C, 16 hour light, 4 replicates, 8 weeks of culture,

SS = single shoot
MS = multiple shoots
C = callus
C + SS = Callus with single shoot
C + MS = Callus with multiple shoots

The inoculated node cultures started to show visible differentiation and growth since the second week of culture. Initially, the nodes swelled, became dark green and the shoot(s) began to emerge from the cut edge of the nodes that was above the contact point with the media. The observation was recorded since the third week when the differentiated parts were visible and distinguished for the observation. The culture was recorded periodically up to the eighth week.

Table no. 5.1.5

Effect of different combinations of BAP and NAA on nodal explants

S.N.	Composition of media	Response	No. of shoot
1	MS	Single shoot. Four leaves sprouted from the tip of the shoot. Stunted growth of the shoot which turned yellow at the end of the fifth week (Photo No. 36).	Single shoot
2	MS + 0.5 ppm BAP	Four shoots emerged from the upper cut edge of the node. The tip of the shoots had dense foliage. The shoots were short and not distinguished into internodes (Photo No. 37).	Four shoots
3	MS + 1.0 ppm BAP	The node swelled initially into creamy and light callus. From the surface of the callus, two long and distinct shoots emerged. Four new shoots had sprouted in the sixth week. Leaves emerged at the base of the shoots	Multiple shoots (four to six)
4	MS + 1.5 ppm BAP	The node swelled and became green callus like mass. From the surface of the callus, three short and thin shoots developed. The shoots showed stunted growth with dry shoot tips after the fifth week.	Multiple shoots (three to four)
5	MS + 2 ppm BAP	Dark green callus was formed. From the side and the surface of the callus, tender green multiple shoots emerged.	Multiple shoots (four to six)

6	MS + 0.5 ppm NAA	A single shoot developed from the upper cut edge of the node. From the base of that shoot, two branches emerged. The shoots have distinct internodes.	Multiple shoots (three)
7	MS + 0.5 ppm BAP + 0.5 ppm NAA	The node swelled but did not transform to callus. Six long, distinct shoots with clear nodes and internodes emerged. These healthy and green shoots developed directly from the node: i.e. without forming branches (Photo No. 39 and 49).	Multiple shoots (six to eight)
8	MS + 1 ppm BAP + 0.5ppm NAA	The node turned into a green callus like mass from where a single shoot emerged. From the base of that shoot, six other shoots sprouted like a trimmed shrub.	Multiple shoots (four to six)
9	MS + 1.5 ppm BAP 0.5 ppm NAA	The node turned into brown callus. From the surface of the callus, thick, short and green shoots emerged. The shoots were healthy, short but not so distinct.	Multiple shoots (four to six)
10	MS + 2.0 ppm BAP + 0.5 ppm NAA	The node transformed into whitish-brown callus from where multiple shoots sprouted. The shoots were thick and indistinct with more dense leaves at the tip.	Multiple shoots (five to seven)
11	MS + 1.0 ppm NAA	A single shoot developed from the upper cut edge of the node. The shoot was thin and distinct with nodes and internodes visible. The shoot was yellowish brown.	Single shoot
12	MS + 0.5 ppm BAP + 1 ppm NAA	Patches of dark brown callus formed. A short, pale and indistinct shoot emerged from the upper surface of the callus. (Photo No. 48)	Single shoot
13	MS + 1.0 ppm BAP + 1 ppm NAA	Hard and friable callus formed with dark green upper surface and brown lower surface. No shoots and leaves observed.	No any shoot

14	MS + 1.5 ppm BAP + 1 ppm NAA	Soft, dark brown callus with dark green upper surface observed. A short, pale and fleshy shoot appeared from one side of the upper part of the callus.	Single shoot
15	MS + 2 ppm BAP + 1 ppm NAA	Soft, light brown callus in the form of patches was observed. The upper dark green callus gave rise to several short, thin, fleshy and leafy shoots. The shoots showed stunted growth. The callus decreased in quantity with the progressive growth of the shoots.	Multiple shoot (three to six)

Culture condition: MS solid media, 25ºC ± 2 °C, 16 hour light, 4 replicates, 8 weeks of culture.

Nodal explants were inoculated in MS (hormone free medium) and in fourteen other different combinations of MS with BAP alone or MS + BAP + NAA in various combinations. The subcultures were maintained in the laboratory conditions of 25±2°C, 16 hours illumination for eight weeks of observation period. The response of the nodal explants was observed on weekly basis until the eighth week. The response of the explants in various combinations was noted (table no. 5.1.5).

The nodal explants cultured in MS + 0.5 ppm BAP + 0.5 ppm NAA gave the best response for shoot multiplication *in-vitro*. The inoculated nodes gradually swelled into a large green mass but did not transform into callus. Four to six healthy, long and distinct shoots sprouted from the cut ends of the swollen node. The shoots had distinct nodes and internodes, with four to six green leaves at the apex of each shoot. The shoots did not bear branches. The green mass diminished in quantity with the elongation of the shoots.

CHAPTER FIVE

DISCUSSION

The morphogenetic response of explants varies with genotype, age, nature and cultural condition. The result obtained from the subculture and culture of various explants of *Valeriana jatamansii* Jones and *Rauvolfia serpentina* L. Benth. ex Kurz have been discussed on the basis of relevant literatures.

6.1 Subculture of *in-vitro* Shoots for Optimum Shoot Multiplication of *Valeriana jatamansii* Jones

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).

Miyagawa *et al.* (1986) carried out shoot multiplication *in-vitro* in *Stevia primorida* by using MS – agar medium containing kinetin (10^{-5} M) at 25°C, 16 hours light a day. The primary shoot tips with one-two leaves were subcultured in BAP 2 ppm and NAA 0.2 ppm and BAP 2 ppm and NAA 2 ppm, with slow rotation of 2 rpm for two months. Subculture was continued by using the shoot premorida (*in-vitro*) for several times. From a small aggregate of shoot primordia, 50 to 60 individual shoot primordial were obtained. Therefore, their propagation rate was about 3 n, where 'n' is the number of weeks. When the pieces of shoot primordia in the media (MS + BAP 2 ppm and NAA 0.2 ppm , and BAP 2 ppm and NAA 2 ppm) were transferred on agar medium B5 containing 0.02 ppm BAP and 2% sucrose in static culture in 2 to 3 weeks, they easily developed into a large number of young plantlets with shoots and roots which could be grown to normal plants.

In the present study, MS medium supplemented with 1 ppm BAP and 0.5 ppm NAA was used for the *in-vitro* shoot proliferation. Repeated subculture of the four-week-old shoot explants was done consecutively for six batches at an interval of four weeks. Within two weeks, green shoots sprouted from the base of the shoot tip explants. Initially, only two or three shoots emerged per explant. However, in the fifth to sixth passage of subculture, 8 – 11 adventitious shoots emerged. The shoots were healthy and sturdy in their organization. There was no callus formation in any of the stages. Kayastha (2000) had obtained 10 – 15 microshoot after the 4^{th} subculture of the shoot tips of two month old *Swertia chirata* (wall). C. B. Clarke. The shoots were subcultured in every 4 – 6 weeks of interval in MS medium supplemented with 1 ppm BAP and 0.01 ppm NAA. The optimum shoot proliferation was observed in the above combinations of medium.

Similary, Rajkarnikar, Saiju and Bhatta (2000) had obtained 12 – 16 shoots of *Rauvolfia serpentina* L. Benth. ex Kurz after the 7^{th} subculture of the shoot tips, used as the explants, in MS medium supplemented with 1 ppm BAP and 0.1 ppm NAA. The subculture was done at the interval of 5 to 6 weeks. Niroula and Saiju (2000) had obtained 20 – 30 shoots of *Valeriana jatamansii* Jones after 4 – 6 subcultures. They had used shoot tip of 0.5 cm length, and had inoculated in MS medium supplemented with 1 ppm BAP and 0.1 ppm NAA. After one – two weeks of culture, the explants showed response and became green. The shoot proliferation was observed after 4 weeks of culture. The shoots were formed directly from the excised segments of shoot tip.

Jain *et al.* (1991) had proliferated the shoot apices of *Rosmarinus officinalis* L. Var *genuine* forma *erectus* on a nutrient agar. Explants consisting of small groups of proliferating shoots, ca. 10 shoots each. About 50 resulted from each explants, which were maintained by periodic subculture, at 30 day intervals, to constitute stock cultures. The proliferating shoots remained green and healthy and kept proliferating without any decline in the regenerative potential over a period of three years. Shoots from such long term cultures retained the

biosynthetic potentiality of original cultures. Similar to the findings of Jain *et al.* (1991), in the present investigation too, the shoot proliferating capacity did not decline for over 3 years now.

Next, Batra *et al.* (2001) obtained healthy and sturdy multiple shoots (8 - 10) of neem, *Azadirachta indica* A. Juss, by using nodal stem segments as the explants in MS medium supplemented with 1 ppm BAP, in 6 weeks of culture. It was observed that with an increase in the BAP concentration from 1.0 to 5 ppm, the number of shoots produced per node decreased considerably. Karki and Niraula (1999) had studied the effects of Cytokinin and Auxin for regeneration of shoot tip explants of *Acacia auriculiformis*. A. Cuss. Ex Benth. They used shoot tip explants excised from the *in-vitro* grown seedlings and cultured the explants in MS medium supplemented with different concentrations of BAP ranging from 0.1 to 5 ppm in combination with 0.1 ppm NAA. The shoots obtained after the first culture were repeatedly subcultured. In MS + 0.5 ppm NAA shoots ranging from 25 – 30 were developed after the fourth and the fifth subculture in 6 – 8 weeks. There was no callus formation in 0.5 ppm NAA and 0.1 ppm NAA. In the present study, there was no callus formation and initiation of shoot was from the base of the first shoot, i.e. the other shoots emerged as adventitious shoots which finally appeared like a shrub or bunch.

Margara and Moncousin (1982) had demonstrated that the degree of rejuvenation is dependant on the size of the isolated meristem, the number of subsequent subculture and the concentration of regulations in the medium. The more the subcultures that have taken place, the quicker is the rejuvenation. Successful rejuvenation of 30-yr-old *Eucalyptus grandis* was achieved after 12 repeated subcultures in 1 year period. Similar results were obtained with 30-50 years old Lilac (*Syringa vulgaris*) shrubs by prerik *et al.* (1986). In both the above investigations, MS medium supplemented with 1 ppm – 2.5 ppm BAP and 0.5 ppm NAA were used throughout the subcultures.

Hatano *et al.* (1988) investigated for clonal multiplication of *Aconitum carmichaeli* by tip tissue culture and verified for the alkaloid contents of clonally propagated plant. Shoot tips and axillary buds of *A. carmichaeli* Debx cultured in MS medium supplemented with 5 ppm BAP at 25±1°C under 16 hours light for six weeks. Multiple shoots at the rate of 7 shoots per culture wer obtained. Multiple shoot forming ability did not decrease for atleast six generation. MS + 0.5 ppm IAA at 20±1°C under continuous light for six weeks resulted in root formation.

The size of the explants have some role in easier regeneration. Van Aartrijk (1984) had concluded that, in some plants, a larger explants are easier to regenerate than the smaller ones which, in his opinion was, due to the presence of more food reserves. Next, in contrast to larger explants, smaller explants have been able to regenerate more adventitious shoots. It is, as per Van Aartrijk, due to relatively larger wound surface in a smaller explants.

Peirik and steegmans (1975) had observed that the orientation of inoculation in media plays an important role with many plants. Apolar inoculation (upside down inoculation of explants) promotes regeneration where as polar inoculation (natural orientation, base down) inhabits regeneration. This is perhaps due to a better oxygen supply above the medium, but can also be linked to accumulation phenomena. However, they have not been able to point out the exact reason of regeneration on a polar inoculation. In the present study, Polar inoculation was done. However, there was no inhibition observed.

6.2 Subculture of Hairy Roots of *Valeriana jatamansii* Jones on the Different Concentration of NAA

In the present study, nodal explants of 0.5-1 cm obtained from *in-vitro* developed shoots were inoculated in MS medium supplemented with four different concentrations of NAA, Viz. 0.5 ppm NAA, 1 ppm, 1.5 ppm NAA and 2 ppm NAA. The observation was made on a weekly basis for six weeks.

Nodal explants in the MS (hormone free) media formed a small, single and greenish shoot within the fourth week. In MS + 0.5 ppm NAA, the node initially swelled became callus-like mass of creamy white color. From the upper and entire surface of the callus, bristle-like hairy roots sprouted. The hairy roots emerged on the surface of the soft, thick and white adventitious roots. The hairy roots were distinct, about 0.5 cm long with the rapid proliferation of white hairy roots. In the later stage, the colour of the hairy roots turned to bright white colour. The roots persisted beyond six weeks. In MS + 1 ppm NAA, hairy roots were shorter and did not extend beyond the surface of the embedded callus. Hairy roots diminished at the terminal ends of the short, thick and soft adventitious roots. The nodal explants turned into creamy brown callus with the lower end of the callus denser brown and the upper exposed surface: creamy white. The callus formed more adventitious roots than hairy roots. In the fifth week, the callus turned light green. In MS + 1.5 ppm NAA, distinct brown callus of friable nature spread on the surface area of the media. Hairy roots were indistinct and they appeared to have been glued uniformly on the surface of brown callus. In MS + 2 ppm NAA, sparse nature of hairy roots was observed on the upper surface of harder friable and brown callus. The callus spread relatively slowly.

Jaya and Ramakrishna (1999) have developed hairy roots of *plumbago indica*. They examined the ability of *Plumbago indica* to give rise to transformed roots after inoculation with *Agrobacterium rhirogenes*. The productivity of hairy roots and callus was compared. The content of the chemical plumbagin was analysed by HPLC method. Young leaves of *P. indica* were inoculated on MS medium with different concentrations of BA, IAA and IBA. The regenerated shoots were used for *Agrobacterium* infection. The micropropagated shoots were transferred to MS basal medium and incubated in dark for 2 days and then inoculated with 3 days old cultures of *A. rhizogenes* and again kept in dark. Within 15 days, hairy roots were developed which were subsequently cultured on liquid MS basal medium with the antibiotic, Carbenicillin (Himedia). Nearly

0.016 mg/g dry weight of plumbagin was extracted from the hairy roots as against 0.0623 mg/g dry weight of plumagin from the callus. In the present investigation, hairy roots emerged without the use of *A. rhizogenes*. The development of hairy roots took place in adequate photoperiod of over 16 hours per day. No antibiotic was used.

Similarly, Tanaka, Takao and Matsumato (1995) have produced multiple shoots from hairy roots of *Vinca minor* L. The shoots originated from the hairy roots contained a pharmaceutically important indole alkaloid, vincamine. A previously established Ri-transformed clone, vm-101, proliferated rapidly *in-vitro,* displayed a high degree of lateral branching and rapid shoot elongation, with a growth index 2.5 times that of an untransformed plant. The addition of 2.2 μM BA to the cultural medium increased the shoot number but did not decrease the growth index. Vincamine context in the leaves on *in-vitro* cultured vm-101 was twice that in the cultured transferred plant. These results suggest that multiple shoot culture obtained from hairy root culture might be an excellent tool for *in-vitro* production of pharmaceutically useful chemicals.

Duproz *et al.* (1994) obtained tropane alkaloids in transformed roots of *Datura quercifolia*. Hairy root cultures of *D. quercfolia* were established following infection with *Agrobacterium rhizogenes* strain LBA 9402. Eight tropane alkaloids were identified in the hairy roots, hyoscyamine being the major constituent. The growth and the hycoscyamine content of transformed roots were investigated under various conditions. Gamborg B5 medium was identified as the best for growth as well as for hyoscyamine accumulation. The influence of sucrose concentration was examined and a 5% concentration was found to be the most appropriate for growth and for alkaloid production.

In general, MS medium supplemented with 0.5 ppm NAA induces root formation. Kukrija and Mathur (1985) induced roots on MS + 0.5 ppm NAA (liquid medium) of *Duboisia myoporoids*. As in the present investigation, most of the works on tissue culture established that auxin like IAA, IBA or NAA

induced root formation including hairy roots in certain species of plants. However, specific literature on hairy root culture is scanty both in the print and the electronic versions. It could be so because the study on hairy roots has a short period of history in the line of *in-vitro* investigation in tissue culture.

6.3 Subculture of Hairy Roots of *Valeriana Jatamansii* Jones on Different Strengths of MS

In the present study on hairy roots, the suitable strength of MS media for the effective proliferation and propagation of hairy roots was investigated. On $\frac{1}{4}$ MS + 0.5 ppm NAA, hairy roots emerged in the beginning but disappeared progressively towards the terminal part of the long, white and soft adventitious roots. On $\frac{1}{2}$ MS + 0.5 ppm NAA, dense hairy roots that emerged from the surface of long, white and soft adventitious roots, persisted for a relatively longer period. There was one to two green shoot like structures originated from the center of the callus. Kukreja and Mathur (1985) induced roots on MS + 0.5 ppm NAA (liquid medium) in *Duboisia myoporoides*. Basnet (2004) obtained high concentrations of roots, (average 8.4 roots) through the subculture of callus of *Citrus aurantifolia* Swingle on $\frac{1}{2}$ MS supplemented with 1.5 ppm IBA. Similarly, a eight-week old callus of the same species produced 10.6 adventitious roots in average when the callus was treated with $\frac{1}{2}$ MS and 1 ppm NAA.

Similarly, Batra, Sardana and Chandna (2001) subcultured the mature *in-vitro* developed nodal segments of *Jatropha curcas* on to rooting medium comprised of full, $\frac{1}{2}$ or $\frac{1}{4}$ MS medium supplemented with various auxins, viz. IAA, IBA and NAA. The best rooting response (60-80%) with a little callus formation was observed in $\frac{1}{2}$ MS medium supplemented with 0.5 ppm NAA. Thakur and

Ishii (1999) carried out rooting experiments of shoot base explants obtained from the *in-vitro* developed shoots of *Pinus gerardiana* wall. The experiments were carried out on $\frac{1}{2}$ strength MS medium involving four different auxins and their combinations. Over 40% rooting was achieved when shoots were kept in $\frac{1}{2}$ MS medium containing 0.25 ppm NAA, and 0.25 ppm IBA.

Pant and Basavaraju (1999) successfully induced rooting in the plantlets of *Morus indica* Linn on $\frac{1}{2}$ MS supplemented with auxins (IBA or NAA) at low concentration (0.5 to 1 ppm). In the present investigations on $\frac{3}{4}$MS + 0.5 ppm NAA, the subcultured node swelled, turned dark green and the light green. The hairy roots that emerged from the surface of the white adventitious roots diminished in quantity with the elongation of the adventitious roots. On full strength of MS medium + 0.5 ppm NAA, the subcultured node swelled and turned to brownish green callus. The initially emerged hairy roots were later on dominated by the brownish callus that occupied the central region of the colloid mass. The limited hairy roots began to clump together and finally disappeared from the surface of the adventitious roots. Thus, increased strength of MS with auxins like NAA induced callus formation and inhibited the proliferation of hairy roots.

6.4 Acclimatization and Hardening of *In-Vitro* Developed Plantlets

Planning on *in-vitro* propagation program for a woody or herbaceous plant must always include the plant rooting and acclimatization phases, which are decisive for the future of the young plants. Final success depends on a succession of good results in the following operation: induction and expression of rhizogenesis, obtaining a rooting system of good quality; transfer and acclimatization; technical success of these operations at the lower cost. In the present study, with the objective of developing a suitable protocol for the

acclimatization and transfer of *in-vitro* obtained plants on the land (soil), a series of procedures was investigated. Three different hardening media were selected for the initial hardening and strengthening of the roots: cocopit, sand, and a mixture of equal volume of sand and soil (1:1). Basic parameters such as percentage of survived plants, length of shoot, density of root and number of leaves per plant were taken. The plants were observed after 22 days of transfer to the three different hardening media. Cocopit favored for the highest percentage of survival (91.6%) and for the elongation of shoot. The density of roots was moderate. There were three leaves per plant in the average. The leaves were dark green and healthy with elongated petiole. In sand, the percentage of survival was 83.3 percent with moderate average length of shoot (70 mm). However, the roots were dense and longer than that of the plants in the other two hardening media. There were four leaves per plant in the average. In the mixture of sand and soil (1:1), the survival percentage was just 54.1 with 55 mm average shoot length. However, the density of roots was slightly lower than that of plants in the cocopit and the sand. There were four leaves per plant in the average. The leaves were weaker in chlorophyll contents as they appeared yellowish green. Karki and Saiju (2000) had successfully transferred the 6-8 weeks shoots of cardamom (*Amomum subulatum* Roxb.) in non sterile sand. Ninety percent of shoots were developed under day and night temperature of 35°C and 15°c under 70 percent humidity and light intensity of 3000-4000 lux. The rooted plants were transplanted in pots. After one year, the mature plants were transplanted in the field.

Similarly, Joshi *et al.* (2000) established the microshoots of *Elaeocarpus sphaericus* (Gaertn) K. Schum in non sterile sand under green house condition for rooting. Next, Pant and Basuvaruju (1999) had first transferred the 30 days old *in-vitro* rooted plantlets of *Morus indica* Linn into the sterilized vermiculite pots for hardening. Polythene cover was used to cover the entire pots with plants to retain moisture. After two weeks the plantlets were transferred into pots containing sterilized sand and soil mixture (1:1). Establishment of plants

in soil in natural conditions was done after 10 days. Thakur and Ishii (1999) achieved 100 percent survival of acclimatized plantlets of *Pinus gerardiana* wall. The rooted plantlets were transferred to a potting mixture of vermiculite and perlite (1:1). The plantlets were regularly sprayed with water and maintained 90-95% humidity. The polythene covers were kept for the first five days. The plants were then shifted to growth cabinet for one month. There was 100 percent survival of the plants in the growth chamber. In the present study, miniature greenhouses were made in order to maintain adequate humidity and uniform temperature. There was over 95 % survival of the plants.

In the same way, Rajbhandari and Bajaj (1991) had proven that rooting *in-vitro* produced shoots in non-sterile sand as inexpensive and efficient technique for en-masse micropropagation. Manandhar and Rajbhandari (1986) obtained successful rooting of *in-vitro* proliferated shoots originated from shoot tips of potato (*Solanum tuberosum* Linn) cv. 'Cardinal' in a non-sterile medium composed of sand and dried leaves mixed in equal proportion by volume. Rooted plants were successfully established in the field and grown to maturity. Also, Amatya and Rajbhandary (1988) successfully induced rooting in the microshoots of eight week old *Ficus auriculata* Lour in a mixture of sand and dry leaf powder (2:1 v/v). Rooted plantlets were established in the field. Similarly, Pradhan and Rajbhandari (1988) transferred the microshoots of *Brassica oleracea* var. Captata to non-sterile sand under greenhouse condition for rooting. In the present study, the acclimatized and hardened plants were transferred to soil packed in polythene for two months. Thereafter, the plants were transferred to the garden soil. These plants have borne one year old rhizomes by now.

Batra *et al.* (2001) hardened the *in-vitro* rooted plantlets of *Azadirachta indica* A. Juss (neem) in pots containing Vermiculite and soil (1:3 v/v) with adequate humidity for 15 days. The plants were exposed to sunlight for further acclimatization. After 2-3 weeks, the plants were finally transferred to pots containing garden soil. In the present investigation, a mixture of sand and soil

(1:1 v/v) was used. However, the survival percentage was smaller (54.1%). Nevertheless, the survived plants adapted to the garden soil fairly rapidly. The growth was slower probably due to poor content of chlorophyll on the leaves. Most of the literatures stress upon the use of non-sterile sand for the rooting of *in-vitro* plants because the plants (rooted in sands) have fairly high survival rate, rapid proliferation of roots and smooth adaptation in the soil. It is also cost effective for mass propagation of *in-vitro* developed plants. Besides, the method is technically less complex and therefore, handy to the non-technical persons including farmers.

There has been scant use of cocopit by investigators. The present investigation revealed that although the *in-vitro* shoots readily got hardened in cocopit with profuse rooting, these roots having been elongated in the soft natured cocopit, found it difficult by the delicate root tips to penetrate the relatively hard soil.

Niroula and Saiju (2000) had successfully transferred the microshoots of *Valeriana jatamansii* Jones in non sterile sand. Ninety percent microshoots rooted in non sterile sand. They had observed the field trial cultivation of the *in-vitro* propagated plants. The yield of rhizome was also analyzed. In the present investigation, the yield of rhizome is being observed. This method can be effective to produce several thousand plants from a few mother plants.

6.5 Shoot Tip and Nodal Culture of *Rauvolfia serpentina* L. Benth. Ex Kurz

Shoot tips and nodes are the commonly utilized explants in tissue culture technology for the micropropagation of plants. In the present investiagion, both shoot tips and nodes were cultured in MS medium alone; in MS medium supplemented with different combination of BAP and the combination of MS with different concentration of BAP and NAA together.

6.6 Shoot Tip Culture

In the present investigation, shoot tips were inoculated in fifteen different combinations of MS (alone), MS supplemented with BAP (0.5 ppm – 2 ppm), MS supplemented with different concentration of NAA and BAP. Shoot tip in MS medium (hormone free) developed into a single shoot but died within fifth week. It was probably due to the lack of growth regulators. In MS with lower concentration of BAP (0.5 ppm – 1 ppm), light creamy callus was formed from the shoot tip. Multiple shoots but without distinct nodes and internodes were emerged. In MS supplemented with higher concentrations of BAP (1.5 – 2 pmm) the shoot tip formed relatively thicker creamy callus. A single shoot emerged. However, dense leaves appeared around the short shoot. MS with 0.5 ppm NAA did not form callus out of the inoculated shoot tip. A single shoot with distinct nodes and internodes emerged. Lower concentrations of BAP and NAA (0.5 ppm each) with MS did not form callus. A single shoot appeared. Higher concentrations of BAP (1 ppm – 2 ppm) in combination with 0.5 ppm NAA + MS medium favored for the proliferation of multiple shoots. MS + 2 ppm BAP and 0.5 ppm NAA proved effective for the multiple shoot formation (four to six per explants) from the shoot tip explants. The multiple shoots emerged from the prominent and brown callus of the inoculated shoot tip. Also, in the present investigation, MS supplemented with lower concentration of BAP (0.5 ppm) along with higher concentration of NAA (1 ppm) formed callus with upper green and lower brown colour. No shoot appeared. However, higher concentrations of BAP (1 ppm – 2 ppm NAA) along with higher concentration of NAA (1 ppm) favoured for the formation of callus-originated multiple shoots. However, the shoots were more leafy. Shahazad *et al.* (2001) obtained 10 – 12 shoots from the 10 days old shoot tip culture of *Mentha arvensis* in MS + 5 ppm BAP + 0.2 ppm NAA. In the present investigation, MS + 2 ppm BAP + 0.5 ppm NAA proved effective for the multiple shoot formation from the shoot tip explants.

Niraula (1994) obtained 2 – 4 shoots from the eight week old shoot tip culture of Gerbera Plants cultured in MS + 1 ppm BAP + 0.1 ppm NAA. Kharel and Karki (1994) inoculated the shoot tips of *chrysanthemum morifulium* varieties. "Giant fishtail violet" in MS + 1 ppm BAP + 0.01 ppm NAA. Microshoots were obtained. Similarly, Rajkarnikar and Saiju (2000) reported the shoot tip explants of *Rauvolfia serpentina* L. Benth. ex Kurz from excised shoot tips on MS + 3 ppm BAP + 0.1 ppm NAA. Next, Karki and Saiju (2000) obtained multiple shoots from the shoot tip explants of *Amomum subulatum* Roxb in MS + 1.0 ppm BAP and 0.1 ppm NAA.

Also Niroula and Saiju (2000) reported that the shoot tip explants of *Valeriana jatamansii* Jones gave multiple shoots in MS + 1 ppm BAP + 0.1 ppm NAA. The microshoots were rooted in non-sterile sand. Joarder *et al.* (1988) reported that vegetative development of explants was strongly influenced by type and concentration of growth substances as well as number of sub-cultures. They had obtained 192 shoots of *Morus alba* L. from a single shoot tip explant within 120 days. MS + 1 ppm BAP result to remarkable increase in leaf and internode extension. High concentration of BAP (2 ppm) decreased leaf and internode extension but enhanced more shoot production giving bushy appearance. BAP 2 ppm caused to obtain 16.65 shoots per culture. Three weeks culture in MS + 2 ppm BAP followed by sub-culture on MS + 1 ppm BAP gave higher number of shoots with extended internode and leaves for multiplication. In the present study, shoot tip explants in higher concentrations of BAP and NAA (MS + 2 ppm BAP + 1 ppm NAA) first formed brown patches of callus. Very short (about 1.5 cm) shoots sprouted from the edge of upper green callus. The shoots were not distinguished into internodes and nodes. They were more leaf like. This observation is very similar to that of Joarder *et al.* (1988). For genetic homogeneity, the shoots obtained from MS Supplemented with 1 ppm BAP and 0.5 ppm NAA were the best since those shoots were developed from relatively less amount of callus than MS

supplemented with 2 ppm BAP and 0.5 ppm NAA, where callus formed prior to sprouting of shoots.

Similar to the findings of Patil and Jayanthi (1997), in the present investigation too, the shoot tip culture of *Rauvolfia serpentina* L. Benth. ex Kurz exuded phenolics slightly, which made it challenging to establish the explants in the laboratory. They also found out that MS + 2 ppm BAP enhanced better axillary sprouting. Unlike their investigations, in the present study, NAA was used with BAP.

Mathur *et al.* (1987) reported that the addition of IAA along with BAP helped in higher multiplication of shoots. However, in the present investigation, IAA was not used. Similar to the present findings, Kulkarni and M.A. Deodhar (2001) obtained multiple shoots of *Garcinia indica* from the *in-vitro* raised buds in MS + 2 ppm BAP + 1 ppm NAA. Maximum increase in stem length occurred with 0.1 ppm BAP and 0.2 ppm NAA. However, NAA caused leaf expansion but did not affect shoot elongation. In the present investigation too, shoots appear more leaf like in MS + 2 ppm BAP + 1 ppm NAA. The caulogenic effect of BAP, observed in the present study, is in consonance with other reports (Joshi *et al.* 1993; Joarder *et al.* 1993; Kumar and Kumar 1997). In the present study, MS medium with 2 ppm formed callus with the shoot tip explants. It then gave rise to shoots and leaves.

6.7 Node Culture

In the present investigation, nodal explants were inoculated in fifteen different combinations: MS (hormone free), MS supplemented with BAP (0.5 ppm – 2 ppm), MS supplemented with BAP and NAA. MS (hormone free) media gave rise to a single shoot with stunted growth. MS + 0.5 ppm BAP treatment enabled the node to induce multiple shoots. The density of leaves was prominent. Joarder *et al.* (1988) reported that low concentrations of BAP induced the dense emergence of leaves in the *in-vitro* culture of *Morus alba* L.

In the present study, higher concentrations of BAP induced callus formation of creamy, white and brown color. Thin multiple shoots emerged from the surface of the callus. Next, in MS + 0.5 ppm NAA, single shoot emerged from the cut edge. Two axillary shoots appeared from that simple shoot. No callus was observed. Similarly, low concentrations of BAP and NAA (MS + 0.5 ppm BAP + 0.5 ppm NAA) proved effective for multiplication of healthy shoots with distinct node, internode and leaves. The swollen node directly gave rise to six shoots without any trace of callus throughout the observation period. The shoots originated directly from the node. No axillary shoots were observed.

Basnet (2004) observed that MS supplemented with 1 ppm BAP + 0.5 ppm NAA, and MS + 2 ppm BAP + 1 ppm NAA seemed to be best for the multiplication of shoots from nodal explant. Similarly, Niroula and Rajbhandari (1992) obtained multiple shoots of *Citrus limon* L. by using nodal explant in MS + 1 ppm BAP and 0.1 ppm NAA. In consonance to the present findings, Batra *et al.* (2001) noted that nodal stem segments of *Azadirachta indica* (neem) cultured on MS + 1 ppm – 5 ppm BAP showed slight swelling of the nodal region prior to the emergence of shoot buds. Similar to that of the present findings, Joarder *et al.* (1993) observed that, with an increased in the BAP concentration from 1 ppm – 5 ppm, the number of shoots produced per node decreased considerably.

Sarker *et al.* (1996) induced multiple shoots from nodal segments and shoot apices of *Rauvolfia serpentina* L. Benth. ex Kurz on MS + 1 ppm BA and 0.1 ppm NAA. Similar to the present observation, callus was formed at the cut bases of the nodal explants which produced shoots. In contrast to the present observation where lower concentrations of BAP and NAA induced healthy multiple shoots, Reddy *et al.* (1988) induced the best multiple shoots proliferation from nodal explants of *Gymnema slyvestre* in higher concentration of BAP (MS + 5 ppm BAP + 0.2 ppm NAA).

In the present investigation, multiple shoots were observed in MS + different concentrations of BAP (0.5 ppm – 2 ppm) alone, and BAP with 0.5 ppm NAA. However, the combination of MS + 0.5 ppm BAP + 0.5 ppm NAA was found to be the best media. Multiple shoots were not observed in MS + 1 ppm BAP + 1 ppm NAA. Instead, callus was formed. Joshi and Singh (2000) verified that MS + 0.5 ppm BAP + 0.1 ppm NAA was the most suitable medium for shoot multiplication by using nodal explants of *Bauhinia variegata*. In the present investigation, the best shoot multiplication with nodal explants was observed in MS + 0.5 ppm BAP + 0.5 ppm NAA. This combination of media has the added advantage in maintaining homogeneity of the progeny because the shoots originated from the nodal explant directly, i.e., without the formation of callus. Further, the shoot induction took place within 4 – 6 weeks. This condition helps to propagate the shoots *in-vitro* and enables to obtain large number of plants at low cost and in a short period of time. Similarly, if the investigation is desired to obtain suitable callus, then the nodal explants cultured in MS + 1.5 ppm BAP + 1 ppm NAA for 3 – 4 weeks is the best. Soft and dark-brown callus with dark green upper surface was obtained in the present investigation. The callus spread on the entire upper surface of the media.

No rooting was observed from the shoots in any of the fifteen different combinations of the media. Rajkarnikar *et al.* (2000) too did not obtain rooted plantlets of *Rauvolfia serpentina* L. Benth. ex Kurz *in-vitro.* The shoots were induced to rooting after acclimatization and hardening in non-sterile sand along with adequate humidity.

CHAPTER SIX

CONCLUSIONS

Tissue culture technique specifically *in-vitro* propagation is the most practicable technique academically and commercially in order to achieve rapid multiplication of those plant species that are extensively exploited for traditional medicinal uses and for the raw materials demanded by burgeoning pharmaceutical industries. The present investigation focused on the proliferation of hairy roots, shoots and rooted plantlets *in-vitro*, is an academic research that enabled to make the following points of conclusion:

For *Valeriana jatamansii* Jones

Shoot multiplication rate did not decline even after two years of subculture.

- Healthy shoots with persistent roots were observed on $\frac{3}{4}$ strength MS medium supplemented with 1 ppm BAP and 0.5 ppm NAA.
- MS supplemented with 0.5 ppm NAA was found to be most effective for the optimum proliferation of hairy roots.
- Similarly, the maximum proliferation of hairy roots was observed in $\frac{1}{2}$ strength of MS supplemented with 0.5 ppm NAA.
- Cocopit, as the initial hardening medium for the *in-vitro* developed plantlets, was found to be effective for the highest percentage of the survival rate of the plantlets *in-vivo* among 3 different hardening media (viz. – cocopit, sand and sand:soil mixture)
- The *in-vitro* developed plantlets achieved their maximum shoot length in cocopit (85 mm in average).

- Non-sterile but sun-dried sand was found effective for the maximum proliferation and propagation of roots *in-vivo*.

- There was almost uniform number of leaves (3-4 leaves per plantlet in average) in the plantlets hardened in all the three media. However, the leaves were healthy and dark green in those plantlets that were hardened in cocopit.

- The plantlets that were hardened in sand were found effective to adapt faster in the soil. The roots of these plants have relatively strong penetrating power into the soil. They had relatively larger size of rhizome.

- The plants hardened in the sand had the highest number of survival and propagation *in-vivo*, that is, in polythene bags and then in garden soil.

For *Rauvolfia serpentina* L. Benth. ex Kurz.

- Micropropagation of *Rauvolfia serpentina* L. Benth. ex Kurz through node culture was easily accomplished.

- Nodal explants were found best for induction of multiple shoots.

- Healthy, distinct and maximum shoot Proliferation (six–eight) was observed in MS medium supplemented with 0.5 ppm BAP and 0.5 ppm NAA.

- Shoot tip explants gave healthy, distinct and multiple shoots (four–six) in MS medium supplemented with 2 ppm BAP and 0.5 ppm NAA.

- MS supplemented with 1 ppm NAA was found most effective for callus formation with shoot tip explants.

- MS supplemented with 1.5 ppm BAP and 1 ppm NAA was found most effective for the proliferation of soft and dark brown callus with nodal explants.

- MS supplemented with lower concentration of BAP seemed suitable for sprouting of multiple shoots.
- MS supplemented with higher concentration of BAP was found effective for the proliferation of leaves but not suitable for the formation of multiple shoots.
- The various combinations of BAP and NAA supplemented with MS medium, seemed ineffective for the differentiation of roots.

CHAPTER SEVEN

RECOMMENDATION

Both the medicinal plants under present investigation have valuable applications for traditional medicinal uses as well as for raw materials in the concerned pharmaceutical industries. Many of the valuable herbs including these species are threatened with extinction due to over harvesting, destructive collection techniques and lack of knowledge for sustainable harvest. The trade of these medicinal plants can be a source of cash income in order to uplift the rural economy and enhance the living standard especially of those low-income farmers. Farming of these medicinal plants will help to solve supply problems, regularize the trade, provide registered products of uniform quality and offer a new source of income to rural poor. Besides, new rural employment opportunities will be possible through the establishment of tissue culture laboratories, nurseries and large scale cultivation of the medicinal plants. To accomplish the sustainable harvest, conserve the wild stock and genetic diversity, and enhance the rural as well as national economy, the following recommendations may be useful:

1. Concerned Ministry, Departments and scholars should work jointly and publish literatures on the importance and conservational utilization of these medicinal plants. The literatures should be simple and suitable to suit the needs of the farmers.

2. Mass propagation of these medicinal plants should be launched through the establishment of Tissue Culture laboratories in the regional levels. For this, suitable protocols should be verified and utilized.

3. Ecologically suitable places for the cultivation of these species should be surveyed and mapped out. Local community should be encouraged,

through demonstration, technical and financial support, to undertake pilot farming and then establishing large scale plantation of these species.

4. Academic curriculum especially at the Bachelor and Masters Level should include the intensive research on the *in-vitro* propagation and field trial of medicinal plants including these species.

5. Legislation should not only include punishable aspects to the defaulters and smugglers of these forest products but also the rewarding measures to those farmers, scholars and institutions who demonstrate their outstanding role in the conservational utilization of these, and other, medicinal plants.

6. Suitable market should be established in the country to buy the crude raw materials supplied by local farmers. However, the source of harvest should be verified and certified prior to buying the supplied materials.

7. Local community should also be authorized and empowered to curb the smugglers who are responsible for illegal harvests of these species. To garner the support of the farmers, certain percentage of the revenue obtained as income tax from the buy and sale of these plants should be given to the community through the local government on transparent and accountable basis. It inculcates the culture of ownership, affinity and conservational ethic to the local people upon their local natural resources.

Photo Plate I

Valeriana jatamansi Jones

1. *In-vitro* raised plant (in MS + 1 ppm BAP) used as the source of explants.
2. Multiple shoot proliferation in MS + 1 ppm BAP after six week of subculture.
3. Multiple shoot proliferation in MS + 1 ppm BAP after four week of subculture.
4. Single shoot formation in MS hormone free media after six week of subculture.
5. Multiple Shoot Formation in MS + 1 ppm BAP and 0.5 ppm NAA.

Photo Plate II

6. Single Shoot in MS hormone free medium after six week of subculture.
7. Proliferation of hairy root mass in MS + 0.5 ppm NAA after six week of subculture.
8. Proliferation of hairy root mass in MS + 1 ppm NAA after six week of subculture.
9. Proliferation of hairy root mass in MS + 1.5 ppm NAA after six week of subculture.
10. Proliferation of hairy root mass in MS + 2 ppm NAA after six week of subculture.

Photo Plate III

11. Proliferation of hairy root mass in full strength of MS + 0.5 ppm NAA after six week of subculture.

12. Proliferation of hairy root mass in $\frac{3}{4}$ MS + 0.5 ppm NAA after six week of subculture.

13. Proliferation of hairy root mass in $\frac{1}{2}$ MS + 0.5 ppm NAA after six week of subculture.

14. Proliferation of hairy root mass in $\frac{1}{4}$ MS + 0.5 ppm NAA after six week of subculture.

Photo Plate iV

15. Multiple shoots *in-vitro* in MS + 1 ppm BAP + 0.5 ppm NAA, after six week of subculture.
16. *In-vitro* plants in the process of acclimatization in the laboratory temperature after six week of subculture.
17. Average size of *in-vitro* developed shoot in MS + 1 ppm BAP + 0.5 ppm NAA after six week of subculture.
18. *In-vitro* raised plants are being hardened in cocopit.
19. Chemical secretion by 10-week-old *in-vitro* plants. Despite the presence of chemical, the shoots had remained healthy.

Photo Plate V

20. *In-vitro* raised plants are being hardened in cocopit (after 21 days of hardenings).
21. *In-vitro* raised plants are being hardened in sand (after 21 days of hardenings).
22. *In-vitro* raised plants are being hardened in a mixture of sand and soil (1:1 v/v) (after 21 days of hardenings).

Photo Plate VI

23. Average size of the *in-vitro* plant after hardening in cocopit for 22 days. The plant is ready to be transferred to polythene bag containing soil.
24. The plants hardened in cocopit and sand are transferred into polythene bags containing humus rich soil.
25. Multiple shoot formation of the plant in the soil after its eighth month of transfer.
26. Roots and rhizome of the plants after their ninth month of transfer into the soil.

Photo Plate VII

27. Rhizome from 10-month-old plants (in the soil) that were initially hardened in sand.
28. Rhizome from 10-month-old plants (in the soil) that were initially hardened in cocopit.
29. Adventitious shoot being emerged from the rhizome from a 10-month old plant in the soil.

Photo Plate VIII

Rauvolfia serpantina L. Benth ex kurz.

30. *In-vivo* plant nurtured in CDB garden, T.U. (source of shoot tip and nodal explants).
31. Shoot tip culture in MS hormone free media after the eight-week of culture.
32. Shoot tip culture in MS + 0.5 ppm BAP after the eight-week of culture.
33. Shoot tip culture in MS + 1.5 ppm BAP after the eight-week of culture.
34. Shoot tip culture in MS + 0.5 ppm BAP + 0.5 ppm NAA after the eight-week of culture.
35. Shoot tip culture in MS + 2 ppm BAP + 0.5 ppm NAA.

Photo Plate IX

36. Node culture in MS hormone free used media after eight-week of culture.
37. Shoot tip culture in MS + 0.5 ppm NAA after the eight-week of culture.
38. Node culture in MS + 0.5 ppm BAP after the eight-week of culture.
39. Node culture in MS + 0.5 ppm BAP + 0.5 ppm NAA after the eight-week of culture.
40. Shoot tip culture in MS + 2 ppm BAP + 0.5 ppm NAA after the eight-week of culture.

Photo Plate X

41. Node culture in MS + 1.5 ppm BAP + 1 ppm NAA after the eight-week of culture.
42. Node culture in MS + 2 ppm BAP + 0.5 ppm NAA after the eight-week of culture.
43. Node culture in MS + 0.5 ppm BAP after the eight-week of culture.
44. Shoot tip culture in MS + 0.5 ppm BAP + 0.5 ppm NAA after the eight-week of culture.
45. Shoot tip culture in MS + 1.5 ppm BAP + 0.5 ppm NAA after the eight-week of culture.
46. Node culture in MS + 2 ppm BAP + 1 ppm NAA.

Photo Plate XI

47. Shoot tip culture in MS + 2 ppm BAP + 1 ppm NAA.
48. Node culture in MS + 0.5 ppm BAP + 1 ppm NAA after the eight-week of culture.
49. *In-vitro* developed shoots from node culture in MS + 0.5 ppm BAP + 0.5 ppm NAA (12 week old).

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