IN VITRO STUDY OF MEDICINAL PLANT (*RAUVOLFIA SERPENTINA* (L.) BENTH. EX. KURZ.)

A DISSERTATION

SUBMITTED TO CENTRAL DEPARTMENT OF BOTANY IN

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By:

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CERTIFICATE

This is to certify that the thesis entitled ***In vitro* Study of Medical Plant (*Rauvolfia serpentina* (L.) Benth. ex. Kurz.)** submitted by **Saraswoti Aryal** for the partial fulfillment of Master's Degree in Botany is based on the results of experiment carried out by her under my supervision. This thesis has not been previously submitted for any other degree.

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APPROVAL LETTER

The dissertation work submitted by Ms. Saraswoti Aryal entitled ***In vitro* Study of Medical Plant (*Rauvolfia serpentina* (L.) Benth. ex. Kurz.)** has been accepted as a partial fulfillment of M.Sc. in Botany.

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ABBREVIATIONS

2, 4-D	:	2, 4- Dichlorophenoxyacetic acid
BAP	:	6- Benzylaminopurine
BM	:	Basal Medium (Murashige and Skoog Medium 1962)
Cm	:	Centimeter
DPR	:	Department of Plant Resource
g/ l	:	Gram per liter]
IAA	:	Indole-3- Acetic Acid
IBA	:	Indole-3- Butyric acid
Kn	:	Kinetin
Mg/l	:	Milligram per liter
MS	:	Murashige and skoog's Medium (1962)
NAA	:	α- Nethaleneacetic Acid

CHAPTER ONE

INTRODUCTION

1.1 Background

Nepal is rich in biodiversity owing to the variation in physiographic structure and climate. The country boasts over 10,669 species of flora ranging from fungi to angiosperms (Hara and Williams, 1979) Hara et. al.1978, 1982, Chaudhary, 1998). The total number of flowering plants recorded in Nepal is 6501 including 5636 species, 206 sub-species, 599 varieties and 60 form (DPR, 2001) in which about 15% (i.e. about 1600 species) of plant species are considered to be medicinally important in Nepal (Shrestha et. al.2000).

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 living in and around the urban areas has access to modern medical facilities, the majority (80-85%) depend on traditional medicine. The use of these medicinal plants in traditional medical care in the rural areas of the country is an age-old practice with many of the ethnic groups having their own system of traditional and indigenous healing methods.

The continuous over exploration of these resources have accelerated its depletion in the wild population growth and expanding trade practices locally and internationally has further deteriorated the natural condition of this important natural resource. For this reason conservation and rational utilization of medicinal plants are considered to be current national key issues.

Tissue culture is the development of new plants in an artificial nutrient medium under aseptic conditions from an explant. The explant

can grow either as an unorganized mass of cells i.e. callus or can be differentiated into a plant. The explant is a small piece of plant used to start a culture such as embryos, seeds, stem, shoot tips, meristem, root tips, callus, single cells and pollen grains.

Plant tissue culture is an important aspect of biotechnology. It has great potential for rapid, large scale and true to type multiplication. Plant tissue culture has been investigated as an alternative means to produce industrially important natural products such as flavors, insecticides and drugs. Many medicinal plant species are disappearing due to rapid agricultural and urban development, deforestation and indiscriminate collection. Therefore successful application of plant tissue culture technology may help to conserve rare and endangered medicinal plants include regeneration of similar clones from the culture cells at high frequency with natural product in similar or higher quantities.

Plant tissue culture is presently of great interest to molecular biologists, plant breeders and industrialists. Tissue culture methods have been employed as an important aid to conventional methods of plant improvement. They have also been used as a tool for the propagation of genetically manipulated superior clones and for *ex-situ* conservation of valuable germplasm. It also helps in the production of disease free and disease resistance plants as well as in the synthesis of many important secondary metabolites.

1.2 Description of Plants

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

Common Name: Sarpagandha (Nep.), Sarpagandha (Sans.). Rauvolfia, Serpent wood (Eng.)

Family: Apocynaceae

Chromosome no. (s): 2n = 20, 22, 24, 44.

The plant *Rauvolfia Serpentina* (L.)Benth.ex.Kurz. is an erect, glabrous, perennial shrub attaining to 75 in height, tap root, tuberous, soft sometimes irregularly soft nodules, leaves arranged in whorls of 3 to 4 to 10 cm long and 5 cm broad gradually tapering into a short petiole bright green above and pale green beneath. Flowers are white pink with deep red peduncle around 1.5 cm long arranged in small clusters. Fruit drupe 5 cm in diameter, dark, purple or blackish when ripe.

Distribution

Rauvolfia Serpentina is widely distributed in the Sub-Himalayan trace from Punjab eastwards to Nepal, Sikkim, Asam, the lower hills of the Gangetic plains, Eastern and Western Ghats, Bhutan, East Pakistan, Ceylon, Burma, Malaya, Thailand and Java. It is generally found in moist deciduous forest at altitudes ranging from sea level to 1200m. The plants are more frequent under the shade of Shorea, Ficus, Terminalia, Cassia, Dalbergia and Mangifera.

Nepal: CE, alt: 100 to 900m

Conservation Satuts: Endangered (IUCN Category), under the forest act (1993) HMG banned this plant for its export. However by obtaining permission from the government its processed materials can be exported.

Part(s) used: Root bark

Important Biochemical Constituents: More than three dozen alkaloids contained in the root bark the most common ones are resrpine, rescinnamine and deserpidine serpentine ajmaline, ajmalicine etc.

Uses:

It has anti-hypertension and sedative properties. It is therefore used to treat high blood pressure and as tranquilizer. Also used for the relief of various central nervous system disorders both psychic and motor

including anxiety states excitement, maniacal behaviour associated with psychosis schizophrenia, insanity, insomnia and epilepsy. Extracts of the roots are valued for the treatment of intestinal disorders, particularly diarrhea and dysentery and also as anthelmintic. Mixed with other plants extracts they have been used in the treatment of cholera, colic and fever. The root was believed to stimulated uterine contraction and recommended for use in childbirth in difficult cases. The juice of the leaves has been used as a remedy for opacity of the cornea. (U.S.D., 1955, 1826, Schlittler in Manske, VIII 288, Mukerji, J. Sci. inductr. Res., 14 A (7), Suppl., I, Kirt. And Basu, II 1550).

1.3 Justification

Increase in population demand of the plant products along with illegal trade is causing the depletion of valuable of plants and natural habitat in our country. Systematic regeneration system for their conservation is lacking. In context of Nepal, commercial cultivation on large scale has not been practiced, only started at very low scale.

Many valuable plants species are being destroyed in nature without their proper use. If all the available plant resources could be used properly Nepal would develop its economic status a lot within few years. Due to the destruction of natural habitat by human encroachment, lack of knowledge and technology, many plant species are being destroyed day by day.

The plant taken for present investigation was *Rauvolfia serpentine* (L). Benth. ex kruz, which is most important medicinal plants cited in IUCN endangered, category. But the attention is not given for its proper improvement, management, conservation and utilization. Besides this causes due to over population of human beings, environmental degradation, over exploration, habitat destruction, introduction of exotic

species and collection of root part for medicinal purposes causes depletion of this plant from its natural habitat.

1.4 Objectives of the Study

R. serpentina can be propagated by conventional methods such as seeds, root cutting, root stumps and stem cuttings. But it is more susceptible to various fungus diseases such as leaf spot, root knot, leaf blotch which causes great loss of yields so for such an endangered plant species tissue culture method is an alternative method for its germplasm conservation and for rapid clone multiplication. With the consideration of above background the following objectives have been selected for the present study.

i. To determine best explant for callus induction e.g. stem, leaf, root etc.

ii. To find out best medium for regeneration of plantlets from the callus.

iii. To determine concentration of hormone best for shoot multiplication and elongation.

iv. To determine proper hormone and its concentration best for root multiplication and elongation.

v. To acclimatize the *in vitro* grown plants through callus and multiple shoots.

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 relevant literature has been mentioned here.

Harberiandt (1902) for the first time proposed the *in vitro* cultivation of plants and introduced the word totipotancy of plant cell. Lamprecht (1918), Knudson (1919), Nemec (1924) made many attempts to find suitable media and optimal conditions for growing organs tissue and cells excised from whole plants.

Takayuki et. al. (1979) obtained callus from leaves and stem of *Mentha Spicata* L. cultured on B_5 media supplemented with 2 ppm and 6 ppm 2, 4 –D, grown in dark at 25°C.

Jha et. Al (1981) reported that for the establishment of calli in umbelliferous species, different organs like petiole, stem segments, young ovaries, roots, immature seeds have been used. In *Apium graveelens*, calli were raised and maintained on MS medium having 2,4-D with kinetin. In *Foeniculum vulgare* BM with 2, 4-D. Kinetin and /or CM proved to be effective for the production of healthy calli, while in cumin, modified BM medium with various concentrations of 2, 4-D, Kinetin, NAA and BAP were used for induction and maintenance of calli. The same medium was suitable for morphogenesis calli were raised from different parts of 15 days old aseptic calli grown cumin seedlings. Hypocotyl, leaf and root segments showed distinct and different responses for the induction of calli.

Karki and Rajbhandary (1983) observed plant regeneration from leaf cultures of *Solanum lanciniatum* Ait on MS medium supplemented with cytokines. The proliferation potential of the culture was maintained

for 5 years by regular sub-cultures. The proliferated shoots were rooted in half concentration of MS media with growth hormones (IBA, NAA).

Kitamura et. al. (1989) observes plants regeneration from the callus of *swertia pseudochinesis* Hara. The callus formation was observed in the seedling cultured on MS media with 0.1 to 10 mg/l NAA with or without BAP. Gurung and Rajbhandary (1989) obtained plantlets from the shoot tips and young leaves excised from traces of *Eucalyptus camaldulensis*. Shoot bud formation was achieved on MS media supplemented with 100 mg/l and casein hydrolysate BAP and NAA.

Banthorpe and Brown (1989) obtained the callus of *Mentha spicata* L. by culturing with 10% coconut milk, 2, 4-D (6mg/l) the callus subculture at periods of 3-4 weeks, on MS media with agar (2-4 g per 100 ml medium) yielded viable and fine cell suspensions.

Roy and Sarker (1991) studied rapid propagation by the formation of shoot calli of *Aloe vera*. Callus formation was induced in stem segments of young axillary shoots grown on underground rhizomatous stem.

Cacho et. al. (1991) studied morphogenesis from leaf hypocotyls and root explants of *Digitalis thapsi* L. The explants could from calli even on the hormones free medium. Addition of 2, 4-D, NAA or BA on the medium increased the growth rate of calli NAA induced root and BA induced shoot formation BA, IAA and Kn combinations were more effective in inducing organogenesis in leaf explants.

Cellarova and Honcariv (1991) studied the morphogenetic capacity of leaf callus of *Digitalis purpurea*. They observed that the growth of callus was fast in the presence of exogenously applied auxin and cytokine in the ratio of 2:1.

Shrestha and Joshi (1991) cultured stem explants of *Swertia chirata* (Roxb. ex. Fleming) Karsten. obtained from *in vitro* growth plants on Murashige and Skoog basal medium produced the calli on MS + 2, 4-D (5 mg/l) + BAP (5 mg/l). These calli when sub cultured on MS + BAP (5 mg/l) produced numerous embryoids and shoot buds. Numerous roots were differentiated in these shoot buds when they were transferred to MS medium supplemented with IAA (5 mg/l).

Varghese et. al. (1992) studied the formation and differentiation of *Vitex negundo*. The best callus formation obtained in short explants on MS medium supplemented with 2,4-D and Kn. Adventitious root formation and callus initiation were observed on MS medium with NAA. The shoot buds developed from nodal explants were found to be maximum in medium supplemented with low concentration of Kn and high concentration of BAP.

Khadka (1993) reported that the leaf explants and anther of *Digitalis purpurea* when cultured on MS medium containing different hormone combinations the leaf explants produced callus on MS + 2,4-D + IAA (1 ppm each) and MS + 2, 4-D + Kn (1 Mg/l each) but it could not differentiate shoot buds and roots while another produced callus on BM + 2, 4-D 5 mg/l and BM + 2, 4-D + Kn (5mg/l each).

Purohit et. al. (1994) achieved *in vitro* clonal multiplication of 'safed musli' (*Chlorophytem borivilianum*) a medicinal herb on MS medium supplemented with 22.2 micro M BAP using young shoots. All shoots produced roots on ¾ strength MS medium containing 9.8 µ M IBA. 60 % can be surviving on pots and soil. Multiplication was also achieved through somatic embryogenesis.

Kharel and Karki (1994) cultured the shoot tips of *chrysanathemum morifolium* variable "Gaint fishtail violet" on MS medium supplemented with BAP 1.0 mg/l and NAA 0.01 mg/l. Mircorshoots were developed

successfully rooted in sand and grown into normal plants. Shreatha and Joshi (1999) investigated the root, leaf, nodal part, shoot tips of *Guizotia abyssinica* produced callus in MS medium, however, proliferation of the explants into callus was more effective in the presence of hormones in the medium. Culture of shoot tips in BM + BAP (3 ppm) + NAA (0.1 ppm) showed the maximum number of shoot multiplication. Excised shoots differentiated roots half strength MS liquid medium containing IBA (1 ppm). The plantlets raised were acclimatized.

Krishnan and Seen (1994) regenerated shoots in scheme and Hilderandt (1972) medium from field grow flowering plant of *Woodfordia fruiticosa*. A medium supplement of 6 Benzylaminopumne (0.2 mg/l) induced high frequency (88%) development of auxiliary shoot buds (3.2) in 4-5 weeks. Subculture of the explants with multiple new shoots in fresh medium for 30 days yielded an even larger number (9.7) of shoots. Highest multiplication (26-35 shoots) was recorded when using culture initiation media with 0.5 mg/l each of BAP and NAA followed by subculture in 0.2 mg/l BAP. The shoot multiplication rate was further accelerated by reculturing 0.4 – 0.6 cm nodal segments of regenerated shoots in media with 1.0 mg/l BAP. Shoot cuttings (3.5-7.0 cm) were rooted in 0.2 mg/l IAA regenerated plants.

Pant et.al. (1995) induced multiple shows from one 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% gelrite. Subsequent transfer of the regenerated shoots on MS media supplemented with NAE 10^{-7} M with BAP 10^{-7} M resulted in root formation.

Pant et.al. (1996) induced multiple shoots from the apical domes of shoot tip of *Cnidium officinle* Makino (Apiaceae) by culturing them on MS media solidified with 0.2% gelrite and supplemented with BAP 10^{-7}

M. An average of 5.3 shoots per segment was obtained within 6 weeks and this ability did not decline even after two years of sub culture. Subsequent transfer of these regenerated shoots on MS media supplemented with NAA 10^{-7} M and BAP 10^{-7} M resulted in root formation. Rooted plantlets were able to grow in soil after a shoot period of acclimatization.

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

Twyford and Man tell (1996) studied morphologically competent cell clusters that were induced from root segment excised from *in vitro* shoot cultures of the *Dioscorea alata* L. The explants were planted in a liquid modified MS medium supplemented with 2, 4-D. Embryogenic cell clusters released from subtropical region of the root explants proliferated further into proembryonic cell masses when transferred to auxin free medium differentiated into somatic embryos. Vishnoi et.al. (1996) Cultured immature inflorescence on MS medium supplemented with 2,4-D ,Picloram or NAA in combination with Kn. Formation of embryos was observed on embroygenic callus on transfer to hormone free MS medium. Calli gave rise to plantlets at varying frequency.

Nayak et.al. (1998) Developed a standard protocol for rapid propagation of *Asparagus robustus* from callus has been developed callusing and regeneration was maximum using segment of shoot tissue. Basal medium supplemented with 3 mg 2, 4-D and 1 mg Kn were most effective in formation of callus. Short formation was optimum on medium

containing 0.1 mg/l NAA, 1 mg BAP and 40 mg adenine sulphate. Root induction was maximized in half strength MS basal medium with 0.5 mg IBA. The number and length of roots are greater under an 8 hours photoperiod as compared with a 16 hrs photoperiod.

Ranjit (1999) obtained callus from the different explant of *Rhus parviflora* on different concentration of growth hormones but in *Bauhinia variegate,* the nodal explant gave multiple shoot on MS media supplemented with BAP 1ppm and NAA 0.5 ppm while leaf, root and stem explant could not give any response.

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

Niroula (1999) regenerated the shoots from the aseptically germinated seedlings of *Rheum emodi* wall as an explant when cultured in MS medium supplemented with BAP 1 mg/l and NAA 0.1 mg/l. The roots were initiated when the micro shoots were transferred in non-sterile stand.

Wakhlu and Sharma (1999) developed a protocol for Micropropagation of *Heracleum candicans* by auxiliary shoot proliferation. Maximum shoot proliferation was obtained on MS medium supplemented with 0.5 mg/l 6-Benzyl adenine and 0.1 mg/l nepthaleneacetic acid. Regenerated shoots were rooted on MS medium fortified with 1 mg/l IBA, complete plants were transferred to soil and all of these plants were morphologically and cytologically identical to the mother plants. Erated shoots were rooted on MS medium fortified with 1

mg/.l IBA, complete plants were transferred to soil and all of these plants were morphologically and cytologically identical to the mother plants.

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

Huang et.al. (2000) cultivated shoot tip, leaf, inflorescence and node explants of *Lomonium wrigntii* (Hance.) Ktze, an ethno medicinal plant on MS media supplemented with 8.87 μm BA and 1.07 μm NAA and obtained induction of adventitious shoots from the shoot tip, inflorescence, node and middle and basal parts of leaf explants after 60 days of culture.

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.) by enhanced auxiliary bud proliferation in nodal segments isolated from mature plants. Although bud break was dependent on BA supply the synergistic combination of 22.2 μM BA and 2.68 μM NAA induced the optimum frequency (91%) of shoot formation as well as shoot number (4-5 shoots/node) sub-culturing nodal segments harvested from the *In vitro* derived axenic shoots on the multiplication medium supplemented with 6.7 μm BA and 2.88 μm IAA was found most suitable for short elongation. Rooting was highest (90%) on full strength. MS medium containing 2.46 μm BA micropropagated plants established in garden soil were uniform and identical to the donor plants with respects to growth characteristics.

Joshi et. al. (2000) observed the nodal cutting of *Elaeocarpus Sphaericus* (Gaerth.) K. Schum was appropriate in liquid MS medium.

Otherwise there was a great possibility of necrotic exudation of phenolic compound. The established green explants were then inoculated in MS medium with the combination of different concentration of BAP and NAA as well as in MS medium with different concentration of BAP alone. It was tried in MS medium with less ammonium nitrate (300 mg/l). The MS medium supplemented with BAP 0.5 gm/l and NAA 0.01 mg/l as well as in MS medium with different concentration of BAP alone. It was also tried in MS medium with less ammonium nitrate (300 mg/l). The MS medium supplemented with BAP 0.5 gm/l and NAA 0.01 mg/l as well as in MS medium with BAP 0.25 mg/l were observed good for proliferation of micro shoots. 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 micros shoots develop roots successfully in non-sterile sand within 15 to 20 days in house condition.

Kayastha (2000) culture the shoot tips of two months old plant of *Swertia chirata* (wall) C.B. Clarke was cultured in Murashige and Skoog (MS) medium supplemented with 1 mg/l of 6-benzyl amino urine (BAP) and 0.01 mg/l of Naphthalene acetic acid (NAA) for multiplication. Ten-fifteen micro shoots were developed after 4^{th} subculture. These micro shoots were transferred in non-sterile sand for rooting. The roots were developed within two to three weeks. The rooted plants have been established successfully in the field.

Joshi et. al. (2000) observed liquid MS medium and the choice of explants as nodal cutting (from 2 to 5 yrs. plants) was appropriate for *Eleocarpus sphacricus*. The established green plants were then inoculated in MS medium with the combination of different concentration of BAP and NAA as well as in MS medium with different concentration of BAP alone.

Niroula and Saiju (2000) regenerated multiple shoots from the young shoot tips of *Valeriana jatamansii* in Murashige and Skoog medium supplemented with Benzyl Amino Purine (BAP) 1 mg/l and Naphthalene Acetic Acid (NAA) 0.1 mg/l 90% micro shoots rooted in non sterile sand and these plants were successfully established in the field.

Rajkarnikar et.al. (2000) obtained the explants *Rauvolfia serpentina* L. Benth. ex. Kurz. response in all concentration of auxin and cytokine initially (up to 1-2 week). After 3 to 4 weeks the MS medium with BAP 1 mg/l , 2 mg/l, 3 mg/l along with 0.1 mg/l NAA showed micro shoots sprouting from the base of explant but the medium with BAP 3 mg/l +0.1 mg/l NAA was seen to be best for micro-shoots sprouting little bit sooner than other medium. The sprouting micro-shoots again sub cultured in various concentration of hormone were present one. It shows not so difference in BAP 1 mg/l and 2 mg/l. In higher concentration of BAP and NAA callusing occurred at the base of explants. The micro-shoots were regularly sub cultured in the best medium i.e. BAP 1 mg/l and NAA 0.1 mg/l at interval of 5-6 weeks. After 7 sub-cultures, the multiplication rate of micro shoots were increased upto 12-16 from individual shoot.

Ranjit et.al.(2000) reported that multiple shoots were induced from nodal explants of *Foeniculum vulgare* on MS medium supplemented with BAP (1 ppm) An average of six shoots was developed from a single shoot after 4 weeks of culture roots were developed on shoots on MS medium supplemented with NAA (2 ppm).

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 on 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 on MS medium supplemented with IBA/Kn/BAP at various concentrations.

Bhuya et.al. (2001) studied regeneration of an important medicinal plant *Wedelia chinensis* Menill. (Family Asteraceae) on MS medium supplemented with Kn (2 mg/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 BAP and Kn in MS medium induced the formation of undifferentiated globular structures which on transfer to MS medium with kinetin (Kn) and IAA produced differentiated shoots.

Ray and Jha (2001) produced multiple shoot from the single shoot tip explant of *Withania somnifera* grown on MS medium supplemented with 0.1 ppm BA.

Chandra et al (2002) obtained shoot bud from nodal segmentderived callus tissue of *Flacourtia jangomas* (Lour) Raeusch, a woody medicinal plant of dioecious habit. Induction of callus was obtained in MS basal medium supplemented with 2.0 mg/l 2, 4-D and 0.5 mg/l BAP. Highest number of shoot bud (7.4 $\pm$ 0.20) was noted in 2.0 mg/l BAP.

Ranjit et. al. (2002) induced shoots from nodal explants of *Foeniculum vulgare* on MS medium supplemented in BAP 1 ppm and NAA 0.5 ppm after for weeks of culture. Averages of six shoots were developed from single shoot after from weeks of culture. The multiple shoot formation has not been declined after one year of subculture. Nodal explants were taken from *in vitro* germinated plantlets as hormones free MS medium. Roots were developed on shoots on MS medium supplemented with NAA 2 ppm.

Anand et. al. (2002). achieved *Adhatoda vasica* Nees by culturing excised nodes on MS and B_5. Media supplemented with Kinetin (4.65 μm) & naphthalene acetic acid (2.69 μm). The micro shoots rooted in half strength B_5 Plantlets were successfully soil transferred.

Bais et. al. (2002) developed a clonal propagation protocol of *Spilanthes mauritiana* DC. Where Juvenile plants uses as starting material. The addition of benzylaminopurine BA (0.1 μm) and napthalencacetic acid NAA (0.1μm) to the culture medium resulted in maximum shooting response with minimal callusing. Shoot rooted best *in vitro* in MS medium supplemented with Indole – 3 – acetic acid (IAA) 0.2 μm and plants that had already developed roots showed better growth with maximum survival rate in the greenhouse after an initial hardening.

Pereira et. al. (2003) induced multiple shoots of *Anenopaegmg arvense* (vell.) stellfeld ex de Souza, an endangered medicinal plant by using nodal segments as explants on MS media supplemented with 4.4 μm of Kinetin Acclimatization of un-rooted plants into soil was successfully achieved.

Joshi et al. (2003) induced multiple shoots from nodal explants of *Foeniculum vulgare* Mill. on Murashige and Skoog (MS) medium supplemented with 1 ppm benzylaminopurine (BAP) and 0.5 ppm naphthalene acetic acid (NAA). For this experiment the nodal explants were taken from *in vitro* germinated plantlets on hormone free MS medium. Roots were developed in ½ strength MS liquid medium supplemented with 1 ppm NAA.

Koroch et. al. (2003) developed a method for the induction of adventitious shoots from leaf tissue of *Echinacea pallida* with subsequent whole plant regeneration proliferating callus and shoot culture were derived from leaf tissue explants placed on Murashige and Skoog medium

supplemented with 6-Benzylaminopurine and Napthaleneacetic acid combinations. The optimum shoot regeneration frequency (63% and number of shoots per explant was 2.3) was achieved using media supplemented with 26.6 μm 6-benzyl aminopurine and 0.11 μm NAA. Rooting of regenerated shoot explants was successful on MS medium both with and without the addition of indole 3-butyric acid. All plantlets survived acclimatization producing phenotypically normal plants in the green house. This study demonstrates that leaf tissue of *E. Pallida* is competent for adventitious shoot regeneration and establishes a useful method for the micro-propagation of this important medicinal plant.

Govindaraju et. al. (2003) developed a high frequency and rapid regeneration protocol via callus and directly from various explants were developed in *Withania somnifera* (L.) Dunal Callus was initiated from inter nodal segments, leaf, root and petiole explant 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 alone with kinetin (0.5-1.0 mg/l). Regeneration was observed from callus of all the except root on MS medium fortified with BAP (0.5-2.5 mg/l) or in combination with IAA (0.5 mg/l). Direct differentiation of multiple shoots from leaf nodal segments and shoot tips occurred within two weeks on MS medium supplemented with BAP (0.5 - 3.0 mg/l) in combination with IAA (0.5 mg/l). The number and height of the shoots per explant varied with different concentration of BAP with low concentration of IAA. Shoots that were dwarf were elongated on MS medium fortified with GA_3 (0.5 mg/l). These plantlets were than rooted successfully in half strength MS media (both liquid and solid) with IBA (0.5-1.0 mg/l) alone or along with IAA (0.5 mg/l) Plantlets were hardened for two weeks and successfully transferred to field with 80-85% survival.

Agrawal and Subhan (2003) induced multiple shoots of *Centella asiatica* (linn.) urban from lamina explants excised from *in vitro* raised shoots through callus phase on Murashige and Skoog (MS) medium

supplemented with cytokine alone and in combinations. Amongst all the cytokinins namely N^6-benzyl adenine (BA), Kinetic (Kn), N^6-2-isopentenyl adenine (2 ip) and zeatin tried in various concentrations (0.1, 1, 5, 10, 20, 30 μm) 10μm Kn proved optimum per differentiating an average of 15.25 ± 4.72 shoots in 75% cultures within 6 weeks. Excised shoots rooted best in half strength MS adjuvant with 1μm indole – 3-butyric acid (IBA) within 15 days of transfer. Nearly 95.83% shoots organized an average of 6.33 ± 0.67 roots per shoot. The tissue culture derived plantlets have been successfully transferred to field.

Tiwari et. al. (2003) cultured nodal sections comprising of auxiliary buds of *Rauvolfia serpentine* to test their growth performance and regeneration potential on three different basal nutrient medium viz. Murashige and Skoog (MS) Gamborg's (B_5) and White's (Wh) medium without growth hormones and with 3.0 mg/l 6-BAP and 0.5 mg/l α-NAA denoted as MSBN, B_5BN and WhBN respectively constituting overall six shoot induction media. Maximum multiple shoot regeneration was observed on B_5BN as compared to other five media. Maximum multiple shoot regeneration was observed on B_5 BN as compared to other five media on MSBN medium the regenerated shoots attained maximum length however the callus formation was less than that on B_5 BN. Maximum shoots with optimal nodes were obtained on medium B5BN. However after 30 days of initial culture the shoot length was highest on MS BN, over all four to five fold improvements was observed for multiple shoot production. *in vitro* with B_5BN medium and two to three fold enhancements on MSBN as compared to WhBN. *in vitro* rooting was obtained efficiency prior to transplanting.

Rani et. al. (2003) observed callus induction from hypocotyl, root and cotyledonary leaf segments of *Withania somnifera* (L.) Dunal grown on Murashige and Skoog (MS) medium supplemented with various concentration and combinations of 2, 4-D and Kinetin (Kn). 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 when sub cultured in the same medium showed profuse callusing. However, these calluses remained recalcitrant to regenerate regardless of the quality and combinations of plant growth regulators in the nutrient pool. When hypocotyl segments were used as explants, callus induction was noticed in 91% of cultures, which showed shoot regeneration on MS medium supplemented with 2mg/l 2, 4-D and 0.2 mg/l Kn. These shoots were transferred to fresh medium containing various concentrations and combinations of 6-Benzyladenine (BA) and combinations of 6-Benzyladenine (BA) and N^6 -2-isopentenyl adenosine (2-ip). Maximum shoot multiplication was observed after 60 d of the second subculture on MS medium containing 2 mg/l (9.9 μm) indole 3-butyric acid (IBA). The plantlets were transferred to the field after acclimatization and showed 60% survival.

Maruthi et. al. (2004) was established *in vitro* regeneration protocol through the stem callus culture of *Celastrus peaniculatus* willd. The explants induced callus on LS medium supplemented 2% of fructose, 6μm Kn and 1 μm IBA. Interaction of the same hormones at lower concentrations (1.5 to 3.5 μm Kn and 0.1 to 0.4 μm IBA). The micro shoots rooted on LS 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.

Joshi and Purohit (2004) achieved *in vitro* propagation protocol of rare Indian medicinal herb "safed musci" (*Chlorophytum borivilianum*) was achieved using encapsulated shoot buds. 4 mm long shoot buds encapsulated in 3.0% sodium alginate matrix polymerized by 100 μm solution of $CaCl_2.2\ H_2O$ yielded best results. Storage conditions gel

matrix media and period in storage influenced the *in vitro* regrowth potential of shoot bud when transferred on standard multiplication (SM) medium containing MS salts and 5.0 mg/l BAP. Encapsulated shoot buds stored on wet agar-gel and kept under culture room conditions of light and temperature (45μ mol m^{-2} S^{-1} and 25 ± 2°C respectively) showed more than 80% sprouting within 3 weeks. Dark stored (4°C) encapsulated shoot buds on agarglled wet medium showed more than 90% sprouting after 7 days of storage. The percent response in terms of regrowth potential declined to 60% when buds were stored for more than 3 day attaining a low of 20% in another 30 days supplementing alginate matrix with sucrose and MS salts (AL_{MSS}) produced better results on compared to that with MS salts alone (AL_{MSS}) or without any of them (AL_W). All the sprouted shoot buds irrespective of their storage condition, produced normal shoots on SM and multiplied at a rate of 2.5 – 70% micro-s hoots could be rooted on the medium containing ¾ MS salts and 2.0 mg/l BA. Rooted plantlets were hardened successfully and growth under green house conditions where they produced normal tuberous roots. This method of Micropropagation opens possibilities for storage of shoot buds during off-season of production and facilitates transport of germplasm with ease.

Tiwari et. al. (2004) inoculated nodal segments of *Pterocarpus marsupium* Roxb. on seven different media compositions viz MS, B_5 and white's without growth hormones ($MS_{00,}$ B_500 and WH_{00}) each supplemented with 3.0 mg/l IBAC (MSIB). Seed germination improved in all the media studied however, MS combination were the best (95-100%) maximum nodes per shoot let were observed in medium MSIB (4.95) while shoot length was maximum in MSIB (2.92 cm) followed by MS_{00} (2.41). Regenerated plants were acclimatized and successfully transferred under field conditions.

Chand et. al. (2004) developed a protocol for callus induction, regeneration and micropropagation of *Ocimum basilicum* (lamiaceae). Callus initiation from the leaf and internodal explants were observed after 15 days from the cut faces of the explants inoculated on MS basal media supplemented with 2 mg/l 2, 4-D and 1 mg/l kinetin. Various types of calli formed viz. white friable, compact and nodular calli were observed from the nodal explants auxiliary bud break was observed and the buds developed into shoot lets used for micro propagation, apical buds showed minimum response where as the nodal explants exhibited maximum response. Bud break (90%) with callusing was observed. From nodal explants where as apical buds showed a slow growth and some callusing of the two cytokinins used both BAP (2 mg/l) and kinetin (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 where the BAP supplementation with 2 mg/l and kinetin concentration was 1.5 mg/l. NAA (3 mg/l) was found to be more suitable for rootlet formation.

RajKarnikar and Bhatt (2004) culture the node and leaf explants of *Azadirachta indica* A. Juss. Tree, the node explant responded in MS medium supplemented with 0.25 mg/l BAP, 0.01 mg/l NAA 0.5 mg/l charcoals and 50 mg/l Adenine sulphate as most suitable for the regeneration of multiple shoots. In *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 micro shoots of *Cephaelis ipecacuanta* from *in vitro* regenerated node segments in MS medium supplemented with 2 mg/l BAP and 0.1 mg/l NAA. These micro 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) cultured the explants (1-2 mm shoot tips and leaves) of *Swertia ciliate* were cultured in MS medium medium supplemented with 1 mg/l BAP and 0.01 mg/l NAA for multiplication of micro shoots. The developed micro shoots were transferred in non-sterile sands for rooting. The roots were initiated within 2 to 3 weeks of transplantation. The rooted plants were established successfully in field.

Rajkarnikar and Bhatt (2004) obtained the regeneration of micro shoots of *Neopicrorhiza scrophulariiforia* (Pennell) Hong from shoot tip explants was found to be best in 0.8% agar solidified MS medium (Murashige and Skoog's, 1962) supplemented with 0.5 mg/l BAP 0.01 mg/l NAA and 10% coconut water. The micro shoots regenerated roots on MS medium with 0.5 mg/l or with 0.1 mg/l NAA. The experiment is still in investigation for successful establishment of plantlets in the filed.

Bhatt (2004) obtained multiple shoots from nodal explants and plantlets grown on MS medium of *Oroxylum indicum* supplemented with BAP.

Liao et. al. (2004) established a rapid Micropropagation protocol for *Aloe vera* L. Var. Chinensis (Haw) Berger. The effects of three factors namely BA, NAA and sucrose on bud initiation were evaluated by L9 (3^4) orthogonal design. Among the three factors sucrose was the most important for bud initiation followed by BA and NAA has the weakest effect. The best medium for bud initiation was semi-solid MS supplemented with 2.0 mg/l BA and 0.3 mg/l NAA on which Chinese Aloe could multiply 15 times in 4 weeks. Some shoots rooted spontaneously on ½ strength MS medium but the rooting percent was improved in the presence of 0.2 mg/l NAA. Rooted plants were acclimatized to green house conditions. The young plantlets from tissue culture were transplanted successfully.

Das et. al. (2005) have been formulated a successful protocol for *in vitro* regeneration of *Vitex negundo* (Verbenaceae) from nodal explants. Plant lets were directly regenerated from the nodal explants on MS medium supplemented with different concentrations of BAP, NAA and Kn multiple shoots were developed on MS medium supplemented with 1mg/l BAP. Roots were initiated within 15 days on half strength MS medium supplemented with 0.1 mg/l IBA. *in vitro* flower heads were also noticed in the matured plantlets in the same medium. The rooted plantlets wee successfully established in the field.

Jain and Chaturvedi (2005) plant regeneration was achieved is a multiple shoots in *Hyptis suaveolens* (Labiatae). The apical buds of the three days old seedlings could be stimulated to undergo multiple shoot formation on Murashige and Skoog's medium supplemented with 1 mg/l BAP + 1 mg/l Kn + 0.2 mg/l NAA. The multiple shoots then separated and cultured in Erlenmeyer flasks containing same fresh medium. On the same media rhizogenesis was noticed after 15 days of culture initiation. The regenerated shows yield essential oil as the intact plant. The plantlet subsequently transferred to soil and kept in green house conditions.

Sivanesaer and Murugesan (2005) were developed an efficient protocol for high frequency plant regeneration from leaf explants of *Withania somnifera* (Ashwagandha) on Murashige and skoog medium supplemented with different concentrations of auxin and cytokines. Frequency of shoot bud regeneration varied with dose of plant growth regulators in the medium. Highest frequency of shoot buds was obtained at a concentration of 1.0 mg/l Kn. *in vitro* rooting of micro shoots were obtained by growing them in half strength MS medium supplemented with 2.0 mg/l IBA. Rooted plantlets were successfully transferred to the field after acclimatization in the net house.

Azad et. al. (2005) has been achieved shoot organogenesis and plant establishment for *phellodendron amurense* Rupr. from excised leaf explants. Young leaf explants were collected from *in vitro* established shoot culture and used for the induction of direct shoot regeneration callus and subsequent differentiation into shoots on MS medium. Direct shoot regeneration was achieved by culturing 1 cm^2 sections of about 10-day-old leaves on MS medium enriched with 4.4 μm BAP and 1.0 μm NAA after 4 weeks of culture. The leaf explants produced callus from their cut margins within 3 weeks of incubation on medium supplemented with 2.0 μm TDZ and 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 NAA. The highest rate of shoot multiplication was achieved at the third subculture and more than 65 shoots were produced per callus clamp. For rooting the *in vitro* proliferated and elongated shoots were excised into 2-4 cm long micro-cuttings which are planted individually on a root induction MS medium containing 2.0 μm IBA with in 3 weeks of transfer to the rooting medium all the cultured micro cuttings produced 2-4 roots. The *in vitro* regenerated plantlets were transferred to kanuma soil and the survival rate ex *vitro* was 90%.

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 ppm BAP + 1 ppm NAA while for root explants was MS + 2 ppm BAP + 1 ppm NAA. Nodal explants were most suitable for shoot multiplication on MS + 1 ppm BAP. For *in vitro* rooting of shoots MS medium supplemented with 1 ppm NAA was found most effective.

CHAPTER THREE

MATERIALS AND METHODOLOGY

3.1 Materials

The present investigation was carried out in Central Department of Botany, Tribhuvan University Kathmandu, Nepal.

The plant materials used for present investigations were *in vitro* grown plants of Rauvolfia serpentine (L) Benth. ex. Kurz. from the tissue culture room of CDB.

Stock	**Constituents**	**Quantity (mg/l)**
A. Macronutrients	Potassium nitrate (KNO_3) Ammonium nitrate (NH_4NO_3) Calcium chloride ($CaCl_2.2H_2O$) Magnesium sulphate ($MgSO_4.7H_2O$) Potassium sulphate (KH_2PO_4)	 1900.00 1650.00 440.00 370.00 170.00
B.Micronutrients	Manganese sulphate ($MnSO_4.4H_2O$) Zinc sulphate ($ZnSO_4.4H_2O$) Boric acid (H_3BO_3) Potassium iodide (KI) Sodium molybdate ($Na_2MO_4.2H_2O$) Copper sulphate ($CuSO_4.5H_2O$) Cobalt chloride ($CoCl_2.6H_2O$) *KI was prepared and stored separately	**mg /100ml** 20.300 8.600 6.200 0.830 0.250 0.025 0.025
C.Iron source	Sodium EDTA (Na_2-EDTA) Ferrous sulphate($FeSo_4.7H_2O$)	**mg/100ml** 273 278
D. Vitamins and amino acids	Glycine Nicotinic acid Pyridoxine HCL Thiamine HCL Myo-inositol * Myo- inositol was added freshly.	**mg/100ml** 200 50 50 10 100
Carbon source	Sucrose	3%(**w/v)**
Solidifying agent	Agar (Bacto agar)	0.8%(**w/v)**

3.2 Methodology

For the investigation MS medium (Murashige and skoog, 1962) was used as basal medium (BM) Supplemented with different growth regulator in different concentrations. One liter of MS medium consists of following chemical composition.

3.2.1. Preparation of Stock Solution

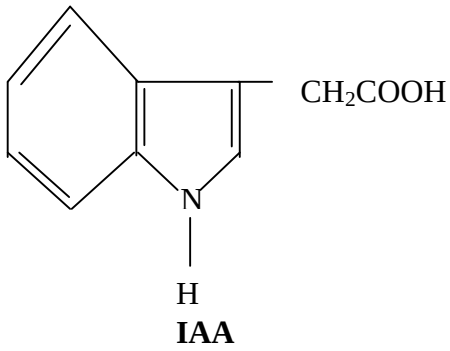
Above mentioned constituents were weight accurately using digital balances. For the preparation of stock A "Macronutrients" the weight chemicals were dissolved completely one by one separately in sterile distilled water in one liter conical flask and final volume was made one liter by adding sterile distilled water. To dissolve the chemical completely, the solution was stirred by magnetic stirred.

Same processes were repeated for the preparation of stock B "Micronutrients" Stock C" Iron source" and stock D "Vitamins amino acids" but the final volume was adjusted to 100ml for each of the stock. All prepared stock solution were stored in clean oven dried brown bottle with labeling and preserved in refrigerator at 4^{o}c. Myo- inositol was generally added freshly.

3.3. Hormone Used for investigation

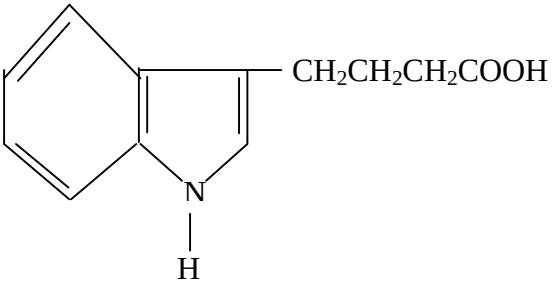
Auxins:

i. Indole-3-acetic acid



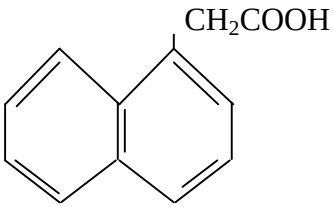
IAA

ii. Indole-Butyric acid



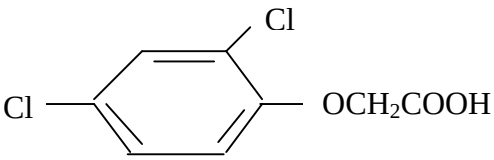
IBA

iii. Naphthalene acetic acid



NAA

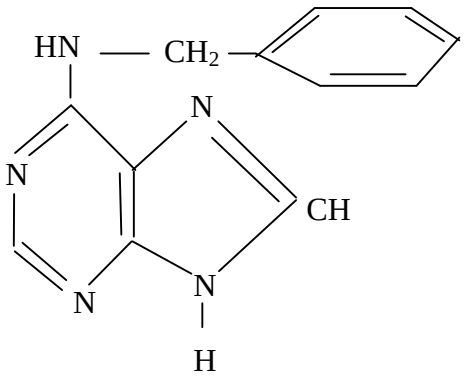
iv. 2,4-Dichlorophenoxy acetic acid



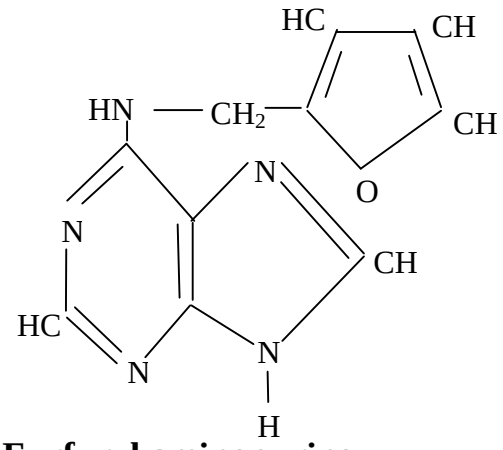
2, 4, D

Cytokinins:

i) 6-Benzyl amino purine



ii. Kinetin



6-Furfuryl aminopurine

3.3.1 Preparation of Hormone Stock Solutions

The auxin used for the present investigation were IAA, NAA, IBA and 2-4-D. For the preparations of stock solutions of 100mg /100ml IAA, IBA and 2,4-D are first dissolved in separate beaker in 2.5ml of 95% ethyl alcohol and added sterile distilled water to final volume (100ml) and adjust pH 5.8. NAA can be dissolved in 2.5 ml of 1N NaOH or KOH and added sterile distilled water to final volume (100ml) and adjust pH 5.8. IAA stock solutions can be stored no longer than a week. It was better to be prepared fresh solution.

Cytokinins used for the present investigations were BAP and kinetin. For the preparation of stock solution of 100mg/l 100ml, first dissolved cytokinin in 2.5ml of 0.5 N HCL or 0.5 N NaOH and heat gently added stored distilled water to made final volume.(100ml). Adjust PH to 5.8.

All preparation stock solutions were stored on sterilized brown bottle with labeling and preserved in refrigerator at 4^0c.

3.4. Sterilized of Glass Water and Metal Instrument

All glasswares and metal instrument were dipped in detergent water solution for 24 hours and washed with the help of bottlebrush. They were cleaned with tap water and finally rinsed with distilled water. They were sterilized in hot-air oven at 120^0C for 30 minutes. Metal instrument like

forceps, scalpels and surgical blades were wrapped with aluminum foil then autoclaved at 121^0 C for 15 minutes at 15 lb /sq. inch.

3.5. Preparation of Media

For the preparation of 1 liter MS medium, 400ml of distilled water was taken in dry and clean conical flask (2 liter). Then added 100ml stock A (Macronutrients), 1 ml stock B (Micronutrients), 1 ml stock c (Iron EDTA) and 1 ml of Vitamins with continuous stirring with the help of magnetic stirrer, 0.1 mg/ l Myo-inositol was added. After complete dissolved, added 30 gm sucrose in the same solution. The volume was made 1000 ml by adding distilled water. The pH adjusted 5.8 by adding 0.1 N NaOH or 0.1 N HCl. For MS supplemented with different concentration and combination hormone media,

Following formula is used:

$$S_1V_1=S_2V_2$$

Where,

S_1 = concentration of hormone in 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 made.

Then, 8 gm (i.e. 0.8 gm in 100ml media) agar was dissolved in hot media. The media was dispersed into clean and sterilized culture tubes they were capped with aluminum foil and rubber with labeling. It was autoclaved at 121°C for 15-20 minutes at 15-lb/ sq. inch. (Bhattarai, 2000). At room temperature the sterilized media was taken and kept slanting position (i.e. around 45°c). If there was no sign of contamination within 1 week, it was ready to inoculation of explants.

3.6. Preparation for Inoculation

For inoculation, the laminar airflow chamber or clean bench was thoroughly cleaned by 95% ethanol or sprit. All the necessary materials (such as autoclaved glass wares and metal instruments, culture tubes with media, sterile distilled water, rubber bunds, aluminum foils, match box, marker pen) except explants were exposed under ultra- violet (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.

3.7 Inoculation of Explants

In present study, explants used were *in vitro* grown plant parts such as stem, leaves and roots. These explants were inoculated separately in MS medium supplemented with 2, 4-D 1 mg/l and Kn 1 mg/l. The size of explant taken were 1.2 ± 0.2 cm. All the culture tubes were maintained at 21 ± 2°C temperature under the photo period of 16 hours daily.

3.8 Subculture of Callus and Inoculation of Shoot on Multiplication Medium

Callus obtained from explant was subculture aseptically on MS medium with different concentration of 2, 4-D and in hormone free medium. Callus also culture in MS medium containing 10% coconut milk.

The shoot regeneration from node and shoot tip explants were sub-culture on MS medium with different concentration 0f BAP and on hormone free medium. All the cultures were maintained at 21 ±2°C and eight hour light daily. The responses were observed after 4 weeks.

3.9 Rooting of Shoots

For *in vitro* rooting the differentiated shoots were exercised and transferred aseptically to the MS media with different rooting hormones

(IAA, IBA and NAA) and MS media containing different concentrations of IAA.

3.10 Acclimatization

A substantial number of micropropagated plants don't survive transfer from *in vitro* conditions to green house or field environment. The green house and field have substantially lower relative humidity higher light level and septic environment that are stressful to micropropagated plants as compared to *in vitro* conditions. Therefore for acclimatization of the *in vitro* grown plants sequential procedures were followed. The plants wee removed from the culture tubes and washed agar thoroughly with distilled water and transferred to pots containing Cocopit. The pots were watered everyday and covered with polythene bag for maintaining humidity. After 3-4 weeks plants were transferred to the environment.

CHAPTER FOUR

RESULT

4.1 Root Culture

About 0.2 – 0.3 cm long root explants from *in vitro* grown plant let were excised aseptically and inoculated in MS medium supplemented with 2, 4-D 1 mg/l and kinetin 1 mg/l. The root explants did not show any response and remains as such for long time.

4.2 Leaf Culture

The leaf explants culture on MS supplemented with 2, 4-D 1 mg/l and Kn 1 mg/l. The leaf explant also did not show any response and tuned brown and finally senesced after four weeks of culture.

4.3 Stem Culture

About 0.5 to 1 cm long stem explant from *in vitro* grown plantlet were excised aseptically and inoculated in MS medium supplemented with 1 mg/l 24-D + 1 mg/l Kn.

After 6 weeks of inoculation stem start to swollen fig. (3) cellular mass appeared around the cut edges of the stem after 7 weeks. After 8 weeks callus was appeared around the cut edges of stem fig. (4) callus was grown vigorously covering the stem after 10 weeks fig. (5), which was whitish and hard.

4.4 Sub-Culture of Stem Callus

Callus, thus obtained were culture on MS medium supplemented with different concentration of 2, 4-D. The effect on callus differentiation was shown in table 4.1.

Table No. 4.1

Effect of Different Concentration of 2, 4-D on Stem Callus

MS + 2, 4-D (mg/l)	Response			
	4 weeks	6 weeks	8 weeks	10 weeks
0.5	(4/4) C_g	(4/4) V_g	(4/4) C_B	(4/4) C_{BB}
1	(4/4) C_g	(3/4) V_g, S_p	(3/4) C_B, Sp	(3/4) PC
1.5	(4/4) C_g	(4/4) V_g	(4/4) C_B	(4/4) C_{BB}
2	(3/4) C_g	(3/4) V_g	(3/4) C_B	(3/4) C_{BB}
2.5	(4/4) C_g	(4/4) V_g	(4/4) C_B	(4/4) C_{BB}
3	(4/4) C_g	(4/4)V_g, S_p	(4/4) Sp, increase in length	(4/4) Pl
MS + 10% coconut milk	(4/4) C_g	(4/4) V_g, S_p	(4/4) Sp	(4/4) Pl

Condition: Temperature 21 ± 2°C, replicates = 4

C_g = green callus V_g = vigorous growth

Sp = small plantlet C_B = Callus brown

C_{BB} = Callus Blackish Brown PC = Plantlet Colorless

Pl= plant

On MS + 0.5 mg/l 2, 4-D, whitish callus turn greenish and grown vigorously fig. (7) after 4 weeks of culture. Similar result was observed on MS + 1.5 Mg/l 2, 4-D, MS +2 mg/l 2, 4-D and MS + 2.5 mg/l 2, 4-D. This callus remains greenish upto six weeks but organogenesis did not occur after eight weeks callus senesced on MS +1 mg/l 2, 4-D and MS + 3 mg/l 2, 4-D also whitish callus turn greenish and grown vigorously fig (8) and (9) after 4 weeks of culture.

After 6 weeks of culture minute shoots were started to regenerate from this callus on both concentration fig (10 and 11). The minute shoot regenerated on MS + 1.0 2, 4 –D mg/l turn whitish after 8 weeks of culture. But on MS + 3.0 mg/l 2, 4-D these minute shoots were grown slowly. These shoots were again cultured on fresh MS + 3 mg/l 2, 4-D upto 10 week fig (12). Here growths of shoots were also slow. Small green callus was also produced at the base of the shoots fig (13).

Similarly the callus was also cultured on MS + 10% coconut milk here also whitish callus turns greenish after 4 weeks of culture fig (14). After 8 week of culture numerous bud, like structure were seen. From these bud like structure only few shoots were regenerated and grown normally fig (15). These plants were cultured on rooting medium (1 mg/l IAA). The induction of root occurred after 2 weeks of culture after weeks only few (11) roots were induced fig (17).

4.5 Shoot Multiplication

The shoots regenerated from node culture were subculture on MS supplemented with different concentration of BAP and MS Basal medium. No response was observed on MS basal medium. On MS + 0.5 mg/l BAP, observed only elongation of shoot length and green callus was seen at the base of the shoot after 4 week of culture fig (19).

On MS + 1 mg/l BAP shoot multiply only few in number (2-4) after 4 week of culture fig (20). Elongation and branching of shoot also occurred. Similar result was observed on MS + 1.5 mg/l BAP. On MS + 2 mg/l multiplication shoot was little bit higher than on MS + 1 mg/l BAP fig (22). On MS + 2.5 mg/l BAP only auxiliary branching of shoot occurs fig (23). On MS + 3 mg/l BAP maximum number of shoot multiplication occurred after 4 weeks of culture fig (24), (25), (26). Effect of different concentration of BAP on shoot multiplication could be tabulated as below:

Table 4.2

Effect of Different Concentration of BAP on Shoot Multiplication after 4 Weeks of Culture

MS + BAP (mg/l)	No. of Shoots per Culture (mean ± SE)	Length of shoot (cm) (mean ± SE)
MS	-	-
0.5	1.17 ± 0.15	4.54 ± 0.45
1	2 ± 0.41	2.92 ± 0.21
1.5	3.17 ± 1.0	0.714 ± 0.39
2.0	5.5 ± 1.2	1.24 ± 0.19
2.5	1.5 ± 0.38	1.75 ± 0.42
3.0	7.83 ± 1.8	2.48 ± 0.48

* S.E. = Standard Error

The table 4.2 shows that the rate of shoot multiplication and elongation on different concentration of BAP after 4 weeks sub culture. For maximum shoot multiplication MS + 3.0 mg/l BAP was found to be based. The mean number of shoot proliferation per culture was more than 7. But multiplication of shoot on MS + 2.5 mg/l BAP was found poor. The mean number shoot per culture was 1.5. While the elongation of shoot on MS + 0.5 mg/l was found best and elongation of shoot on MS + 1.5 mg/l was poor. After 10 weeks of culture maximum shoot multiplication (about 25-30 shoots) was observed on MS + BAP 3 mg/l. (fig 26). Similarly shoot multiplication also observed on MS + 2 mg/l BAP (fig 27).

4.6 Rooting of Shoots

For root initiation, *In vitro* regenerated shoots were cultured on MS medium supplemented with different rooting hormones viz. IAA, IBA, NAA (1 mg/l each). The effect of these hormones on root initiation on shoot could be tabulated below:

Table 4.3

Effect of Rooting Hormones on Root Initiation after 4 Weeks of Culture

Control	Concentration of Rooting Hormones					
	IAA (1 mg/l)		IBA (1 mg/l)		NAA (1 mg/l)	
	No. of root/culture (mean ± S.E.)	Length of roots (mean ± S.E.)	No. of root/culture (mean ± S.E.)	Length of roots (mean ± S.E.)	No. of roots (mean ± S.E.)	Length of roots (mean ± S.E.)
No roots	7.83 ± 0.98	7.38 ± 0.23	1.5 ± 0.67	3.89 ± 0.29	2.5 ± 0.46	1.08 ± 0.07

On MS +1 mg/l IAA, initiation of root started after 2 weeks of culture. The roots were small, heary and few in number (3). After 4 weeks of culture about 11 roots were induced which were thin and long (fig. 28). After 8 weeks, 10 to numerous roots were induced (fig. 29). These were also thin, long and branching. The elongation of root also found better on MS + 1 mg/l IAA.

On MS + 1 mg/l IBA, initiation of root started after 3 weeks of culture. The roots were small and few in number. After 4 weeks of culture, 4-6 roots were induced fig. (32), fig. (33).

On MS + 1 mg/l NAA initiation of roots started after two weeks of culture but the number was very few (1). After four weeks of culture, vigorous growth of callus was found, which was green. Large numbers of roots were also induced. Roots were thick, short and branching (fig. 34). After 6 weeks, callus start to browning, no any differentiation was observed. But numbers of roots were increased (10-18). Fig (35)

The shoots were also culture on different concentration of IAA (0.5 to 3 mg/l) and on MS. The initiation of roots on MS occurs after 5 weeks

of culture but the number was few (3). On MS + 0.5 mg/l IAA, thin, long roots were induced after 4 weeks of culture fig. (36). Similar results were observed on MS + 1.5 mg/l IAA, MS + 2.0, g/l IAA and MS + 2.5 mg/l IAA. But the numbers of roots were increases with increase in concentration of IAA fig. (37), (38) and (39). The maximum number of root induction was observed on MS + 3 mg/l IAA (10-14 numbers) fig. (40). The effect of MS supplemented with different concentration of IAA on rooting after 4 weeks of shoot culture could be tabulated as follows:

Table No. 4.4

Effect of MS Supplemented with Different Concentration of IAA on Rooting of Shoots after 4 Weeks of Culture

MS + IAA (mg/l)	No. of Roots per Culture (mean ± SE)	Length of Roots (cm) (mean ± SE)
0.5	4.8 ± 1.1	4.1 ± 0.21
1.5	5.2 ± 0.6	4.7 ± 0.68
2.0	5.6 ± 1	3.6 ± 0.77
2.5	5.5 ± 0.38	3.1 ± 0.35
3.0	9.6 ± 1	5.1 ± 0.19

* S.E. = Standard Error

From above table 4.4, it was clear that for maximum root induction MS + 3 mg/l IAA medium was found to be best. The mean number of root per culture was more than 9. But MS + 0.5 mg/l IAA was found poor. The mean number of root per culture was 4. The elongation of root also better on MS + 3 mg/l IAA while poor on MS + 2.5 mg/IAA.

After 8 weeks of culture, numbers of roots were increased in each concentration. But maximum number of roots was observed on MS + 3 mg/l IAA. fig. (40), (41), (42), (43), (44) and (45).

The rooted plantlets (both from callus regenerated and shoot multiplied) were acclimatized to the pots containing Cocopit fig. (49).

CHAPTER FIVE

DISCUSSION

The morphogenetic response of the explant varies with genotype, age, nature and culture condition. The result obtained from the culture of various explants of *Rauvolfia Serpentina* (L) Benth. ex. Kurz has been discussed on the basis of relevant literatures.

In present study, cultured of leaf, stem and root explants of *Rauvolfia Serpentina* (L). Benth ex. Kurz on MS basal medium and MS + 1 mg/l 2, 4-D + 1 mg/l Kn. MS basal medium was found non-effective in callus induction and other differentiation. The leaf explant did not show any response on MS + 1 mg/l 2, 4-D + 1 mg/l Kn it was turned brown and finally senesced often four weeks of culture. But Parveen (1978) reported callus formation of *Rauvolfia* leaf explants in the presence of 2, 4-D. Similarly, Pokhrel (1998) found that the callus of leaf explants of *Madhuca butyrace* proliferated into callus on MS supplemented with 2, 4-D 1 ppm and BAP 1 ppm. The nature of callus was green and friable only callus was produced on subsequent cultures. Ranjit (1999) obtained that the leaf explant of *Rhus parviflora* produced callus on MS + Kn 1 ppm + 2, 4-D 1 ppm as well as MS + BAP 1ppm and MS + BAP 2 ppm. In the same media leaf explants of *Bauhinia Variegate* failed to produce callus. Sulaiman and Babu (1993) produced callus from leaf explant of *Meconopsis simplifolia* (D. Don.) walp. on the agar solidified MS medium with Kn and NAA. Ranjit (1999) has reported similar type of result in *Bauhinia variegata*.

Cellarova et. al.(1991) obtained fast growth of leaf callus of *Digitalis purpurea* MS medium with in the auxins and cytokines in the ratio of 2:1. Kayastha and Joshi (1980) investigated the induction and differentiation of callus from root, stem and leaf of *Pisum sativum* in synthetic media containing different concentration of IAA, 2, 4-D and Kn.

Jha et. al.(1981) reported healthy calli in *Apium graveelens* on MS medium having 2 4-D with Kinetin and in *Foeniculum Vulgare* BM with 2 4-D Kn and or CM. While in *Cumin* modified BM medium with various concentrations of 2, 4-D, kinetin, NAA and BAP were used for induction and maintenance of calli. Khadka (1993) reported leaf explants produced callus on MS + 2, 4-D + IAA (1 ppm each) and MS + 2 4-D + Kn (1 mg/l each). Similarly, lyer et al. (1998) initiated green callus from leaf explants of *Nyctanthes arbor-trisiris* cultured on MS medium supplemented with 2, 4-D (5 ppm), NAA (1 ppm) and coconut milk 15%. Takayuki et. at. (1979) obtained callus from leaves of *Menthe spicatra* L. cultured on B_5 media with 2 ppm 2, 4-D. Malabadi and Nataraja (2002) obtained callus from leaf explant of *Clitoria ternatea* on MS + IAA (5.72 μm) + Kn (9.3 μm). Manandhar (2002) obtained callus from leaf of *Heracleum wallichii* on MS + BAP + NAA (0.5 ppm each). Bhatt (2004) obtained whitish compact callus mass from the leaf explant of Oroxylum indicum cultured on MS + BAP (3 ppm) after four weeks. Similarly, Chand (2006) produce green mass of amorphous callus from leaf explant of *Clinopodium umbrosum* on MS + BAP (1 ppm) + NAA (1.5 ppm).

In present study culture of stem explant on MS + 1 mg/l 2, 4-D + 1 mg/l Kn obtained vigorous growth of callus after 9 weeks. The callus was hard and whitish. Similarly, Roy and Sarker (1991) studied rapid propagation by the formation shoot calli of *Aloe vera*. Callus formation was induced in stem segments of young auxiliary shoots grown on underground rhizomatous stem. Nayak et. at. (1998) obtained callus of *Asparagus robustus* using segment of shoot tissue on MS supplemented with 3 mg/l 2, 4-D and 1 mg/l Kn. Chand and Sing (2004) induced callus of *ocimum basilicum* from inter- nodal explants on MS basal media supplemented with 2 mg/l 2, 4-D and 1 mg/l Kinetin.

In present study, the callus obtained from stem explant was sub cultured on MS medium supplemented with different concentration of 2

4-D (i.e.0.5 mg/l to 3 mg/l) and MS+ 10% coconut milk. Organogenesis of callus occurred on 3 mg/l 2, 4-D and MS + 10% coconut milk. Similarly, Kitamura et. al (1989) observed plants regeneration from the callus of *Swertia pseudochinesis* Hara. The callus formation was observed in the seedling cultured on MS media with 0.1 to 10 mg/l NAA with or without BAP. Bahadur et al. (1992) regenerated plants from leaf callus of castor (*Ricinus communis* L.). on MS with Kn and IBA 1 mg/l each. Shrestha (1991) demonstrated that the equal proportion of auxin and cytokinin induced callus and organogenesis in *Swertia Chirata* from its seeds. Similarly, Karki and Rajbnhandari (1983) observed plant regeneration from leaf culture of *Solanm lanciniatum* Ait culture on MS medium supplemented with cytokinins. Maruthi et. al. (2004) regenerate plantlets from stem callus culture *Celartrus paniculatus* willd on LS medium supplemented 2% of fructose, 6 μ M Kn and 1μ IBA.

In presents study, root explant culture on MS+ 1 mg/l 2, 4-D + 1 mg/l Kn did not show any response and remains as such for long time. But Bhuju (1996) obtained callus formation from root explant of *Sesamum indium* var. ciano 16 " white seed" on MS supplemented with 2, 4-D 1 ppm and MS + 2,4-D + Kn ppm Similarly, Ranjiit (1999) obtained callus from root explant of *Rhus parviflora* produced callus on MS + Kn 1 ppm + 2, 4- D, 1 ppm. Shrestha (1999) also induced callus from root explant of *Guizotia abyssinica* on MS medium.

In present study, the shoot regenerate from node and shoot tip explants was culture on MS and MS medium supplemented with different concentration of BAP (0.5 mg/l to 3mg/l) for shoot multination. There was no response on MS basal medium. It was observed that shoot length elongation occurred on MS + BAP 0.5 mg/l while maximum shoot multiplication was observed on MS+BAP 2 mg/l and MS + BAP 3 mg/l. Sarkar et. al. (1996) induced multiple shoot from nodal segments and shoot a pieces of *Rauvolfia serpentine* in MS medium containing 1.0 mg/l

BAP and 0.1 mg/l NAA was found to give the best shot proliferation rate. Rajkarnikar and Saiju (1999) regenerate the multiple shoots of *Rauvolfia serpentina* (L) Benth. ex. Kurz. from excised shoot tips on MS medium supplemented with 3 mg/l BAP and 0.1 mg/l of NAA. Niroula and Saiju (2000) obtained multiple shoots from young shot tips of *Valeriana jatamansii* in MS medium supplemented with BAP 1 mg/l and NAA 0.1 mg/l. Similarly Joshi et. al (2003) induced multiple shoots from nodal explants of *Foeniculum Vulgare* Mill. on MS supplemented with 1 ppm BAP and 0.5 NAA.

Agrawal and Subhan (2003) induced multiple shoots of *Centella asiatica* (L). Urban from lamina explants excised from *in vitro* raised shoots on MS medium supplemented with cytokines alone and in combinations. Tiwari et. al. (2003) obtained maximum multiple shoot regeneration on Gamborg medium supplemented with 6 mg/l BAP. Rajkarniker et. al. (2004) regenerated multiple shoot of *Swertia chirata* from shoot tip culture on the MS + 1 mg/l BAP +0.01 mg/l NAA. Bhatt (2004) regenerated maximum multiple shoots of oroxylum indicum from nodal explant MS + BAP 2 ppm.

Rajkanikar and Bhatt (2004) regenerated multiple shoot of *Neopicroruiza scrophulariifolia* by using shoot tip explants on MS medium supplemented with 0.5 mg/l BAP, 0.01 mg/l NAA and 10% coconut water. Similarly, Chand (2006) induced multiple shoot of *Clinopodium umbrosum* from nodal explant on MS + BAP 1 ppm.

In present study, shoots were transferred on MS medium supplemented with different rooting hormones such as IAA, IBA and NAA (1 mg/l each). On MS+ 1 mg/l IAA the initiation of root start after two weeks o culture the roots were than and heary. After 4 weeks of culture numerous normal roots were initiated. The roots were thin, long. Shrestha and Hoshi (1991) induced roots on MS medium supplemented

with IAA (5 mg/l) in *in vitro* regenerated shoots of *Swertia Chirata*. Similarly, Krishna and Seen (1194) induced root on MS+ 0.2 mg/l IAA in *in viro* regenerated shoots of *Woodfordia fruticosa* (L.) Kurz. Kaur et. al. (1991) found best rooting in Valeriana Jatamansii Jones on MS media supplemented with BA and IAA or NAA after 3-4 weeks of culture. Bais et. al (2002) found best rooting in *Spilanthes mauritiana* DC. On MS medium supplemented with IAA 0.2µ M. Similarly Govindaraju et. al. (2003) rooted *In vitro* grown shoots of *Withania somnifera* (L) Dunal in half strength MS media with IAA (0.5 mg/l).

On MS + 1mg/l IBA showed response only after 4 weeks of culture. The number of roots per culture was very few which were normal and short. But Wakhlu and Sharma (1999) found fortified rooting in *Heracleum candicans* on MS medium with 1 mg/l IBA. Kharel and Karki (1994) induced roots in *Chrysanthemum morifolium* on half strength MS liquid medium with IBA (1 ppm). Similarly, Purohit et al (1994) produced roots in *Chlrophytem borivilianum* on ¾ strength MS medium containing 9.8 microns IBA. Nayak et. al. (1998) induced maximum root in *Asparagus robustus* on half strength MS medium with 0.5 mg IBA. Similarly, Koroch et. al. (2003) induced root in *Echinacea pallida* on MS medium both with and without the addition of IBA. Bhatt (2004) obtained maximum roots on *Oroxylum indicum* in half strength MS medium supplemented with 2 ppm IBA.

On MS+ 1 mg/l NAA initiation of root also start after two weeks of culture roots were induced along with vigorous callus growth. The roots were thick, short, Callus was soft and creamy white. Ranjit et. al. (2000) induced roots in *Foeniculum vulgare* on MS medium supplemented with NAA (2 ppm). Similarly, Chand et. al.(2004) found best rooting in *Ocimum basilicum* on NAA (3 mg/l), Rajkarnikar and Bhatt (2004) regenerated roots in *Neopicrorhiza scrophulariifolia* (Pennell.) Hong. on

MS medium with 0.5 mg/l or with 0.1 mg/l NAA. Chand (2006) found best rooting in *Clinopodium umbrosum* on MS +1 ppm NAA.

In vitro grown shoots were also cultured on MS and MS medium with different concentration of IAA (0.5 to 3 mg/l). The regeneration of root occurred on all concentration of IAA Regeneration root also observed on MS medium but roots were only few in numbers (3). Maximum number of regeneration and elongation of root occurred on MS + 3 mg/l IAA.

CHAPTER SIX

SUMMARY

R. serpentina can be propagated by conventional methods such as seeds, root cutting, root stumps and stem cuttings. But it is more susceptible to various fungus diseases such as leaf spot, root knot, leaf blotch which causes great loss of yields so for such an endangered plant species tissue culture method is an alternative method for its germplasm conservation and for rapid clone multiplication. In this investigation the explants (root, leaf and stem) were culture on MS medium supplemented with 1 mg/l 2, 4-D + 1 mg/l Kn for callus induction. Only stem explant showed responded.

The callus thus obtained was sub cultured on different concentration of 2, 4-D (0.5 mg/l to 3 mg/l) and MS + 10% coconut milk. The proliferation of callus was vigorous on low concentration of 2, 4-D but plant regeneration occurred on higher concentration of 2, 4-D (3mg/l). Plants regeneration was also observed on MS media supplemented with 10% coconut milk.

In vitro grown shoots obtained from node culture were cultured on MS media with different concentration of BAP for shoot multiplication. Maximum number of shoot multiplication was observed on BAP 3 mg/l. The elongation of shoot length occurs on low concentration of BAP (0.5 mg/l).

The auxins IAA, IBA and NAA 1 mg/l (each) were used for root induction. Among them IAA was found most effective for induction of root *in vitro* maximum number of root induction was also observed on NAA but there was large amount of callus was developed and the roots were thick. IBA was found less effective for root induction.

The *in vitro* rooted plants (both through callus and shoot multiplication) were successfully acclimatization on Cocopit.

CHAPTER SEVEN

CONCLUSION AND RECOMMENDATION

7.1 Conclusion

The present investigation was focused on production of callus mass from suitable explants and developed a protocol for micropropagation on *Rauvolfia serpentian* (L.) Benth ex. Kurz. Following points were concluded.

- Stem explants had good response for callus induction than root, leaf explants.
- Best medium for callus induction was MS +1 ppm, 2, 4-D + 1 ppm Kn.
- The obtained callus was sub cultured on MS supplemented with different concentration of 2, 4-D (0.5-3 mg/l). The proliferation of callus was vigorous on low concentration of 2, 4-D but organogenesis occurred on higher concentration of 2, 4-D (i.e. 3 mg/l) plant regeneration was also observed on MS media supplemented with 10% coconut milk.
- Maximum shoot multiplication observed on MS medium supplemented with 3 mg/l BAP.
- For the elongation of shoot length MS medium in combination with 0.5 mg/l BAP was found most effective.
- Root differentiations in shoots were found on NAA, IBA and IAA. The root differentiated in NAA was thick, short with callus while in IAA roots were thin, long and normal. IBA was found less effective to induced root on shoots. After long period of

culture only few number of roots were induced which are thin and short.

- Maximum number of root induction was found in MS medium containing 3 mg/l IAA.

7.2 Recommendation

From the above study, following recommendations are made.

- Although tissue cultures are much expensive and time consuming process. It needed more investment for research programme to develop standard protocol for its commercialization. But by this method large number of plants obtained within short period of time. So, the government should make policy to promote the research programme by providing constant financial and technical supports.

- *Rauvolfia Serpentina* (L) Benth. ex. Kruz. Is most important medicinal plant, cited in IUCN endangered category. Due to over exploitation causes the depletion of this plant from natural habitat. So, it should be conserved by *in situ* and *ex situ* measures.

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PHOTOPLATE I of *Rauvolfia Serpentina* (L.) Benth. ex. Kurz.

Fig 1: Plant in natural habitat.

Fig 2: *In vitro* grown plant used for callus induction.

Fig 3: Swollen stem explant on MS + 2, 4-D 1 mg/l +Kn 1 mg/l after 6 weeks of culture.

Fig 4: Callus developed from stem explant on MS + 2, 4-D 1 mg/l + Kn 1 mg/l after 8 weeks of culture.

Fig 5: Callus developed from stem explant on MS + 2, 4-D 1 mg/l + Kn 1 mg/l after 10 weeks of culture.

Fig 6: Callus developed from stem explant on MS + 2, 4-D 1 mg/l + Kn 1 mg/l after 12 weeks of culture.

Fig 7: Callus culture on MS + 2, 4-D 0.5 mg/l after 4 weeks of culture.

Fig 8: Callus culture on MS + 2, 4-D 1 mg/l after 4 weeks of culture.

Fig 9: Callus culture on MS + 2, 4-D 3 mg/l after 4 weeks of culture.

Fig 10: Callus culture on MS + 2, 4-D 1 mg/l after 6 weeks of culture.

PHOTOPLATE II

Fig 11: Callus culture on MS + 2, 4-D 3 mg/l after 6 weeks of culture.

Fig 12: Callus culture on MS + 2, 4-D 3 mg/l after 8 weeks of culture.

Fig 13: Callus culture on MS + 2, 4-D 3 mg/l after 10 weeks of culture.

Fig 14: Callus culture on MS + 10% coconut milk after 4 weeks of culture.

Fig 15: Callus culture on MS + 10% coconut milk after 8 weeks of culture.

Fig 16: Callus culture on MS + 10% coconut milk after 10 weeks of culture.

Fig 17: Regeneration of root on plant regenerated from MS + 10% coconut milk on MS + 1 mg/l IAA after 4 weeks of culture.

Fig 18: Regeneration of root on plant regenerated from MS + 10% coconut milk on MS + 1 mg/l IAA after 8 weeks of culture.

Fig 19: Shoot culture on MS + 0.5 mg/l BAP after 4 weeks of culture.

Fig 20: Shoot culture on MS + 1 mg/l BAP after 4 weeks of culture.

PHOTOPLATE III

Fig 21: Shoot culture on MS + 1 mg/l BAP after 8 weeks of culture.

Fig 22: Shoot culture on MS + 2 mg/l BAP after 4 weeks of culture.

Fig 23: Shoot culture on MS + 2.5 mg/l BAP after 4 weeks of culture.

Fig 24: Shoot culture on MS + 3 mg/l BAP after 4 weeks of culture.

Fig 25: Shoot culture on MS + 3 mg/l BAP after 8 weeks of culture.

Fig 26: Shoot culture on MS + 3 mg/l BAP after 10 weeks of culture.

Fig 27: Shoot culture on MS + 2 mg/l BAP after 10 weeks of culture.

Fig 28: Regeneration of root from shoot on MS + 1 mg/l IAA after 4 weeks of culture.

Fig 29: Regeneration of root from shoot on MS + 1 mg/l IAA after 8 weeks of culture.

Fig 30: Regeneration of root from shoot on MS + 1 mg/l IAA after 10 weeks of culture.

PHOTOPLATE IV

Fig 31: Regeneration of root from shoot on MS + 1 mg/l IAA after 12 weeks of culture.

Fig 32: Regeneration of root from shoot on MS + 1 mg/l IBA after 4 weeks of culture.

Fig 33: Regeneration of root from shoot on MS + 1 mg/l IBA after 6 weeks of culture.

Fig 34: Regeneration of root from shoot on MS + 1 mg/l NAA after 4 weeks of culture.

Fig 35: Regeneration of root from shoot on MS + 1 mg/l NAA after 6 weeks of culture.

Fig 36: Regeneration of root from shoot on MS + 0.5 mg/l IAA after 4 weeks of culture.

Fig 37: Regeneration of root from shoot on MS + 1.5 mg/l IAA after 4 weeks of culture.

Fig 38: Regeneration of root from shoot on MS + 2 mg/l IAA after 4 weeks of culture.

Fig 39: Regeneration of root from shoot on MS + 2.5 mg/l IAA after 4 weeks of culture.

Fig 40: Regeneration of root from shoot on MS + 3 mg/l IAA after 4 weeks of culture.

PHOTOPLATE V

Fig 41: Regeneration of root from shoot on MS + 0.5 mg/l IAA after 8 weeks of culture.

Fig 42: Regeneration of root from shoot on MS + 1.5 mg/l IAA after 8 weeks of culture.

Fig 43: Regeneration of root from shoot on MS + 2 mg/l IAA after 8 weeks of culture.

Fig 44: Regeneration of root from shoot on MS + 2.5 mg/l IAA after 8 weeks of culture.

Fig 45: Regeneration of root from shoot on MS + 3 mg/l IAA after 8 weeks of culture.

Fig 46: Regeneration of root from shoot on MS + 2 mg/l IAA after 10 weeks of culture.

Fig 47: Regeneration of root from shoot on MS + 2.5 mg/l IAA after 10 weeks of culture.

Fig 48: Regeneration of root from shoot on MS + 3 mg/l IAA after 10 weeks of culture.

Fig 49: Plantlets transferred in cocopit.