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.

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 treatments (Singh 1997). Out of the 264 endemic species of flowering plants of Nepal (IUCN), 101 species are endangered and eight under feared extinct groups (Shrestha & Joshi 1996). Most of these endangered species are the plants of medicinal and aromatic values, which are either confined in small areas or are collected recklessly from their natural habitats for commercial purposes. Medicinal plant based industries are few in the country. Over 90% of the commercial collection in Nepal is exported to India and is the major source and steady basis for the Indian pharmaceutical and aroma industries. The trade in medicinal and aromatic plants is an important source of income to the government and a major source of cash income to the rural people of Nepal's impoverished villages.

Medicinal properties of plants are due to the active chemical constituents present in different parts of the plant. Medicinal plants are important natural resources for primary health care as well as commercial commodities for income generation activities for a vast majority of the rural people in Nepal. It is estimated that only 15-20% of the population in and around the urban areas has access to modern medicinal facilities, the majority (80-85%) depend on traditional medicine. The use of these medicinal plants in

traditional medicinal care in the rural areas of the country is an age-old practice with many of the ethnic groups having their own systems of traditional and indigenous healing methods.

Due to rapidly increasing population and unmanageable collection, medicinal plants are in danger. As the result, Nepal is facing challenges to sustain its medicinal plant resource base and safeguard its rich biodiversity. Recently attempts for systematic study of wild medicinal plants in the country have been done through national and international efforts. Based on field studies, potential and viable recommendations to develop and manage the country's rich medicinal plant diversity have been provided to the government.

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

The quality, quantity and controlled production of desired chemicals substances have been possible in tissue culture technology. In a single species, there have been proven evidences of the differences in the contents of chemicals between the *in-vitro* developed and the *in vivo* plants. Similarly, the chemical constituents in callus, hairy roots, leaf primordia, tender stem, rhizome etc vary in the same plant 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 a lot of 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 to meet the demand of raw materials placed by the ever flourishing pharmaceutical industries for the requisite raw materials especially from flowering plants.

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 rare in the wild and are being scarce with time due to several causes such as 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.

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 for medicinal plants includes regeneration of clones from the cultured cells at high frequencies. Long term storage and conservation of the rare and endangered species, manipulation of DNA and protoplast culture to form genetically engineered plants also comes under tissue culture technology. We have no doubts that the feasibility of cultivating suspensions of isolated cells of higher plants in-vitro will widen the scope of this method even more. Methods for growing cell suspensions are being rapidly developed.

1.2 Description of Plant

Botanical Name: *Rauvolfia serpentine* (L.) Benth. Ex. Kurz

Common Name: Chadmarua, Sarpagandha (Nepali), Sarpagandha (Sanskirt), Serpentina (English)

Family: Apocynaceae

Chromosome no: n = 10, 11, 12, 22

Rauvolfia, the genus named in honor of Leonhard Rauwolf, a German Physicia of the sixteenth century, has 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.

Botany of the plant

R. serpentina (L.) Benth.ex. Kurz is an erect, evergreen, glabrous under-shrub usually to 75cm tall with white bark, tuberous tap roots of 0.2-2cm width and 4-12cm length. It has characteristic slightly wrinkled and coarse surface. The root bark is grayish yellow to brown and displays irregular longitudinal fissures. The leaves are simple, glabrous, lanceolate and are generally in whorls of three to four, crowding the upper part of the stem, bright green above and pale green beneath. The inflorescence is corymbose with white or pink flower. Fruit is drupe that is 5cm in diameter and dark, purple or blackish when ripe.

Distribution

The plant grows in tropical and sub-tropical regions, benefiting from the monsoon rains. It may be grown almost anywhere at low or medium elevations where rainfall is not less than 75cm. It flourishes in hot and in partial shade. Soil with plenty of humus and a pH of 4.0-6.3 is desirable for luxuriant growth.

It is widely distributed in the sub-Himalayan tract (up to an elevation of 1000 to 1150m in Nepal). In Nepal, it 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. The plants are more frequent under the shade of *Ficus, Terminalia, Cassia, Dalberbia, Magnifera* and *Dendrocalomus (*Fig-1).

Conservation Status:

Endangered (IUCN category). The plant is enlisted in Appendix II in CITES. Under the Forest Act (1993) Nepal Government has banned this plant for export. However by obtaining permission from the government, its processed materials can be exported.

Part(s) used: Root bark.

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 shedding of leaves is far richer than the root of the green plants i.e. during June- August.

Bio-chemical constituents:

A large number of alkaloids (50 or more) have been isolated from various species of *Rauvolfia*. Reserpine ($C_{33}H_{40}N_{2O}$) is pharmacologically the most potent. Other important alkaloids are: reserpinine, rescinnamine, deserpidine, deseerpideine, serpentine, serpentinine, ajmaline, ajnalinine,ajmalicine, isoajmaline,rauwolfinine and yohinbine.

Medicinal Importance:

Reserpine is a universally known medicine for hypertension. The utility of this plant in treating mental disease and snake-bites was 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 labor 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 bowels.

1.3 Objectives of the Study

1) To find best medium for *in-vitro* multiplication of shoot using nodes as explants.
2) To find the effect of kinetin on shoot tip explants.
3) To find the effect of phytohormone on leaf explants.
4) To compare the callus inducing capacity of nodes, roots and leaves as explants.
5) To compare the morphogenetic nature of different callus produced in different medium.
6) *In-vivo* rooting of *in-vitro* produced shoot.

1.4 Justification of the Study

1) It is a fairly exploited medicinal plant for traditional medicinal use and for the export of crude raw materials. It is enlisted in the commercially threatened species of plants of Nepal under Forest Act, 1993 (Shrestha & Joshi 1996),endangered (IUCN) and Appendix II in CITES.
2) *In-vitro* grown plants are generally disease free and disease resistant resulting in more productivity.
3) 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.
4) The commercial demands of these species can be met without affecting their wild relatives in their natural habitat.

1.5 Limitation of Study

Due to time barrier and technical issues, comparative analysis of phytochemicals between *in-vitro* produced callus and wild plants in *R.serpentina* could not be tested. Similarly, field trial of acclimatized plantlets could not be carried out due to constraints of time and financial support.

CHAPTER - TWO
LITERATURE REVIEW

Large numbers of works have been carried out regarding the tissue culture. There are numerous literatures on *in-vitro* culture of medicinally important plants. Some latest literature has been mentioned here.

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

Sarkar et.al.(1996) induced multiple shoots from nodal and shoot apices of *Rauvolfia serpentine* on MS medium containing 1.0 mg/l BAP and 0.1 mg/l NAA. The best shoot proliferation was found at cut ends of the explants. The *in-vitro* proliferated shoots produced root and later transferred to the soil.

Rajkarnikar and Sanju (1999) working on *R. serpentina* found that excised shoot tips on MS media supplemented with 3 mg/l BAP and 0.1 mg/l of NAA produced micro - shoots.

Rajkarnikar et.al. (2000) showed the micro-shoots sprouting from the base of shoot tip explants in the MS medium with BAP (1, 2, 3 mg/l) along with 0.1 mg/l NAA in *R. serpenfina*.

Joshi and Singh (2000) propagated shoots from nodal explants of *Bauhinia Variagata* and *Bauhinia purpurea* using MS medium supplemented with 1 mg/l BAP and 0.5 mg/l NAA.

Joshi et.al. (2000) proliferated the micro-shoots from the nodal explants of *Elaeocarpus sphaericus* (Gaerth) K. Schum in the MS medium supplemented with BAP (0.5 mg/l) and NAA 0.1 mg/l. The proliferated micro-shoots when sub-cultured 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

micro-shoots. The micro-shoots developed root successfully in non-sterile sand within 15 to 20 days in green house condition.

Karki and Saiju (2000) developed a protocol for large scale production of plantlets of *Amomum subulatum* Roxb from the two cultivars Viz Ramsai and Golsai. The shoot tips (1-2mm) were excised from the mother plants and cultured in MS solid medium supplemented with 10 mg/l BAP and 0.1 mg/l NAA. Multiple shoots with roots were produced by separated sub-culture in same quantities of liquid medium. Rooted plants were successfully planted in the field.

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

Ranjit et.al.(2000) reported that multiple shoots were induced from nodal explants of *Foeniculum vulgare* on MS medium supplemented with BAP (1 mg/l). An average of six shoots were developed from a single shoot after 4 weeks of culture. Nodal explants were taken from *in-vitro* germinated plantlets as hormone free MS medium. Roots were developed on shoots in MS medium supplemented with NAA 2 mg/l.

Tejavathi and Purushothama (2000) reported that in *Guolvulus alsinoides,* callus formation, direct and indirect rhizogenesis and multiple shoot formation were found to common in all the explants. Multiple shoots were obtained in MS medium supplemented with IAA + BAP/Kn at various concentrations. In addition to multiple shoots *in - vitro* flowering was observed on shoot tip and nodal segments in MS medium supplemented with IBA/Kn/BAP at various concentrations.

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 micro-shoots were developed after fourth subculture. These micro-shoots were

transferred in non-sterile sand for rooting. The roots were differentiated within two to three weeks.

Zhu and Jian (2000) dealt with the influence of phytohormone on organogenesis of *Hypericum perforatum* and revealed that organogenesis, bud differentiation occurred on MS medium with BA and NAA to a great extent.

Tiwari et.al. (2000) derived a protocol for the rapid and large scale *in-vitro* clonal propagation of the valuable medicinal herb *Centella asiatica* L. He used axillary bud proliferation in nodal explants isolated from plants. Although bud break was dependent on BA supply, the synergistic combination of 22.2µm BA and 2.68µm NAA induced the optimum frequency (91%) of shoot formation as well as shoot number (4.5 shoots/node).

Bhuya et al. (2001) studied regeneration of an important medicinal plant *Wedelia chinensis* Menil on MS medium supplemented with Kn (2mg/l) and IAA (0.05 mg/l). Regenerated shoots were cultivated on MS medium with 0.1 mg/l IAA for rooting. The rooted plantlets were successfully established in soil. Combination of undifferentiated globular structures which on transfer to MS medium with Kn and IAA produced shoots.

Chandra et.al. (2002) obtained shoot bud from nodal segment derived from callus tissues of *Flacourtia jangomas* (Lour) Raeusch a woody medicinal plant of dioecious habit. Induction of callus was obtained in MS basal medium supplemented with 2,4-D (2.0 mg/l) and BAP (0.5 mg/l). Highest number of shoot bud (7.4 ± 0.2) was noted in BAP (20.0 mg/l). Rizogenesis was achieved in NAA (1.0 mg/l) from both inter-node and leaf explant.

Anand et al (2002) achieved *Adhatoda vasica* Nees by culturing excised nodes on MS and B_5 media supplemented with Kinetin (4.05µm) and NAA (2.69µm). The micro-hoots rooted in half strength of B_5 media. Plantlets were successfully acclimatized.

Bais et.al. (2002) developed a clonal propagation protocol of *Spilanthes mauritiana* DC where juvenile plants were used as starting material. The addition of BA (0.1 µm) and NAA (0.1 µm) to the culture medium resulted in maximum shoot response

with minimal callusing. Shoot rooted best *in-vitro* in MS medium supplemented with IAA (0.2 μm) and plants that had already developed roots showed better growth with maximum survival rate in the green house after an initial hardening.

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

Shah (2002) obtained multiple shoots is *Asparagus racemoscus* Willd when nodal explants and shoot tip cultured in combination of BAP (1 mg/l) and NAA (0.5 mg/l) respectively. The explants were taken from *in-vitro* grown plantlets. The callus was produced from cut ends. When callus cultured on Ms + BAP (1 mg/l) + NAA (1 mg/l) differentiated into shoots as well as roots. The proliferated shoots were thicker than normal plants. The rooting was observed best in NAA (0.5 mg/l).

Pereiva et.al. (2003) developed multiple shoots of *Anenopaegmg awense* Vell and *Stellfeld exde* Souza an endangered medicinal plant by using nodal segments as explants on MS medium supplemented with 4.4 μm of kinetin. Acclimatization of un-rooted plants into soil was carried out successfully.

Poudel (2003) obtained maximum number of multiple shoots of *Mentha spicata* L on MS medium supplemented with BAP (1 mg/l) for both node and shoot tip explants.

Niroula et.al. (2003) micro-propagated *Dioscorea bulbifera* from nodal explants. The best medium for regeneration was MS + 0.5 μm IAA + 20 μm Kn + 500 mg/l CH + activated charcoal (20%)

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

Joshi et.al. (2003) induced shoots from nodal explants of *Foeniculum vulgare* Mill on MS medium supplemented with BAP (1 mg/l) and NAA (0.5 mg/l). For this experiment the nodal explants were taken from *in-vitro* germinated plantlets on hormone free MS medium. Roots were developed in 1/2 strength MS liquid medium supplemented with NAA (1 mg/l).

Koroch et.al. (2003) developed a method for the induction of adventitious shoots from leaf tissues of *Echinacea pallida* with sub- sequent whole plant regeneration, proliferating callus and shoot culture were derived from leaf tissue explants placed on MS medium supplemented with BA and NAA combinations. The optimum shoot regeneration frequency (63%) and number of shoots per explants was 2.3) was achieved using MS media supplemented with BA (26.6 μm) and NAA (0.11 μm). Rooting of regenerated shoot explants was successful on MS medium both with and without the addition of IAA.

Govindarau et.al. (2003) developed a high frequency and rapid regeneration protocol via callus and directly from various explants was developed in *Withania somnifera* (L) Dunal. Callus was initiated from inter-nodal segments, leaf, root and petiole explants on MS and B_5 media supplemented with 2,4-D (0.5 -3.0 mg/l) and NAA (0.5-3.0 mg/l) either alone or along with kinetin (0.5-1.0 mg/l). Regeneration was observed from callus of all the explants except root on Ms medium fortified with BAP (0.5-2.5 mg/l) or in combination with IAA (0.5 mg/l)..

Agrawal and Subhan (2003) induced multiple shorts of *Centella asiatica* (Linn) Urban from lamina explants excised from *in-vitro* raised shoots through callus phase on

MS medium supplemented with cytokinin alone or in combinations. Amongest all the cytokinins, kinetin (10 µm) proved optimum for differentiating an average of 15.25$\pm$472 shoots in 75% cultures with 6 weeks.

Tiwari et.al. (2003) cultured nodal sections composing of axillary buds of Rauvolfia serpentia (L) Benth ex Kurz on three different nutrient medium viz. MS, Gamborg's (B5) and White's (wh) medium without growth hormone and with 3.0 mg/l BAP and 0.5 mg/l NAA denoted as MSBN, B_5BN,whBN respectively constituting overall six shoot induction media. Maximum multiple shoot regeneration was observed on B_5BN as compared to other five media on MSBA medium the regenerated shoots attained maximum length. However the callus formation was less than that on B_5BN. Maximum shoots with optimal nodes were obtained on medium B_5BN. However, after 30 days of initial culture the shoot length was highest on MSBN. Overall four to five fold improvement was observed for multiple shoot production *in-vitro* with B_5BN medium and two to three fold enhancement on MSBN as compared to whBN. *In-vitro* rooting was obtained efficiently prior to transplanting.

Rani et.al. (2003) observed callus induction from hypocotyls, root and cotyledonary leaf segments of *Withania somnifera* (L.) Dunal grown on Ms medium + 2, 4 –D and kinetin. Maximum callusing (100%) was obtained from root and cotyledonary leaf segments grown on MS medium supplemented with a combination of 2 mg/l (9.1 µm) 2,4-D and 0.2 mg/l (0.9 µm) kn. The callus obtained from hypocotyl leaf segments when sub-cultured on fresh media of 2, 4- D and Kn shoot regeneration was observed but root callus showed profuse callusing and remained recalcitrant to regenerate plantlets.

Maruthi et.al. (2004) established *in-vitro* regeneration protocol through the stem callus culture pf *Celastrus paniculatus* Willd. The explants induced callus in L_5 medium supplemented 2% of fructose . 6 µm Kn and 1 µm IBA. Interaction of the same hormones at lower concerntrations (1.5 to 3.5 µm) Kn and 0.1 to 0.4 µm) IBA) induced micro-shoots. These micro-shoots rooted on L_5 half strength medium supplemented with 1.0 to 5.0 µm IBA or 1.0 to 3.0 µm NAA. About 98% of the regenerates were successfully acclimatized to the natural environment.

Chand et.al. (2004) developed a protocol for callus induction, regeneration and micro-propagation of *Ocimum basilicum* (Lamiaceae). Various types of calli formed and nodular calli were observed from the nodal explants and buds developed into shoot used for micro-propagation. When these callus were sub-cultured (originated in Ms + 2 mg/l 2, 4-D + 1mg/lkn on BAP (2 mg/l) or Kn (1.5 mg/l) were 100% responsive in shoot-let formation. Length of the shoots were more in kinetin supplied media where as BAP supplemented media showed reduced shoot length. Multiple shoots were also observed in some nodal explants supplermented with BAP, 2 mg/l and kinetin 1.5 mg/l.

Rajkarnikar and Bhatt (2004) culture the node and leaf explants of *Azadirachta indica* A. Juss. tree. The node explants responded in MS medium supplemented with 0.25 mg/l charcoals and 50 mg/l adenine sulphate as most suitable for the regeneration of multiple shoots. *In-vitro* raised micro-shoots developed roots in Ms medium fortified with 0.5 mg/l NAA and 0.5 mg/l charcoal.

Rajkarnikar et.al. (2004) obtained multiple shoot of *Cephaelis ipecacuanha* from *in-vitro* regenerated node segments in MS medium supplemented with 2mg/l BAP and 0.1 mg/l µm NAA. These shoots produced roots in non-sterile sand within 2-3 weeks. The regenerated plants were successfully grown in soil in the green house.

Rajkarnikar et al. (2004) showed the multiplication of microshoots from the explants (1-3mm shoot tip and leaves) of *Swertia ciliata* cultured in MS medium supplemented with 1 mg/l BAP and 0.1 mg/l NAA. The developed micro-shoots were transferred in non-sterile sand for rooting. The roots were initiated within 2 to 3 weeks of transplantation. The rooted plants were successfully established in field.

Bhatt et al. (2004) showed the development of plantlets within 5 to 6 weeks from the nodal explants of hybrid Asiatic lily cultured in the MS medium supplemented with 0.5 mg/l BAP and 0.1 mg/l NAA the established shoot tips were sub-cultured in the name medium for four weeks. 8 to 10 micro-shoots developed in the 3rd subculture. These micro-shoots rooted in non-sterile and within 3 to 4 weeks.

Basnet (2004) developed a protocol for the *in-vitro* propagation of *Coffee arabica* L. and *Citrus aurantifolia* Swingle. Explants were taken from *in-vitro* grown seedling. In *C. Arabica,* the best medium for callus proliferation from root explants was Ms + 2 mg/l

2, 4-D and 1 mg/l Kn where callus re-differentiated into root. For leaf explants the best medium for callus proliferation was MS + 2 mg/l 2,4-D and 1 mg/l Kn. No organogenesis occurred on sub-culturing this callus. MS medium supplemented with BAP (1 mg/l) and NAA (0.5 mg/l) induced maximum multiple shoots from nodal explants. The shoots were rooted in half strength of MS medium supplemented with 1.5 mg/l IBA.

Bhatt (2004) obtained multiple shoots from nodal explants obtained from *in-vitro* grown seedling on MS-medium supplemented with 2 mg/l BAP in *Oroxylum indicum* where as MS medium supplemented with 1 mg/l BAP was best for elongation of multiple shoots.

Devkota (2004) investigated the appropriate combinations of plants growth regulators for the micro-propagation of *Valeriana jatamansii* Jones by using node and shoot tip as explants. Ms medium. supplemented with BAP(1 mg/l) was suitable for shoot initiation and MS medium with IBA (0.5 mg/l) for rooting.

Sivanesaer and Murugesan (2005) developed an efficient protocol for high frequency plant regeneration from leaf explants of *Withania somnifera* on MS medium supplemented with different concentration of auxins and cytokinins. Highest frequency of shoot buds was obtained at a concentration of 1.0 mg/l Kn. *In-vitro* rooted plantlets (rooted in 2.0 mg/l IBA) were successfully transferred to the field after acclimatization in the net house.

Das et.al. (2005) have formulated a successful protocol for *in-vitro* regeneration of *Vitex negundo* (Verbanaceae) from nodal explants. Plantlets were directly regenerated from the nodal explants on MS medium supplemented with different concentration of BAP, NAA and Kinetin.

Azad et al. (2005) has achieved shoot organogenesis and plant establishment for *Phellodendron amurense* Rupr from excised leaf explants. Direct shoot regeneration was achieved by culturing 1cm^2 sections of about 10 days old leaves on MS medium enriched with 4.4 µm BAP and 10 µm NAA after 4 weeks of culture. Leaf explants produced callus from their cut margins within 3 weeks of incubation on MS + 2.0 µm TDZ + 4.0

µm 2, 4 –D or 4.0 µm NAA. The maximum number of adventitious shoots was regenerated from the leaf derived callus within 4 weeks of culture on MS medium containing 1.5 µm BAP and 1.0 µm MAA. The micro-shoots were rooted in MS + 2.0µm 1BA. within 3 weeks of transfer which were then transferred to kanuma soil and the survival rate in *ex-vitro* was 90%.

Bhadel (2005) obtained callus from *in-vitro* culture of *Ephedra girardiana* on MS + 2, 4-D and Kinetin (2 mg/l). In the same medium multiple shoots were produced. Root initiation was observed after sub-culture of callus on the medium with NAA (1 mg/l).

Nguyen et al. (2005) developed a protocol for micro-propagation of *Curcuma zedoaria* Roscoe by rhizome sprout culture. Maximum shoot induce from rhizomes on MS medium supplemented with BAP (0.5-5.0 mg/l), well developed shoots were rooted on MS medium with NAA (2 mg/l).

Chand (2006) cultured the explants (root, shoot tip, leaf and node) of *Clinopodium umbrosum* on MS medium supplemented with different concentration of BAP. The best medium for callus proliferation from leaf was MS + 0.5 mg/l BAP + 1 mg/l NAA while for root explants was MS + 2mg/l BAP + 1 mg/l NAA. Nodal explants were most suitable for shoot multiplication on MS + 1 mg/l BAP. For *in-vitro* shoot multiplication on MS+1 mg/l BAP. For *in-vitro* rooting of shoot MS medium supplemented with l mg/l NAA was found most effective.

Sharma (2006) inoculated shoot tips and nodes from the *in-vivo* plants of *Rauvolfia serpentria* (1) Benth ex Kurz on MS medium with fifteen different combination and concentration of BAP and NAA. Shoot tip responded with healthy and distinct multiple shoots on MS +2 mg/l BAP + 0.5 mg/l NAA. Multiple shoots were differentiated from the callus. The nodal explants differentiated into healthy and relatively study shoots MS + 0.5 mg/l BAP + 0.5 mg/l NAA. There was no callus formation.

Aryal (2006) obtained callus from stem explants of *Rauvolfia serpenfina* (L.) Benth ex Kurz on MS medium supplemented with 1 mg/l 2, 4-D + 1 mg/l kinetin. The subculture of this callus on MS medium supplemented with 3 mg/l 2, 4 –D or 10% coconut milk regeneration of plant occurred. Maximum number of shoot was observed on MS + BAP (3 mg/l) and the elongation of shoot length occurs on low concentration of BAP (0.5 mg/l) when shoot tips were inoculated. Explants were taken from *in-vitro* grown plantlets. The micro-shoots rooted best on MS medium with IAA (3 mg/l).

Shrestha (2006) cultured the explants (root, inter-node, node and shoot tips) of *Neopicrorhiza scrophularifolia* (Pennell) Hong obtained from *in-vitro* grown seeds on MS medium supplemented with different concentration of BAP and NAA. Shoot tip explants gave the most satisfactory result for shoot multiplication. MS medium supplemented with BAP (1 mg/l) and NAA (0.5 mg/l) was found to be best for callus induction and shoot multiplication where as elongation of shoot was done in hormone free MS media. MS medium without auxin and with auxins (1AA, NAA and IBA) were used to induce rooting. Among these auxin free medium was found to be best for rooting.

Xu, Tiefang et.al. (2005) developed a protocol for efficient *in-vitro* plant regeneration of *Pinellia ternate* (Thunb) Breit, an important wild Chinese herb. Tubers as explants were superior to leaves and petioles and MS medium was better than N_6 and while's media. It was found that 9.1 μm kinetin and 2.3 μm 2,4-D in the medium was best hormone combination for promoting callus induction. The highest shoot regeneration frequency (99%) was achieved from callus cultured on MS medium supplemented with 9.3 μm kinetin and 2.3 μm 2,4-D. The rooted shoots were successfully transplanted in soil, with over 95% survival.

Dai Wenha et al. (2005) developed a protocol for micro propagation of 'Amethyst' purple raspberry. Shoots were produced from explants in both MS and Driver Kuniyuki Walnut (DKW) media supplemented with different concentration of thidiazuran (TDZ) and benzyladenine (BA) solely or combined. Shoot can be rooted both *in-vitro* and *ex-vitro* with or without IBA at 0 to 1.0 μm.

Khanna et.al. (2006) developed a protocol for high frequency plant regeneration from immature embryos of *Argyrolobium roseum* Jaub and Spach an important medicinal legume. Green nodular calli were initiated from immature embryos excised from 10d- old pods in 70% of culture within 3 weeks when grown in MS medium supplemented with BAP (0.5 mg/l) + IAA (0.25 mg/l). Subsequent sub-culture of callus pieces to MS medium supplemented with BAP (0.5 mg/l) alone or in combination with IAA (0.25 mg/l) facilitated regeneration of multiple shoots. Organogenic calli bearing multiple shoots when transferred to MS medium supplemented with BAP (0.5 mg/l) + IAA (0.25 mg/l) supported rapid shoot elongation. *In-vitro* rooted plantlets were successfully acclimatized in a sterile mixture of sand and garden soil (1:1) under green house and there after transformed to field beds.

Uddin et.al. (2006) developed a protocol for *in-vitro* culture of *Steria rebaudiana* Bert an important non-caloric sweetening herb to explore its potential for micro-propagation. Leaf, nodal and inter- nodal segments of the selected herb as explants were cultured on MS medium containing 2, 4-D at 2, 3, 4 and 5 mg/ l for callus induction. Inter-nodal segment initiated callus earlier than node and leaf. The highest amount of callus was found on MS medium with 3.0 mg/l 2, 4 -D and MS medium with 5.0 mg/l 2, 4 –D gave the poorest callus.

Verma et.al. (2006) developed a protocol for micro propagation of *Gerbera jamesonii* Bolus. The *in-vitro* developed calli of *G. famesonii* were kept in three culture media viz. MS, B_5 and N_6 and the number of plantlets produced were kept in three culture media viz. MS, B_5 and N_6 and the number of plantlets produced were recorded at an interval of 5 days for 30 days. The findings indicate maximum number of plantlets from the call placed on MS medium supplemented with IAA (1 mg/l) and kinetin (5 mg/l) followed by N_6 and B_5.

Yang et al. (2006) developed a protocol for an efficient *in-vitro* regeneration system is *Fortunella crossifilia* Swingle. It was found that the optimum explants and its orientation was epicotyl planted vertically with upper part upward and a short regeneration frequency of 1.48 shoots per explants was obtained on MS medium supplemented with 22.19 μm 6-BA. A rooting percentage as high as 74% was obtained

on ½ MS supplemented with 0.54 μm, NAA, 9.29 μm Kn and 0.5 g dm (-3) activated charcoal.

Misic et.al. (2006) developed a protocol for *in-vitro* propagation of Balkan endemic plant *Salvia brachyodon* Vandas from nodal segments. 6- Benzyl-aminopurine (BA) was more effective in axillary buds promoticn when compared to thidiazuroa. The rooting of regenerated shoots was induced by transferring them to the media supplemented with auxins. The acclimatization of *in-vitro* rooted shoots was successful.

CHAPTER - THREE
MATERIALS AND METHODS

3.1 Materials

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

The initiating materials used for the investigation of *R. serpentina* (L) Benth ex Kurz was *in-vitro* grown plants on MS media established in the tissue culture laboratory of Biotechnology unit of CDB (Fig. - 2).

3.2 Methodology

For the investigation, Murashige and Skoog's medium (M.S., 1962) was used as the basal medium either alone or in combination with different concentrations of hormones. One liter of MS medium consists of the following ingredients.

1) Macronutrients (A)

Components	Composition of MS (Final cone. Mg/l)	(10X) gm/l stock concentration	Volume to be taken for 1 litre medium
Potassium nitrate (KNO_3)	1900	19.0	100ml
Ammonium nitrate (NH_4NO_3)	1650	16.5	
Magnesium sulphate (Mg SO_4. $7H_2O$	370	3.70	
Calcium chloride ($CaCl_2$.$2H_2O$	440	4.40	
Potassium dihydrogen phosphate (KH_2PO4)	170	1.7	

(2) Micronutrients. (B)

Components	Composition of MS (Final conc. Mg/l)	(1000X) mg/ 100ml stock concentration	Volume to be taken for 1 liter medium
Boricacid (H_3BO_3)	6.20	620	1 ml
Mangnese sulphute ($MnSO_4$. $4H_2O$)	22.30	2230	
Znicsulphtae ($ZnSo_4.7H_2O$	8.60	860	
Sodium molybdate (Na_2- $MoSO_4$. $2H_2O$)	0.25	25	
Copper sulphate ($CuSO4.5H_2O$)	0.025	2.5	
Cobalt chloride ($CoCl_2.6H_2O$)	0.025	2.5	
Potassium iodide (KI)	0.83	83	

KI was prepared and stored separately

(3) Iron source (c)

Components	Composition of MS (Final conc. Mg/l)	(100X) mg/100ml stock concentration	Volume to be taken for 1 litre medium
Sodium ethylene diamine tetra acetate (Na_2-EDTA)	37.3	373	10 ml
Ferrous sulphate ($FeSO_4.7H_2O$)	27.8	278	

(4) Vitamins (D)

Components	Composition of MS (Final conc) mg/l)	(1000X) mg/ 100ml stock concentration	Volume to be taken for 1 litre medium
Glycine	2.0	200	1 ml
Nicotinic acid	0.5	50	
Pyridoxin HCl	0.5	50	
Thiamin HCl	0.1	10	
Myo-inositol	100	10,000	

Myo-inositol is generally added fresh in the medium.

(5) Carbon Source

Chemicals	gm/l
Sucrose	30 gm

(6) Gelling agent

Chemicals	gm/l
Agar	8 gm

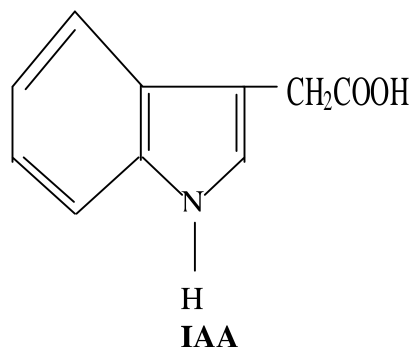
3.2.1 Preparation of Stock Solution for MS Medium

Each stock solution was prepared in the given concentration by first weighing the requisite components using digital balance and dissolved in distilled water in the sequence of the components 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 4^0C'

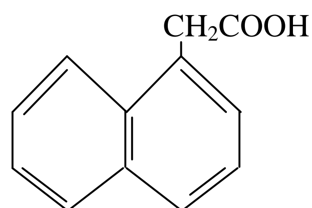
3.3 Hormone Used for Investigation

Auxins :

i. Indole -3- Acetic acid (IAA)

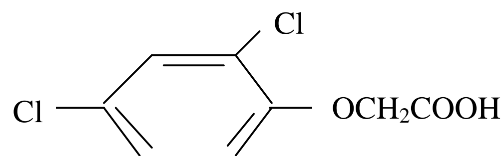


ii. Naphthalene acetic acid



NAA

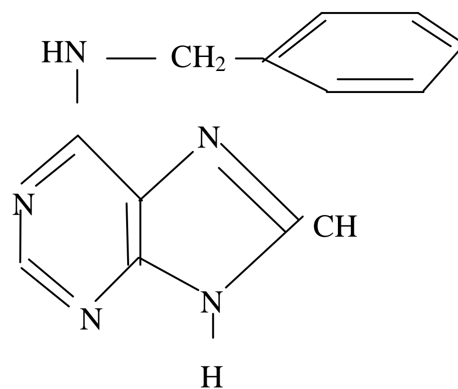
iii. 2,4-Dichlorophenoxy acetic acid



2, 4-D

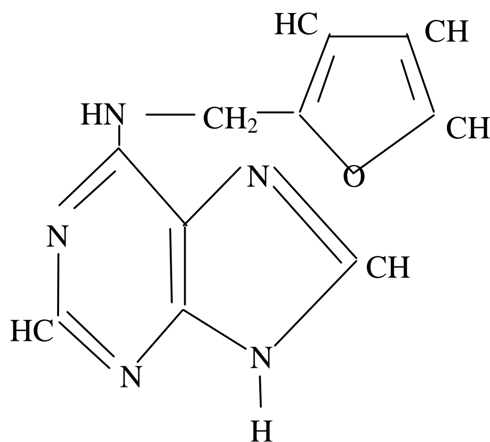
Cytokinins:

i) 6-Benzyl amino purine



BAP

ii. Kinetin



6-Furfuryl aminopurine

3.3.1 Preparation of Hormone stock Solutions

Preparation of desired Auxin (2.4-D, NAA & IAA): 10 mg of auxin was dissolved in few drops of 95 % ethyl alcohol. Then final volume was made 100 ml by adding distilled water. It becomes the stock of 100 ppm. For preparation of cytokinins (BAP& Kinetin) 10mg of cytokinin was first dissolved in few drops of 95 % ethyl alcohol and made final volume 100 ml. It becomes the stock of 100 ppm.

However, in both 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 kept in sterilized brown bottles, labeled them including the date of preparation and then preserved in refrigerator at 4^0C.

3.4 Sterilization of Glasswares and metal Instruments

All Glasswares and metal instruments used for the experiments should be well sterilized. For this, they were dipped in detergent water solution for 24 hours and washed with the help of bottle brush and cleaned with tap water. Finally the glasswares were rinsed with distilled water and sterilized in hot air oven at 120^0 for 3-4 hours. Metal instrument like forceps, scalpels and surgical blades were wrapped with aluminium foil then autoclaved at 121^0C for 15 minutes at 15lb/sq. inch.

3.4.1 Preparation of Medium

For the preparation of 1 liter of MS medium, about 400ml of distilled water was taken in a dry and clean conical flask. Required amounts of stock solutions were added to it with continuous stirring with the help of magnetic stirrer. Next, 30 gm of sucrose (3%) was dissolved little by little with the motion set by the magnetic stirrer. The final volume was made 1000 ml by adding distilled water. The pH was adjusted 5.8 by adding 0.1N NaOH or 0.1N HCl when required. For MS medium supplemented with different concentrations and combinations of hormone, the following formula was used.

$S_1V_1 = S_2V_2$

Where,

S_1 = Concentration of hormone in the stock solution

V_1= Volume of hormone 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

Then the prepared hormone (s) was added in the dispensed volume of the MS medium in different beakers as desired by the procedure. The 8gm (i.e. 0.8gm in 100ml media) agar was dissolved in hot media. The media was dispensed into clean and sterilized culture tubes. They were capped with aluminum foil and tied with durable rubber. The medium was labeled specifically with the type and the concentration of hormone. The medium filled culture tubes were autoclaved at 121^0C for 15-20 minutes at 15 lb/sq-inch. The autoclaved media was allowed to cool to normal atmosphere pressure and the culture tubes with media were taken out. They were kept in the culture tube stands and were finally placed in the aseptic chamber of tissue culture laboratory at 45^0 inclinations 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.

3.5 Preparation for Inoculation

For inoculation, the laminar airflow chamber was thoroughly cleaned by spirit. All the necessary materials such as autoclaved glassware, metal instruments, culture tube with media, sterile distilled water, rubber bands, aluminum foils, match box, marker pen except explants were exposed under ultraviolet (UV) light for 45 minutes. Air blower was opened after closing UV light for 30 minutes. Then, inoculation of explants was carried out aseptically with continuous air blowing.

3.6 Inoculation of Explants

Healthy nodes and root tips (3-5mm) were taken from *in-vitro* raised plants similarly shoot tips (3-5mm) and leaves were taken from *in-vitro* raised plantlet. Then, the explants were cultured in MS media with or without hormones.

The mode and leaf explants were inoculated in the MS medium (free of hormones) as well as in all the nine other various combination of MS+BAP and NAA. Similarly node, leaf, and root were inoculated separately is MS medium supplemented with various concentrations of 2,4–D Shoot tips (3-5mm) were also inoculated in different concentration of kinetin. All the cultures were maintained at 25 ± 2^0 C and 16 hour photo-period daily.

3.7 Subculture of Callus

In the process of callus subculture, the callus grown *in-vitro* was taken out aseptically and transferred to medium with or without various combinations of growth hormone. The cultures were maintained at 25 ± 2^0C and 16 hourphoto-period daily.

3.8 Acclimatization and in-vivo Rooting of in-vitro Multiplied Shoots

3.8.1 Preparation of Plants for Plantation

- Six weeks old *in-vitro* grown shoot was selected and the caps of culture was opened and exposed for three days at laboratory or room temperature.
- The media was washed from the plantlets with clean water first and then distilled water and Fungicide(0.01% Bavistine) was spread uniformly.
- The basal part of 30 plants shoots were dipped in IAA(3 mg/l) for 1 hour and other 30 plants were not treated with hormone.
- While treating hormone the leaves and the shoots must not be immersed in the hormone.

3.8.2 Preaparation of Bed

- Coco-pit was soaked overnight in water. Two clean trays were taken and soaked coco-pit was spread uniformly.

3.8.3 Plantation and Caring of Plants

- Plants with or without treating hormone were planted separately in the above prepared beds of coco-pit.
- These trays were covered with white polythene sheets.
- Plants were watered regularly twice a day.
- 5% NPK as a fertilizer was given once a week.
- Observation for rooting was done after one week of plantation until six weeks.

3.9 Statistical Analysis

Standard error(S.E.) and mean value were determined applying raw data through the use of SPSS computer programming.

CHAPTER FOUR
RESULT

4.1 Culture of Leaf Explants

Leaf explants were excised from *in-vitro* grown plantlets (Fig.-2) and cultured in MS medium supplemented with various combination of BAP (0-2 mg/l) & NAA (0-0.5 mg/l). They were also cultured on MS medium with 2, 4-D (0-3 mg/l) concentration of hormones.

The leaf explants on MS+2, 4-D started callusing from cut ends on 8 days of inoculation in all concentrations. The callus was whitish in color. The growth of callus was so vigorous that in the 4 weeks of culture, the identity of explants was lost (Fig.-3)

The explants cultured on MS + 0.5 mg/l NAA produced callus at the cut end. Nature of callus was whitish and hard. Within 3 weeks many white roots were differentiated from callus (Fig.-4). The white roots turned into brown color in the 5 weeks (Fig.-5). In MS + 0.5 mg/l BAP + 0.5 mg/l NAA the explants produced green and hard callus from cut end(Fig.-6). In the 3 weeks of culture callus differentiated a single white root (Fig.-7). The callus proliferated into red colored callus and network of white roots (Fig.-8). The newly proliferated red callus remained unchanged which was also hard in nature. The explants cultured on MS + 1 mg/l BAP + 0.5 mg/l NAA also produced callus and callus differentiated into roots but the nos of roots were less in no (Fig.-9,10,11,12). The explants on MS + 1.5 mg/l BAP + 0.5 mg/l NAA produced green and hard callus which differentiated roots (Fig.-13,14). The explants on MS+ 2 mg/l BAP + 0.5 mg/l NAA produced brown callus with shoot primodia from cut end in the 4 weeks of culture (Fig.-15). Further growth of callus was not seen but the no of shoot was increased which was light green in color (Fig.-16,17).

The leaf explants cultured on MS medium alone and in combination with BAP of 0-2 mg/l hormone concentration were swollen only and senesced after 4 weeks.

4.2 Culture of Root Explants

The root explants measuring 0.2-0.3cm were excised from *in-vitro* grown plant and inoculated in MS medium with 2, 4-D of various concentrations (0-3 mg/l).The explants swelled and started callusing on 9 days from the cut ends. On MS+ 0.5 mg/l 2, 4-D all the explants inoculated showed response (Fig.-18) while in other concentration response was not observed in all the explants inoculated (Fig.-19). The growth of callus was so vigorous that the identity of explants was lost in the 4 weeks of culture (Fig.-20).. The callus was whitish and hard.

The root explants cultured in MS + BAP (1 mg/l) + NAA (0.5 mg/l) produced callus mass with green and red patches in white callus which could differentiated roots (Fig. -21).

4.3 Subculture of Root Callus

The 4 weeks old callus obtained from MS + 2,4-D was sub-cultured on MS medium supplemented with 2,4-D, BAP & NAA separately and in combination.

The callus sub-cultured on MS + 2,4-D (0-3 mg/l) showed only increase in callus mass. The growth of callus was observed good in higher concentration i.e. MS + 3 mg/l 2,4-D than in lower concentration while sub-culturing (Fig.-22). In the 6 weeks of sub-culture, the growth of callus was so vigorous (Fig.-23) that they turned dark brown in the 8 weeks of culture (Fig.-24).

In case of callus sub-cultured in MS medium supplemented with BAP & NAA in combination and separately, rhizogenesis from callus was observed in 2 weeks of sub-culture in MS + 0.5 mg/l NAA & MS + 1.5 mg/l BAP +0.5 mg/l NAA (25,26). Until 6 weeks the callus sub-cultured in other hormone concentrations did not show morphogenetic response but the white mass of callus turned green (Fig.-28).

4.4 Culture of Shoot Tips

4 weeks old shoot tips (0.3-0.5mm) obtained from *in-vitro* plants were cultured on MS medium supplemented with kinetin of various concentrations (0-3 mg/l) which showed following changes as in Table 4.1 & 4.2.

1) MS Basal Medium

The shoot tip cultured on MS medium showed slight elongation of shoot which attained its shoot length 2.16 ± 0.081 cm in 4 weeks. Single root was initiated from basal part of shoot tip after 6 weeks which was about 3cm in length in 8 weeks. The shoot attained 3.76 ± 0.14cm length after 8 weeks of culture (Fig. -29).

2) MS + 0.5 mg/l Kinetin

Shoot multiplication was observed after two weeks. In this case brown callus was observed on the basal part of shoot tip. The number of shoot was 3.0 ± 0.57 and shoot length 1.82 ± 0.35cm in 4 weeks. From the callus single root was emerged in the 6 weeks of culture which was about 2.7cm in length. After 4 weeks the no of shoot was not increased but elongation of shoot occurred which became 2.94 ± 0.40cm in 8 weeks (Fig. - 30).

3) MS + 1 mg/l Kinetin

Shoot multiplication and callus formation at the basal part of shoot tip occurred after 3 weeks of culture. From the callus a small bud sprouted in 4 weeks of culture (Fig. - 31) which multiplied and become 2 in number after 4 weeks. The shoot tip that gave callus turned yellowish and finally senesced with the growth of callus originated plant (Fig.-32). The no of shoot was 1.66 ± 0.33 and its length 2.75 ± 0.25cm in 4weeks. In the 8 weeks of culture the no of shoot was 2.00 ± 0.57 and its length 4.05 ± 0.39cm (Fig.-33).

4) MS + 1.5 mg/l Kinetin

Here, the no of shoot was 3.0$\pm$00 and its length 1.38$\pm$0.13cm in 4 weeks of culture which was started to multiply after 3 weeks with brown callus originating from base of shoot tip (Fig.-34). In the 8 weeks of culture the no of shoot became 5.00$\pm$1.00 and shoot length 3.36$\pm$0.44 cm where callus differentiated root on the same culture (Fig. -35)

5) MS + 2 mg/l Kinetin

The shoot tip started to multiply with brown callus originating from its base after 3 weeks. The no of shoot was 2.66$\pm$0.66 and its length 1.50$\pm$0.21cm in the 4 weeks (Fig.-36). Rhizogenesis from callus on the same culture observed after 6 weeks and was about 1.5cm in length. In the 8 weeks of culture the no of shoot was 3.33$\pm$0.66 and its length 2.0$\pm$0.23cm (Fig.-37)

6) MS + 2.5mg/l Kinetin

The shoot tip gave brown callus as well as multiple shoot after 3 weeks. The no of shoot was 4.33$\pm$1.45 and its length 1.55$\pm$0.14 in the 4weeks of culture (Fig.-38). In the 8 weeks the no of shoot became 5.33$\pm$1.45 and its length 3.31$\pm$0.27cm (Fig.-39). Morphogenesis from callus was not observed on the same culture until 8 weeks.

7) MS + 3 mg/l Kinetin

In the 4 weeks of culture the no of shoot was 1.33$\pm$0.33 with their length 2.42$\pm$0.075cm (Fig -40).The brown callus that was originated from base of shoot tip in the 3 weeks gave single plant in the 6 weeks as in Fig. no 41. The mother plant did not die here as it died in 1 mg/l kinetin. The callus derived plant and mother plant were found healthy (Fig.-42). The no of shoot was 2.33$\pm$0.33 and its length was 3.07$\pm$0.36 in the 8 weeks.

The shoot tips were not good enough to give multiple shoots in MS+ Kinetin as compared to BAP. However average 5.33$\pm$1.45 shoots were produced in MS +2.5 mg/l kinetin and maximum shoots length 4.05$\pm$0.39 in MS+1 mg/l kinetin in 8 weeks of culture. The nature of callus that originated at the bases of shoot tip was brown and hard.

4.5 Culture of Nodal Explants

The nodal explants of o.3-0.5mm from one year old *in-vitro* plants was cultured on MS medium supplemented with or without BAP(0-2 mg/l) and NAA(0-0.5 mg/l) in combination and separately which showed the following changes as in Table 4.3 & 4.4.

1) MS Basal Medium

The nodal part gave 2 plants after 3 weeks with swelling of cut part which did not form callus. The average height of plant was 1.9±0.01cm in the 4 weeks (Fig.-43). After 4 weeks the no of shoot remained constant but the shoot attained its height 2.75± 0.25 in 8 weeks (Fig.-44).

2) MS + 0.5 mg/l BAP

The nodal part gave multiple shoots within 2 weeks with swelling of cut edges The no of shoot was 3.33±0.51 and its length was 2.30±0.30cm in the 4 weeks (Fig. -45). The swelled part of cut edges did not transform into callus. In 8 weeks of culture average no of shoot was 6.33±0.42 while their length was 4.52±0.33 (Fig. - 46).

3) MS + 1 mg/l BAP

The average no of shoots was 4.66±0.20 and shoots length 3.25±0.48cm in the 4 weeks (Fig. - 47) which started to multiply in 2 weeks. In 8 weeks the no of shoot increased to 8.00±0.25 and its length was 6.60±0.48cm (Fig.-48). The swollen part of cut edges remain as it is and formation of callus was not observed.

4) MS +1.5 mg/l BAP

The nodal part which started to multiply in 2 weeks of culture attained the average no of shoots 6.50±0.29 and its length was1.95±0.024 in 4 weeks (Fig.-49). The cut edges gave little brown callus whose growth was not observed on the same culture. Shoot multiplication was observed after 4 weeks also and in 8 weeks no was found 12.00±0.36 with shoot length 7.46±0.62cm (Fig.-50).

5) MS + 2 mg/l BAP

In 4 weeks the average no of shoots was 2.00±0.82 and its length was 1.75±0.43cm (Fig.-51) which multiplied rapidly after 4 weeks. Small green callus on the cut edges was observed whose growth was not observed in the same culture but turned brownish. The average no of shoot in 8 weeks was 16.00±1.10 and its length was 2.64±0.10 (Fig.-52)

6) MS + 0.5 mg/l NAA

Here the cut edges of node starting to callusing without formation of shoot in the 1 week. The explants only swelled until 4 weeks (Fig.-53) In 5 weeks two small plantlets arose from nodal bud with rooting of callus. The no of shoot remained same till 8 weeks with the shoot length of 1.50±0.50cm and whitish root above the media and brownish inside the media was observed (Fig. - 54)

7) MS + 0.5 mg/l BAP +0.5 mg/l NAA

The nodal part started to multiply and cut edges produced greenish callus in 2 weeks of culture. Whitish mass of root was observed in 5 weeks of culture from callus which gave network of roots after 5 weeks of culture. The average no of shoot was 2.00±0.24 and its height was 1.50±0.31cm in 4 weeks (Fig.-55). The no of shoots became 3.66±0.70 and its height was 6.87±0.37cm in 8 weeks (Fig.-56).

8) MS + 1.0 mg/l BAP + 0.5 mg/l NAA

Greenish callus from cut edges and shoots from nodal part was observed in 2 weeks. The no of shoots in 4 weeks was found to be 2.66±0.37 with shoot length of 1.50±0.31cm (Fig.-57). In 8 weeks of culture the no of shoots was 4.00±0.54 with shoot length was 6.87±0.37cm (Fig.-58). The callus started rooting after 5 weeks as well as small plants originated whose growth was not observed in the same culture.

9) MS + 1.5 mg/l BAP + 0.5 mg/l NAA

The average no of shoots was 2.00±0.15 with shoot length of 1.50±0.31cm in 4 weeks of culture (Fig.-59) where multiple shoots from nodal part and greenish callus

from cut edges were observed in 2 weeks of culture The average no of shoots became 6.00±0.85 with shoot length of 3.95±0.20cm in 8 weeks of culture (Fig.-60). The callus differentiated into shoots and roots where only the root length and number were increased in the same culture.

10) MS +2 mg/l BAP +0.5 mg/l NAA

Greenish callus from cut edges of node and multiple shoots from nodal part was observed in 2 weeks of culture whose no was 3.66±0.57 and its length was 0.86±0.070cm in 4 weeks of culture (Fig.-61). The callus differentiated into micro-shoots in 6 weeks of culture whose growth was not observed in the same culture. Rooting from callus in the same culture was not recorded until 8 weeks of culture. In 8 weeks of culture the average number of shoots was 7.33 ± 0.93 and shoot length was 3.79 ± 0.51 cm (Fig.-62).

11) MS+ 2.4-D (0.5-3 mg/l)

The nodal explants produced callus from the cut edges in all concentrations on 8 days. The callus inducing capacity was better in lower concentration of 2, 4-D (0.5 mg/l) than in higher concentration of 2, 4.-D (3 mg/l). The callus was light brown and hard. In MS + 0.5 mg/l 2, 4-D one shoot from nodal bud arose which was about 1.5cm in length in 3 weeks of culture (Fig.-63) whose further growth was not observed .

Regarding the nodal culture, the best concentration of hormone for the shoot multiplication was MS+2 mg/l BAP where average no of shoot in the 8 weeks was 16.00±1.10 and the best shoot elongation was found in the MS + 1.5 mg/l BAP with shoot length of 7.42±0.62 in 8 weeks of culture.

4.6 Subculture of Nodal Callus

The 2-week old callus that was originated in MS + 2, 4-D was sub-cultured in MS medium alone and in combination with BAP of concentration 0.5, 1,1.5 and 2 mg/l. Rhizogenesis from callus in 6 weeks of culture was observed (Figs.- 64, 65, 66, 67).

Similarly 2-week old green callus that was originated from the cut edges of node in MS+ BAP(o.5-2 mg/l) +NAA(0.5 mg/l) were sub-cultured in the same concentrations of fresh medium as it was originated (Fig.-68). In 3 weeks of culture normal and abnormal plants (thicker than normal) were differentiated from callus (Fig. - 69, 70, 71, 72). The average no of shoots in 5 weeks of culture in MS +0.5 mg/l BAP + 0.5 mg/l NAA was 5 with maximum height of shoot was 3.5cm and rooting of callus (Fig. -73). In 5 weeks of culture the average no of shoot that originated from callus on MS + 1 mg/lBAP +0.5 mg/l NAA was 15 with maximum height of shoot was 7.5cm (Fig. - 74). Similarly on MS + 1.5 mg/l BAP+ 0.5 mg/l NAA the average no of shoot was 23 and maximum height of shoot was 9.5cm with rooting of callus (Fig.-75) and in the MS + 2 mg/l BAP +0.5 mg/l NAA the average no of shoot was 18 and maximum height of shoot was 7cm in 5 weeks of culture (Fig. - 76).

4.7 Acclimatizaton and *In-vivo* Rooting of *in-vitro* Multiplied Shoots

The 6 weeks old *in-vitro* multiplied shoots were taken for *in vivo* rooting (Fig.-77, 78, 79, 80). They were planted in the coco-pit bed with or without treating with IAA (3 mg/l) for 1 hour. They showed following response where observation was done after 1 week.

1) Shoots that were treated with IAA:

In this case the shoots started to root after 1 week. The percentage of rooting in 2 weeks was 90% while in 3 weeks of plantation 100% shoots were rooted. The average no of roots were 6 and root length was 5cm (Fig.-81,.82).

2) Shoots without treating with rooting hormone:

Here, the shoots started to root after 2 weeks. In the third week of plantation , only 75% of the shoots rooted. 5% of shoots was found died in this case. In the fourth week of plantation, altogether 90% shoots was found to be rooted. Rest 5% of shoots was found to be rooted after 6 weeks. The average no of roots in 3 weeks of plantation was 2.5 with root length of 2cm (Fig.-83).

Table 4.1

Response of Shoot-tip explants in MS medium with different concertrations of Kinetin in 4 weeks of culture

MS+Kinetin (Mg/L)	Number of shoots (mean±SE)	Response by Shoot tip	
		Length (cm) (Mean±SE)	Callus initiation
MS	1.00 ± 0	2.16± 0.08	***
0.5	3.00 ± 0.57	1.82 ± 0.35	+ +
1	1.66 ± 0.33	2.75 ± 0.25	++
1.5	3.00 ± 00	1.38 ± 0.13	+ +
2	2.66 ± 0.66	1.50 ± 0.21	+ +
2.5	4.33 ± 1.45	1.55 ± 0.14	+ +
3	1.33 ± 0.33	2.42 ± 0.075	+ +

Table 4.2

Response of Shoot-tip explants in MS medium with different concertrations of Kinetin in 8 weeks of culture

MS+Kinetin (Mg/L)	Number of shoots (mean ±SE)	Response by Shoot tip	
		Length (cm) (Mean±SE)	Callus Response originated at base of shoot tips
MS	1.00 ± 0	3.76 ± 0.14	__ __ __
0.5	3.00 ± 0.57	2.94 ± 0.40	Root
1	2.00 ± 0.57	4.05 ± 0.39	Plantlets
1.5	5.00 ± 1.00	3.36 ± 0.44	* * *
2	3.33 ± 0.66	2.00 ± 0.23	Root
2.5	5.33 ± 1.45	3.31 ± 0.27	* * *
3	2.33 ± 0.33	3.07 ± 0.36	Plantlets

Culture condition MS, 25±2^{0}C, 4 replicates, ***=no response, +=Callus initiation, SE=Standard Error

Table 4.3

Effect of different concentrations of BAP and NAA on shoot regeneration from nodal explants in 4 weeks of culture.

Hormone concentration in mg/l		Number of shoots (Mean±SE)	Length of shoot (cm) (Mean±SE)	Callus initiation in cut edges
BAP	NAA			
0	0	2.00±0	1.90±0.01	* * *
0.5	0	3.33±0.51	2.30±0.30	* * *
1	0	4.66±0.20	3.25±0.48	* * *
1.5	0	6.50±0.29	1.95±0.024	+
2	0	2.00±0.82	1.75±0.43	+
0.5	0.5	2.00±0.24	1.68±0.38	+ + +
1	0.5	2.66±0.37	2.13±0.35	+ + +
1.5	0.5	2.00±0.15	1.50±0.31	+ + +
2	0.5	3.66±0.57	0.86±0.07	+ + +
0	0.5	- - - -	- - - -	+ + +

Table 4.4

Effect of different concentrations of BAP and NAA on shoot regeneration from nodal explants in 8 weeks of culture.

Hormone concentration in mg/l		Number of shoots (Mean±SE)	Length of shoot (cm) (Mean±SE)	Callus response initiated at cut edges
BAP	NAA			
0	0	2.00±0	2.75±0.25	-----
0.5	0	6.33±0.42	4.52±0.33	-----
1	0	8.00±0.25	6.61±0.48	-----
1.5	0	12.00±0.36	7.46±0.62	* * *
2	0	16.00±1.10	2.64±0.10	* * *
0.5	0.5	3.66±0.70	4.40±0.34	Rooting
1	0.5	4.00±0.54	6.87±0.37	Rooting +Microshoots
1.5	0.5	6.00±0.85	3.95±0.20	Rooting +Microshoots
2	0.5	7.33±0.93	3.79±0.51	Microshoots
0	0.5	2.00±0	1.50±0.50	Rooting

Culture condition MS, 25±2^{0}C, 4 replicates, ***=no response, +=Callus initiation, SE=Standard Error

CHAPTER FIVE

DISCUSSION

The morphogenetic response varies with genotype, age, nature and culture condition. The results obtained from the culture of various explants and callus on their morphogenetic potentiality of the medicinal plant *R. serpentina* have been discussed on the basis of available relevant liteatures.

In the present study, leaf, node and root explants of *R. serpentina* were cultured on MS +2, 4-D (0-3 mg/l.) for callus induction. It was found that cut part of nodal explants started callusing within 7 days, leaf explants within 8 days & root explants within 9 days. The callus obtained from node was light green which turned brownish after 6 weeks. The callus obtained from root and leaf explants were white in color. The growth of callus was observed good that originated from root explants. However MS medium alone was not effective to induce callus. Similarly node and leaf explants produced callus in MS medium supplemented with BAP and NAA in different combinations.

Parveen (1978) reported callus formation of *Rauvolfia* leaf explants in the presence of 2, 4-D. Similarly, Bhatt (2004) obtained whitish compact callus mass from the leaf explants of *Oroxylum indicum* cultured in MS+BAP (3 mg/l) after four weeks. Chand (2006) produced green mass of amorphous callus from leaf explants of *Clinopodium umbrosum* in MS+BAP(1.5 mg/l)+NAA(0.5 mg/l). Chandra et. al. (2002) induced callus on *Flacourtia jangomas* in MS+2,4-D(2.0 mg/l) +BAP(0.5 mg/l). Shah (2002) induced callus in *Asparagus racesmoscus* from nodal explants in MS+BAP (1 mg/l) + NAA (0.5 mg/l). Shrestha (2000) also obtained callus from nodal explants on MS+BAP (1 mg/l) + NAA (0.5 mg/l) on *Neopicrorhiza scrophularifolia*. Uddin et. al. (2006) working on *Steria rebaudiana* produced callus from leaf and nodal segments in MS+2,4-D at 2,3,4 and 5 mg/l. The highest amount of callus was found in MS+0.5 mg/l 2,4-D and MS+5 mg/l gave the poorest callus. In my investigation also MS+0.5 mg/l 2,4-D gave the highest amount of callus and MS+3 mg/l 2, 4-D gave the poorest callus in primary culture. But opposite was the when callus originated was sub-cultured.

In the present study, the whitish callus obtained in presence of 2,4-D was sub-cultured. Sub-culture of callus was done on MS medium supplemented with BAP and NAA in combination and singly to observe the morphogenetic potentiality of callus. Similarly the callus was also sub-cultured on MS+2,4-D (0.5-3 mg/l). In this case the callus sub-cultured in 2,4-D showed only increase in mass where no change in the color of callus was observed. But the whitish callus sub-cultured on MS+BAP+NAA turned slightly greenish after 2 weeks. Rhizogenesis was observed from this callus but organogenesis from callus was not observed until 6 weeks of culture. On the other hand Rani et. al. (2003) obtained shoot from callus sub-culture of *Withania somnifera* on 2,4-D (2 mg/l)+Kn (0.2 mg/l). Similarly, Xu Tie fang et. al. (2005) obtained shoot from callus subculture in MS+2,4-D (9.1 μm)+6-BA (4.4 μm) that was originated in the same concentration of hormone on *Pinella ternate*.

In the present study, leaf was cultured in MS medium supplemented with BAP and NAA in cambinations and singly in various concentrations. No response was observed in MS medium alone and MS+BAP (0.5-2 mg/l). While in MS+BAP (2 mg/l)+NAA (0.5 mg/l) brownish callus was observed on cut end and lower surface of callus from which plantlets were arised within 4 weeks of culture. After that, the growth of callus was ceased.The average number of plantlets observed within 8 weeks were 7-9 in the same culture. In MS+BAP (0.5-1.5 mg/l)+NAA (0.5 mg/l) only vigorous rooting of greenish callus with increase growth was observed. Similar result was obtained by Azad et. al. (2005) in *Phellodendron amurense* by culturing leaf in MS+BAP (4.4 μm)+NAA (1.0 μm) after 4 weeks and cultured leaf was 10 days old. Leaf cultured in MS+IDZ (2.0 μm)+2,4-D or NAA (4.0 μm) produced only callus from cut marging in 3 weeks of incubation. Sivanesaer and Murigesan (2005) also regenerated plant from leaf explants of *Withania somnifera* in MS medium supplemented with different concentrations of auxins and cytokinin. Highest frequency of shoot buds was obtained at a concentration of 1.0 mg/l Kn. But in the present study, cytokinin alone was not effective for response in leaf explants. Similarly Chand (2006) obtained green callus from leaf explants which produced leafy shoot in MS+BAP (1 mg/l)+NAA(0.5 mg/l). In MS+BAP (2 mg/l)+NAA(0.5 mg/l), rhizogenesis was observed. Similarly, Agrawal and Subhan

(2003) induced multiple shoots of *Centella asiatica* from lamina explants excised from *in-vitro* raised shoots through callus phase on MS+Cytokinins. Amongst all the cytokinins, kinetin (10um) proved optimum for differentiating shoots. Govindaraju et. al. (2003) working on *Withania somnifera* also regenerated plants from callus culture of all the explants by culturing them in MS+BAP (0.5-2.5 mg/l) or in combination with IAA (0.5 mg/l) and NAA (0.5-3.0 mg/l) either alone or along with kinetic (0.5-1.0 mg/l). Chandra et. al. (2002) also achieved rooting from leaf explant of *Flacourtia jangomas* in MS+NAA (1.0 mg/l). Similarly Rajkarnikar et. al. (2004) also obtained multiple shoots from leaf explants of *Swertia ciliata* cultured in MS+BAP (1 mg/l)+NAA (0.01 mg/l).

In the present study, shoot tips cultured on MS+kinetin (1, 3 mg/l) produced callus at the base of shoot tip which produced plantlet. Shoot tips cultured in MS medium alone also produced root with slight elongation of shoot. Similarly, Shrestha (2000) obtained multiple roots on *Neopicrorhiza scrophulriphia* from shoot tip cultured in MS medium alone with the elongation of shoot. Rajkarnikar et. al. (2000) also observed micro-shoots in MS+BAP(1-3 mg/l)+NAA(0.1 mg/l) in *R. serpentina*.

Similarly, in the present study, nodal explants, cultured on MS medium supplemented with BAP (0-2 mg/l) and NAA (0-0.5 mg/l) in combination produced multiple shoots from nodal part without formation of callus. But from cut end, callusing was observed only in combination of BAP+NAA and NAA (0.5 mg/l) singly. The best shoot multiplication was found on MS+BAP (2 mg/l) in 8 weeks of culture while best shoot elongation was observed in BAP (1.5 mg/l). Sarkar et.al.(1996) induced multiple shoots from nodal segments and shoot apices of *R. serpentina* in MS medium containing 1.0 mg/l BAP+0.1 mg/l NAA. The difference may be due to use of little amount of auxin (0.1 mg/l NAA) used by Sakar which produced callus from cut part and presence of cytokinetin (1.0 mg/l BAP) differentiated multiple shoots. The difference may be due to difference of explants taken for investigation. Similar to the present investigation, callus was formed at the cut bases of the nodal explants, which produced shoots on further subculture on fresh media.

Poudel (2003) also obtained multiple shoot on *Mentha spicata* in MS+BAP (1 mg/l). Bhatt (2004) found MS + BAP(2 mg/l) best for multiple shoot proliferation in *Oroxylum indicum* and MS+BAP (1 mg/l) for elongation of multiple shoots. Devkota (2004) working on *Valeriana jatamansii* produced maximum shoot from nodal explants on MS+BAP (1 mg/l). Chand (2006) observed MS+BAP (1 mg/l) best for shoot multiplication using nodal explants of *in-vitro* grown plants in *Clinopodium umbrsum*. Similarly Piveira et. al. (2003) induced multiple shoots of *Anenopaegmg arvense* by using nodal segments as explants on MS+kinetin (4.4 μm). Ranjit et. al. (2000) also reported multiple shoots of *Foeniculum vulgare* on MS+BAP (1 mg/l).

In the present investigation, the 2 weeks old green callus originated from cut edges of nodal explants in MS+BAP (0.5-2 mg/l)+NAA (0.5 mg/l) were sub-cultured on the fresh media of same concentration. It was found that many normal and abnormal (thicker than the normal) plantlets were observed after 2 weeks of subculture. Rooting of callus was also observed. Similar results were found by Shah (2002) in *Asparagus racemoscus*. Shah (2002) obtained callus from *in-vitro* obtained nodal explants in BAP (1 mg/l)+NAA (0.5 mg/l) in cut edges when this callus sub-cultured on MS+BAP (1 mg/l)+NAA (1 mg/l), the callus differentiated into shoots as well as roots. The proliferated shoots were thicker than the normal. Shrestha(2006) also obtained callus from nodal explants of *Neopicrorhiza scrophularifolia* in MS+BAP (1 mg/l)+NAA (0.5 mg/l). Microshoots were initiated from this callus. Similarly, Chandra et. al. (2002) obtained shoot bud from nodal segment derived callus on MS+2,4-D (2.0 mg/l)+BAP (0.5 mg/l) after subculturing on the same concentration of fresh media in *Flacourtia jangomas*.

Regarding, the *in-vivo* rooting of *in-vitro* multiplied shoots using coco-pit as bed, in the present study, 100% rooting was observed on hormone treated (3 mg/l IAA for 1 hour) within 3 weeks while 90% rooting observed on shoots without treating hormones within 4 weeks. Similarly, Pereiva et. al. (2003) successfully acclimatized the *in-vitro* multiplied shoots of *Anenopaegmg arvense* and *Stellfeld exde* in soil without rooting *in-vitro* Dai Wenha et. al. (2005) also rooted Amethyst purple raspberry both *in-vitro* and *in-vivo* with or without IBA at 0 to 1.0 μm.

Joshi et. al. (2000) also rooted *in-vitro* multiplied shoots of *Elaeocarpus sphaericus* in non-sterile sand within 15 to 20 days in green house condition. Niroula and Saiju (2000) also rooted 90% microshoots of *Valeriana jatamansi* in non-sterile sand Rajkarnikar et. al. (2004) also rooted *in-vitro* multiplied shoots of *Cephaelis ipecacuantia* and *Swertia ciliata* in non-sterile sand within 2-3 weeks. The rooted plantlets were successfully established in field.

Similarly, Pokharel (2007) also developed roots and rhizome in *Valeriana jatamansi* in *in-vivo* conditon in cocopit within 4 months. After that they were successfully transferred to the field containing soil and sand in 3:1 ratio.

During the present investigation, the beds that contains plants were covered by constructing miniature green house using white polythene. Since *in-vitro* plants are in 100% humidity inside the culture tube, they have poorly developed epidermis. By covering with polyhouse, humidity can be maintained and loss of plants due to excessive evaporation can be checked. *In-vivo* rooting of *in-vitro* multiplied shoots, reduces the day involved in laboratory, the cost of medium, electricity and space in incubation room. This directly reduces the total cost of production of tissues culture plants.

CHAPTER SIX
SUMMARY

The morphogenetic response and totipotency of various parts like root, node, leaf and callus of *R. serpentina* was studied. *In-vivo* rooting of *in-vitro* multiplied shoots were done in coco-pit bed. The multiple shoots were obtained through shoot tips, node and callus culture.

The leaf explants had better response on callus formation than the root & the node explants as 100% response was observed when it was cultured in MS+2 ,4-D (0.5-3 mg/l). Hormone-free MS medium didn't show any response in callus inductioin. For primary callus induction MS+0.5 mg/l 2,4-D found better than MS+3 mg/l 2,4-D while the opposite was true for callus subculture. Similarly, 2,4-D originated callus on subculturing on MS+BAP (0-2 mg/l)+NAA (0-0.5 mg/l) showed only rhizogenesis and whitish callus turned to greenish until 6 weeks.

The leaf explants cultured on MS+BAP (0-2 mg/l) +NAA (0-0.5 mg/l) in singly and in combination produced greenish callus except in MS medium alone and MS medium with BAP singly. In MS+BAP (2 mg/l)+NAA(0.5 mg/l) plantlets were produced from cut edges with induction of brownish callus whose growth was not observed after plantlets induction. On other combination of hormone rooting of callus with white and red patches on greenish callus was observed.

The nodal explants produced multiple shoots. On hormone free MS medium, two plantlets with single rooting found. MS+BAP (2 mg/l) was effective for high number of shoot induction while MS+BAP (1.5 mg/l) was effective for elongation of shoot. The nodal explants cultured in MS+BAP+NAA at various concentrations produced callus from cut ends. Sub-culture of this callus on fresh media of same concentration produced multiple shoots with normal and thicker plants with rooting of callus.

The shoot-tips cultured in MS+kinetin (0.5-3 mg/l) also produced multiple shoots but the number of shoots was less than that produced in MS+BAP. The bases of shoot tip produced callus which produced plantlets on the same culture with normal growth.

In-vivo rooting of *in-vitro* multiplied shoots were done in coco-pit bed with or without hormone. The average number and length of roots was found better in hormone treated shoots than in untreated shoots. 100% rooting was observed in hormone treated shoots within 3 weeks while only 90% of shoots rooted within 4 weeks in non treated shoots.

CHAPTER SEVEN
CONCLUSION

From the present study of *R. serpentina* the following conclusions have been made:

The node, leaf and root explants started callusing within 7,8 and 9 days of inoculation on MS+2,4-D (0-3 mg/l) respectively.

1. The color and growth of callus was found different in different combinations of hormone. The color of callus that was produced on MS+2,4-D was white while MS+BAP+NAA in combination produced green callus. The calli in both cases were hard.
2. Primary callus induction was better in MS+0.5 mg/l 2.4-D while their growth was found better in MS+3 mg/l 2,4-D on further subcultures.
3. The callus produced in MS+2,4-D was not effective for organogenesis while the callus produced in MS+BAP+NAA was effective for organogenesis.
4. The leaf explants produced plantlets in MS+BAP (2 mg/l)+NAA (0.5 mg/l) while rooting was observed in other hormone concentrations used for present investigation.
5. MS+BAP (2 mg/l) and MS+BAP (1.5 mg/l) was found better for multiple shoot production and better shoot elongation respectively when node from *in-vitro* grown plants was used as explants.
6. The shoot tip cultured in MS+kinetin (0.5-3 mg/l) was not so effective for multiple shoot production while the bases of shoot tips produced callus which gave healthy plantlets in the same culture.
7. *In-vivo* rooting of *in-vitro* multiplied shoots can be done in coco-pit bed with or without hormone.

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