CHAPTER ONE
INTRODUCTION

1.1 Background

Nepal is rich in biodiversity due to its wide range of bioclimatic zones. Of total percent land area, only 20% has been used for the crop cultivation. Nepal's varied agro-climatic environment offers vast quantities of plants to grow. More than 80% of the total population depends upon agriculture for their livelihood (Central Bureau of Statistics, 1994)

Medicinal plants that are defined as plants with healing properties (Oxford dictionary), are 30,000 to 70,000 species across the world; and 80 percent of the worlds rural people meet their needs of primary health care from such plants (Sarkar, 1996). The wealth of medicinal plants in Nepal may be considered as one of the important natural resources for the economic benefit of the nation. Out of 6,500 flowering plants of Nepal, 1,624 are found to have medicinal values of which 1,515 are angiosperms (Shrestha *et al.*, 2000).

The Medicinal plants have been used as the basic raw materials for the preparation of drugs in number of traditional systems including folk herbal tradition, ayurvedic system, unani system and homeopathy as well as in the modern or allopathic system. Today, people are much more interested in ethno medicines than in synthetic drugs. The reasons behind that are most species of medicinal plants are non toxic, easily available, mostly used in crude form (natural form) and therefore give rise to few, if any side effects even when some adverse affect occur, they are much less serious than those caused by chemically synthesized medicines (Anonymous, 1978). Over 60 percent of all pharmaceuticals are plant based (Evans, 1996). The demands of herbs for medicinal purposes are growing day by day. These demands are not only from ayurvedic pharmaceutical companies but also from modern pharmaceutical

multinational companies. These companies are extensively investing and exploring the possibilities of finding active pharmaceutical substance from these herbs.

The commercial exploitation of medicinal plants has been a concern of depletion of some of the important species. Wild medicinal plants were collected especially before the unset of seed setting through untrained and unskilled labourers. This has led to the unscientific extraction of the entire plants, roots, rhizomes, tubers and bulbs. In addition, the over exploitation changed the environmental conditions and original habitats which has led to gradual loss of plants species. In many of the species, exploitation pressure has gone to the extent that some plants are at the verge of extinction and therefore have been declared as threatened, rare, vulnerable or endangered depending on its presence in nature. *Withania somnifera* (L.) Dunal is categorized as an endangered plant.

Tissue culture technique is quite useful and the only alternative to increase production to those plants whose propagation is slow by means of the conventional sexual seed reproduction. *In-vitro* propagated plants of many important medicinal species were found to be uniform, showing less variation in their content of secondary metabolites than their wild/cultivated counterparts (Ramada *et al*., 1991).

Micropropagation is the aseptic culture of cells, pieces of tissue, or organs. It is possible to regenerate new plants from small piece of plant tissue because each cell of a given plant has the same genetic makeup and is totipotent, i.e., is capable of developing along a “programmed” pathway leading to the formation of an entire plant. Using micropropagation, millions of new plants can be derived from the single plant. This rapid multiplication allows breeders and growers to induce new cultivars much earlier than they could by using conventional propagation techniques, such as cuttings. It can also be used to establish and maintain virus free plant stock. This is done by

culturing the plants apical meristem, which typically is not virus infected, even though the remainder of the plant may be. Once new plants are developed from the apical meristem that can be maintained and sold as virus free plants.

1.2 Description of *Withania somnifera* (L.) Dunal

Botanical name	:	*Withania somnifera* (L.)Dunal
Common Name	:	Ashwagandha (Nepali)
		Turangi gandha (Sanskrit)
		Winter cherry (English)
Family	:	Solanaceae
Chromosome number	:	2n = 22, 24

Ashwagandha in Sanskrit means “horse smell”, probably originating from the odor of its root. The species name *somnifera* means “sleep-bearing” in Latin, indicating it was considered a sedative.

Withania somnifera (L.) Dunal is an annual tomentose shrub, 30 to 150cm high. Roots stout fleshy, whitish brown, leaves simple, ovate, glabrous those in the floral region smaller and opposite. Flower hairy, inconspicuous greenish or lurid yellow, in axillary umbellate cymes, fruits berries small, globose orange red when mature, enclosed in the persistent calyx; seed yellow, many heart shaped. Plants are bisexual, flowers in December. Root, the main source of valuable chemicals, remains intact with the plant.

1.2.1 Origin of Plant

The exact origin of this species is unknown but it is probably believed that, it is originated from Nagaur (Rajasthan) India. *Withania somnifera* (L.) Dunal has been extensively domesticated from the wild. In India at least five

different cultivars have been reported for increase root size and adaptation to different climatic zones (Anonymous, 2000).

1.2.2 Distribution

It is cultivated all over India; especially Gujrat, Maharastra, Rajasthan and Madhya Pradesh are leading states of *W. somnifera* cultivation. Its representative collections are also reported from Mustang district of Dhawlagiri zone, at 650 to 1000m height in Nepal. There are over 20 other species of the *Withania* genus, that occur in the drier part of North Africa, Bangladesh, Pakistan, Afghanistan (Helmand province), Shrilanka, and the Mediterranean region. (Anonymous, 1948).

1.2.3 Important Biochemical Constituents

a) Roots Alkaloids

The pharmaceutical activity of roots is attributed to the presence of several alkaloids. The total alkaloid content of the Indian roots has been reported to vary between 0.31 to 4.3 percent. The wide variation in the alkaloid contents might be due to some environmental and wild or cultivated forms (Srivastava *et al*.,1996). Many biochemical heterogeneous alkaloids have been reported in the roots. They include pyridine derivatives, anahygrine, tropine, pseudotropine, ansferine and its all d and l forms. Other components remain unidentified (The wealth of India, 2000).

In addition to alkaloids, the roots are reported to contain starch, reducing sugar, glycosides, dulcitol, twelve withanolids of a, b, c, d and e forms. The free amino acids identified in roots include aspartic acid, glycine, tyrosine alanine, proline, tryptophan, glutamic acid and cystine.

b) Leaves Alkaloids

Leaves of the plant also contain withanolids (C_{28} H_{38} O_5). All twelve withanolids have been isolated from the leaves of the plant. In addition to withanolids, the leaves contain five unidentified alkaloids (yield 0.09%), glycosides, glucose and many free amino acids. Occurrence of chlorogenic acid, condensed tannins and flavonoids are reported from the leaves and stem part of the plant (Dhalla *et al*., 2000).

1.2.4 Medicinal Importance

All parts of the plant are used in herbal medicines. Some herbalists refer to Ashwagandha (winter cherry) as Indian ginseng, since it is used in ayurvedic medicines in a way similar to that ginseng is used in traditional Chinese and Korean medicines. Some of its important uses are pointed below:

- ✓ In Ayurveda winter cherry is used in kapha and vata based disorders. It is used as an adapotogenic, antioxidant or free radical scavenger, anti-stress, immunomodulator aphrodisiac herb; this ayurvedic herb is used with special reputation attached to it very commonly in India, in restoring the strength of body and mind. With Gokshura, it is better as a nervine tonic in hypoglycemia.
- ✓ Winter cherry is a good hypnotic in alcoholism used in arthritis and degenerative diseases, strengthening in children as nutritional supplement, helps in breathing difficulty.
- ✓ Used in tuberculosis along with *Capparis paniculata* powder.
- ✓ Its leaf powder used in skin diseases like leprosy. It has also antihelminthic property. Its powder is also used in reducing obesity problems.
- ✓ Its fruit and seeds are used as diuretic.
- ✓ Its root powder is useful in seminal debility, benefits women in leucorrhea and weakness, also found to be promising in sterility of

women, it increase milk secretions (glactogogue), useful in uterine infection, Increases body weight in emaciated child, used in glandular swellings and surgical dressing. It is a good health restorative for aged persons who want to be younger than their age.

- ✓ A mixture of ashwagandha, liquorice and amla is found to be useful for improving the eye sight.
- ✓ Ashwagandha root powder is a wonderful herbal body building solution for those who desire to build their body in a non-harmful way. It promotes the anabolic activities in the body. Its 3 gm of powder could be taken twice daily with hot milk after the meal. It should be used for more than three months.

1.2.5 Cultivation

Withania somnifera (L.) Dunal is cultivated in soil that is unsuited for other crops and requires little care. Seed are sown by broadcast method in the nursery just before the onset of rainy season. Two to three kilograms of seed are sufficient to provide seedlings for one acre field. When the seedlings are sufficiently tall, they are planted in the field at a distance of 60cm×60cm. Manuring and weeding are not necessary. Nitrogen fertilizer promotes heavy leafy growth with fewer yields of roots. Irrigation is also hardly required. Excessive rain is harmful. The plant bears flowers and fruits in December. Harvesting starts from January and continues till March. Thus this plant can be used in crop rotation practice as well. The entire plant is uprooted for roots, which are separated from the aerial parts by cutting the stem 1-2 cm above the crown. They are then transversely cut into smaller peaces for drying occasionally the roots are dried whole (Wealth of India, 1993).

The yield of roots is three hundred to five hundred kg per acre when fresh and two hundred fifty to three hundred kg per acre when dried. The dried roots were graded, grinded packed and sold to pharmaceutical companies, five to six hundred Rupees per kilogram (Market current survey, 2007)

1.3 Justification of the Study

Existing information on medicinal and aromatic plants (MAPs) in mountain region suggests that the economic future of mountain communities may be secured through MAPs cultivation. If transparent and technically sound system and modalities is put into place to conserve natural resources, adopt sustainable harvesting and processing practices and ensure equitable sharing of benefits. With the growing demand of market for MAPs and limited economic opportunities more people in remote areas of Nepal are getting involved in harvesting these products. Consequently, with growing competition for collection and little regulation for access over these products in public forest, many valuable MAPs species are on the verge of extinction. Cultivation and utilization of MAPs is the effective way of income generation where the utilization of agricultural land is limited.

Withania somnifera (L.) Dunal is one of the very important medicinal plant species cited in IUCN endangered category. Its survival of seeds is very poor in natural condition. Besides, this cause due to over population of human beings near the forest, environmental degradation, over exploitation, habitat destruction, introduction of exotic species and collection of root part for medicinal purposes caused depletion of this plant from its natural habitat.

The most effective way of protection of rare and endangered species is through their protection in the natural habitats. But since this particular plant form a part of the economical network of the region, it is felt that this plant must be propagated so that in due course of the time they can serve as cash crops for the formers and on the other hand will ease the exploitation pressures on the natural population by fulfilling the demand of the traders. Since market value of this plant is higher and it takes little care for cultivation in poor quality soil. Therefore, it was felt essential to choose this multipurpose, valuable plant species to develop a protocol for mass propagation and cultivation.

1.4 Objectives of the Study

With the consideration of above background, the following objectives have been aimed for the present study.

General Objective:

1. To develop a protocol for mass propagation of *Withania somnifera* (L.) Dunal so that people can cultivate this medicinal plant as cash crop.

Specific Objectives:

1. To determine the appropriate explant for rapid proliferation
2. To determine concentration of phytohormone most effective for shoot multiplication and root formation.
3. Callus study of *in-vitro* grown plantlets.

Limitation of study:

1. Best condition for acclimatization.

CHAPTER TWO
LITERATURE REVIEW

Harberlandt (1902) was the first to propose cultivating plant cells *in-vitro* and developed a more versatile tool to explain morphogenesis and to determine the toitpotentiality of plant cells. Lamprecht (1918), Kundson (1919) and Nemec (1924) made many attempts to find suitable media and optimal condition for growing organ, tissue and cells excised from whole plants.

Skoog (1944) and Tsui (1951) reported that in tobacco pith tissue cultures, the addition of adenine and high levels of phosphate increased the callus growth and bud formation in the presence of IAA.

Sclogku *et al*. (1962) studied on seed germination of *Musa species* and found normal seed germination in MS basal medium.

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

Manandhar (1980) noted that *in-vitro* culture of seed, root, stem and leaf segment of *Trigonella foenumgraecum* were reared in order to study their morphogenetic potentialities. He reported that higher concentration of IAA was expressed by the suppression of root growth with the formation of callus along its length. These calli on transferring to the fresh medium failed to undergo organogenesis, but the callus with intact plant differentiated in to roots.

Kitamura *et al*. (1984) observed plantlets regeneration from the callus of *Swertia pseudochinesis* Hara. The callus formation was observed in the seedling cultured on MS medium with 0.1- 10 mg/l BAP.

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

Sengupta and Sharma (1988) induced multiple shoots formation in *Withania somnifera* (L.) Dunal, and under shrub of solanaceae family that contains a narcotic substance known as 'withanolide'. Multiple shoot formation was examined in the plant on MS medium using cytokinins alone or in combination with auxins.

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

Choi *et al*. (1991) observed possibility of rapid multiplication of ginger *Zingiber officinales* Rosc., through *in-vitro* culture of shoots tip. The factor investigated was the effect of various growth regulators in shoot tip culture. The shoot tip cultured on MS medium supplemented with NAA and BAP 0.5 mgl^{-1} each was found to be optimal for growth of *in-vitro* plantlet.

Shrestha (1991) observed the germination of *Swertia chirayita* L. on MS media. after the treatment with GA_3 400 mgl^{-1} for 24 hrs. Excised stem segments from *in-vitro* grown plants were cultured on BM supplemented with

PGRs either singly or in combinations. On BM + BAP (5 mgl^{-1}), shoot buds were differentiated. Rhizogensis and embryogenesis were observed on BM with IAA. As the concentration of IAA increased the tendency of shoot bud regeneration was less vigorous, while in lower concentration, reverse condition was observed. Higher concentration of BAP 5 mgl^{-1} was more favorable in the bud regeneration than lower concentration 2 mgl^{-1}. The shoot buds subcultured on BM could not regenerate roots. The best rooting was found in the medium with 5 mgl^{-1} of BAP and IAA both.

Shrestha (1992) observed that basal medium supplemented with different concentration of IAA induce rooting in *Brassica compestris* L. 1 mgl^{-1} IAA was found to be most suitable concentration for rooting. The presence of IAA in the root inducing medium also affects height of the plantlet. The tallest plantlet was obtained in the medium supplemented with 2 mgl^{-1} IAA.

Krishna and Seeni (1994) worked on rapid micropropagation of *Woodfordia fruticosa* L. kurz,a rare medicinal plant, where they found medium supplemented with BAP 0.2 mgl^{-1} induced high frequency 88% development of axillary shoot buds (3.2) in 4-5 weeks. Sub culture of explants with highest multiple new shoot (26-30) were recorded when using culture initiation media with 0.5 mgl^{-1} each of BAP and NAA followed by subculture in 0.2 mgl^{-1} BAP. The shoots multiplication rate was further accelerated by re-culturing 0.4-0.6 cm nodal segments, of regenerated shoots, in media with 1.0 mgl^{-1} BAP.

Niraula (1994) obtained 2-4 shoots from eight week old shoot tip culture of Gerbera plant in MS + 1 mgl^{-1} BAP + 0.1 mgl^{-1} NAA.

Sarkar *et al*. (1996) induced multiple shoots from nodal segments and shoot apices of *Rauvolfia serpentina* L. Benth. Ex kurz. on MS medium containing 1.0 mgl^{-1} BAP and 0.1 mgl^{-1} NAA. Callus formed at the cut bases of the explants which produced shoots when subcultured on MS media containing

low concentration of BAP 0.5 or 0.1 mgl^{-1} and NAA 0.1 mgl^{-1}. The *in-vitro* proliferated plants were transplanted to the soil.

Takashi *et al*. (1996) studied on *Ephedra gerardiana* and found that excised meristem tissue from aseptic shoots and culture in MS medium with 0.5 to 1 mgl^{-1} kin in 3% sucrose developed shoots. Multiple shoots were also developed on MS medium supplemented with 5 mgl^{-1} BAP.

Ranjit (1999) obtained callus from the different explants of *Rhus parriflora* on different concentration of growth hormones but in *Bauhinia variegata* L., the nodal explants gave multiple shoots on MS media supplemented with 1 mgl^{-1} BAP and 0.5 mgl^{-1} NAA. However, leaf, root and stem explants could not give any response.

Rajkarnikar and Saiju (1999) regenerated the multiple shoots of *Rauvolfia serpentina* L. Benth ex. Kurz from excised shoot tips on MS media supplemented with 3 mgl^{-1} BAP and 0.1 mgl^{-1} of NAA. Subcultures of these shoots were carried out on the same media but with lower concentration of BAP 1 mgl^{-1} and NAA 0.1 mgl^{-1} for shoot proliferation.

Karki and Niroula (1999) obtained the multiple shoots through *in-vitro* culture of *Accacia auriculiformis* A. Cunn excised from 12-16 days old seedlings. Twenty five to thirty multiple shoots were proliferated in MS medium supplemented with BAP 0.5 mgl^{-1} and NAA 0.1 mgl^{-1} after the fourth subculture. The micro-shoots of 3-4 mm long were excised and rooted in non-sterile sand. The roots were initiated after 13-21 days. The rooted plants were successfully established in soil.

Karki and saiju (2000) developed a protocol for large scale production of plantlets of *Amomum sublatum* Roxb. form the two cultivars viz. Ramsai and Golsai. The shoot tips of 1-2 mm length were excised from the mother pants and cultured in MS solid medium (1962) supplemented with 1.0 mgl^{-1} BAP and 0.1 mgl^{-1} NAA. Multiple shoots with roots were produced by repeated

subcultures in same quantities of liquid medium. Rooted plants were successfully plated in the field.

Joshi *et al*. (2000) observed the nodal cutting of *Elaeocarpus sphaericum* (Gaerth) K. schum was appropriate in liquid MS medium. Otherwise there was a great possibility of necrotic exudation of phenolic compounds. The established green explants were then inoculated in MS medium with the combination of BAP and NAA as well as in MS medium with different concentration of BAP alone. The MS medium supplemented with BAP 0.5 mgl^{-1} and NAA 0.01 mgl^{-1} as well as in MS medium with BAP 0.25 mgl^{-1} were observed good for proliferation of micro-shoots.

Niroula and Saiju (2000) regenerated multiple shoots from the young shoot tips of *Valeriana Jatamansii* in MS medium supplemented with 1 mgl^{-1} BAP and 0.1 mgl^{-1} NAA, 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 1 mgl^{-1} BAP. Averages of six shoots were developed from a single shoot after 4 weeks of culture.

Rajkarnikar *et al*. (2000) showed the micro-shoots sprouting from the base of shoot-tip explant in the MS medium with 1 and 2 mgl^{-1} BAP and 0.1 mgl^{-1} NAA in *Rauvolfia serpentina* (L.) Benth. Ex. Kurz.

Pant *et al*. (2001) induced multiple shots from nodal explants of *Foeniculum vulgare* mill on MS medium supplemented with BAP 1 mgl^{-1} and NAA 0.5 mgl^{-1} after 4 weeks of culture. An average of six shoots was developed from a single shoot after 4 weeks of culture. Roots were developed from shoots on MS medium supplemented with NAA 2 mgl^{-1}.

Ray S. and Jha, S. (2001) produced multiple shoot from the single shoot tip explant of *W. somnifera* grown on MS medium supplemented with 0.1mgl^{-1} BAP.

Luan and Lin (2001) explained that NAA and BAP combination favours for callus induction in *Platycodon gradiflorum.*

Batra *et al*. (2001) noted that nodal stem segment of *Azadirachta indicum* (neem) cultured on MS medium supplemented with 1-5 mgl^{-1} BAP showed slight swelling of the nodal region prior to the emergence of shoot buds

Pereiva *et al*. (2002) induced multiple shoots of *Anenopaegmg arvense* (vell) 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 successfully achieved.

Karanjit (2002) examined the seed germination of *Capsicum annum* on MS basal medium. The seeds were pretreated in 50% GA_3 prior to inoculation. 99% of seeds were germinated. The seedlings were obtained after one week of culture. Plant regeneration was obtained from the nodal explant of *C.annum* on MS medium supplemented with 1 mgl^{-1} BAP + 0.5 mgl-^{1} NAA after five weeks of culture. Regeneration of the plant was also obtained from the nodal explants on MS medium without any growth hormones. The nodal explant induced callus and proliferated leaves on MS medium supplemented with 0.5 mgl^{-1} NAA.

Ranjit *et al*. (2002) induced shoots from nodal explants of *Foeniculum vulgare* on MS medium supplemented with 1 mgl^{-1} BAP and 0.5 mgl^{-1} NAA after four weeks of culture. Averages of six shoots were developed from single shoot after four 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 from shoots on MS medium supplemented with NAA 2 mgl^{-1}.

Zamir *et al*. (2003) obtained best condition for shoot proliferation form shoot tip explant of guava cultivars in MS medium supplemented with 1 mgl^{-1} BAP and 250 mgl^{-1} L-glutamine. Rooting of the cultured shoots was observed on half strength MS medium supplemented with IAA and IBA.

Rao and Prasad (2003) while working on *Strychnos potatorum* L., an endangered medicinal plant, observed that when nodal segment cultured on MS medium supplemented in varying concentration of BAP 2.5 mgl^{-1} produced multiple shoots.

Jayram *et al*. (2003) studied on rapid *in- vitro* propagation of *Drosera indica* and found that MS + 0.2% agar and M.S. + GA_3 gave no seed germination and also MS + 2, 4-D and kinetin failed to produce callus where as multiple shoots were induced in medium containing 0.5 mgl^{-1} kinetin/zetin.

Raju Prasad (2003) worked on *Celastrus paniculatus* and found that nodal explant gave good response in MS medium fortified with 3% sucrose, 0.6% agar supplemented with 0.5 mgl^{-1} and 2 mgl^{-1} BAP produced callus and when nodal segment and shoot tip subcultured on BAP 1 mgl^{-1} produced large number of multiple shoots.

Neeraja and Prasad (2003) while working on *Withania somnifera* (L.) Dunal concluded that shoot tip cultural in MS basal medium fortified with BAP 1 mgl^{-1} can induce multiple shoots.

Rani *et al*. (2003) observed callus induction form hypocotyls, root and cotyledonary leaf segment of *Withania somnifera* (L.) Dunal grown on MS medium supplemented with various concentration and combinations of 2, 4,-D and kinetin (kin).

Maximum callusing (100%) was obtained from root and cotyledonary leaf segments grown on MS medium supplemented with a combination of 2 mgl^{-1} 2, 4-D and 0.2 mgl^{-1} kin. The callus when subcultured 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 hypocotyls segments were used as explants, callus induction was noticed in 91 % of cultures, which showed shoot regeneration on MS medium supplemented with 2 mgl^{-1} 2, 4-D and 0.2 mgl^{-1} kin. These shoots were transferred to fresh medium containing various concentration and combinations of 6 BAP and N^6-2 isopentyl adenosine (zip). Maximum shoot multiplication was observed after 60 days of the second subculture on MS medium containing 2 mgl^{-1} IBA. The plantlets were transferred to the field after acclimatization and showed 60% survival.

Poudel (2003) cultured shoot tip and nodal segment of *Mentha spicata* L. on MS medium supplemented with 0.5, 1 and 2 mgl^{-1} BAP formed multiple shoots. Thirty-nine shoots per explant were formed on MS media with 1mgl^{-1} BAP within 10 weeks of culture. Explants cultured on MS medium with 0.5 mgl^{-1} NAA + 0.5, 1 and 2 mgl^{-1} BAP also formed multiple shoots. The micro-shoots developed roots on the same medium and formed plantlets after 4-6 weeks of culture, NAA in combination with BAP showed inhibitory effect on multiple shoot formation when present in higher concentrations. The presence of BAP alone on MS medium proved best condition for the multiple shoot formation.

Doe *et al*. (2003) observed that highest efficiency of shoot formation of *Ocimmum basilicum* L. occurred in MS medium supplemented with 5 mgl^{-1} BAP and 2 mgl^{-1} NAA. The presence of NAA inhibits root formation, but when it is combined with different concentration of cytokinin (BAP 1 to 5 mgl^{-1}) it induce an increase in the number of shoot.

Govindraju *et al*. (2003) proposed a high frequency and rapid regeneration protocol via callus and directly from various explants of *Withania somniferous* (L.) Dunal. Callus was initiated from inter-node segment, leaf, root and petiole explants on MS and B5 media supplemental with 2, 4-D (0.5 to

3 mgl^{-1}) and NAA (0.5 to 30 mgl^{-1}) either alone or along with kinetin (0.5 to 1 mgl^{-1}). Regeneration was observed from callus of all the explants except roots on MS medium fortified with BAP (0.5 to 2.5 mgl^{-1}) or in combination with IAA 0.5 mgl^{-1}.

Direct differentiation of multiple shoot from leaf, node segments and shoot tips occurred within two weeks on MS medium supplemented with BAP (0.5 to 3 mgl^{-1}) in combination with IAA 0.5 mgl^{-1}. The number and height of the shoots per explant varied with different concentration of BAP and with low concentration of IAA. Shoots that were dwarfs were elongated on MS medium fortified with GA_3 0.5 mgl^{-1}. These plantlets were then rooted alone or along with IAA 0.5 mgl^{-1}. Plantlets were hardened for two weeks and successfully transferred to field with 80 to 85 % survival.

Sarowar *et al*. (2003) developed on efficient *in-vitro* micro-propagation protocol for direct shoot growth of interspecific cucurbita hybrid variety using shoot-tips of 5 day old explants. The excised shoot tips were cultured on M.S. medium supplemented different combination and concentration for the study of shoot induction. The best condition for shoot growth was MS medium supplemented with 6 BAP. The shooting frequency was 84% and five shots were obtained from each explant after 30 days of culture. Shoots were rotted most efficiently in 1mgl^{-1} IBA.

Korch *et al*. (2003) developed a method for the induction of adventitious shoots from leaf tissue of *Echinaeea pallida* (an important medicinal plant) with subsequent whole plant regeneration. The optimum shoot regeneration frequency (63% and number of shoots per explants was 2.3) was achieved using media supplemented with 26.6 µM BAP and 0.11 µM BAP. Rooting of regenerated shoot explants was successful on M.S. medium both with and without the addition of IAA. All plantlets survived acclimatization producing phenotypically normal plants in the green house.

Agrawal and Subham (2003) induced multiple shoot of *Centella asiatica* from lamina explants excised from *in-vitro* raised shoots through callus phase on MS medium supplemented with cytokinin alone and in combinations. Amongst all the cytokinins BA, Kin, zip and zetin tried in various concentration (0.1, 1, 5, 10, 20, 30 μM) 10 μM kin proved optimum for differentiation of shoots on an average of 15-25 ± 4.72 shoots in 75% cultures within 6 weeks. Excised shoots rooted best in ½ strength MS adjuvant with 1μM IBA within 15 days of transfer. Nearly 95.83% shoots organized on an average of 6.33 ± 0.67 roots / culture. The tissue culture derived plantlets have been successfully transferred to field.

Jayaprakasam *et al.* (2003) isolated and studied the effect of all 12 withnolids from *W. somnifera* and suggested that incorporation of withanolids in the diet may prevent or decrease the possible growth of tumor cells in human beings.

Pandy (2003) observed that rooting of the excised shoots of *Helianthus annus,* found best in medium containing MS + 1 mgl^{-1} IAA.

Ipekici *et al*. (2003) induced multiple shoots from the nodal explants of *Paulownia elongata* by culturing them on MS medium supplemented with 1 mgl^{-1} BAP and 0.1 mgl^{-1} NAA. The *in-vitro* induced plants were rooted and letter transferred to the soil.

Bhadra *et al*. (2004) achieved highest rate of elongation of shoot in *Bulbophyllum liliacinum* on MS medium fortified with 2 mgl^{-1} BAP and 1mgl^{-1} IAA. Strong and stought root system were developed when 3-4 cm shoots were transferred on MS + 3% (w/v) sucrose + 0.5 mgl^{-1} IAA.

Fragas *et al*. (2004) developed *in-vitro* protocol for micro propagation in *Ficus carica* L. They observed the best condition in callus formation along with micro-shoots in MS medium supplemented with BAP and activated charcoal.

Liao *et al*. (2004) established a rapid micro-propagation protocol for *Aloe vera* L. var chinensis (Haw) Berger. The effect of three factors namely BAP, NAA and sucrose on bud initiation were evaluated by L9 (3^{rd}) orthogonal design. The best medium for bud initiation was MS + 2 mgl^{-1} BAP + 0.3 mgl^{-1} NAA on which chinensis Aloe could multiply 15 times in 4 weeks.

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

Agrawal *et al*. (2004) conducted field experiment to study the effect of showing dates and spacing on the root yield of *Withania somnifera* (L.) Dunal (Ashwagandha).

Roce *et al*. (2004) observed the medicinal importance of *Withania samnifera* as anti-inflammatory, cardio active and angiogenic.

Patel *et al*. (2004) conducted a field experiment to study the influence of method of showing, time of harvesting and nitrogen application on dry root yield of ashwagandha (*W. samnifera*). The result indicated that the total dry root yield was not significantly influenced by method of showing and nitrogen application. However, it was marginally higher under application of 25 kg/ ha. Nitrogen than other levels. From foregoing result it is concluded that ashwagandha crop can be grown in Gujrat by Broad casting method with 25 kg N/ha getting maximum dry root yield.

Devkota (2004) investigated the appropriate combination of plants growth regulator's for the micropropagation of *Valeriana Jatamansii* Jones by using node and shoot tip as explant. They had found that MS medium supplemented with BAP 1 mgl^{-1} was suitable for shoot initiation, and MS media with NAA 0.5 mgl^{-1} for rooting.

Basnet (2004) investigated *in-vitro* multiplication of *Citrus aurentifolia,* nodal explants were appropriate than shoot tip explants. Both explants were

cultured in MS media supplemented with BAP at 0, 0.5, 1.0, 1.5 and 2 mgl^{-1} in combination with NAA at 0, 0.5 and 1 mgl^{-1}. In all hormone supplemented media, shoot tip explants developed soft and pale yellow callus after four weeks of culture, which turned into hard callus during the course of subculture. No organogenesis was obtained in further sub culture of shoot tip explants in the same culture conditions. However, when nodal explants were cultured in same culture condition it produced multiple shoots. Maximum shoot number (6.4shoots / explant) was obtained on a medium supplemented with BAP 1 mgl^{-1} + NAA 0.5 mgl^{-1} within six weeks of culture. For *in-vitro* rooting, shoots were cultured on ½ strength of MS medium supplemented with 0.5, 1, 1.5, and 2 mgl^{-1} of IAA, IBA and NAA. All the auxins showed different responses on rooting depending upon their concentration.

Munshi *et al*. (2004) developed protocol for *in-vitro* mass-propagation of turmeric *Curcuma longa* L. using bud from rhizomes. These buds were cultured on MS medium supplemented with different concentration of cytokinins and auxins for shoot multiplication. Best multiple shoot regeneration was found on MS medium fortified with 2 mgl^{-1} BAP and 0.5 mgl^{-1} NAA. In this combination 10-15 initial shoot buds developed within two to three weeks on repeated subculture these shoot buds produced 2-3 folds multiple shoots on the same medium.

Singh *et al*. (2004) developed protocol for mass-multiplication through nodal explant in *Vitis vinifera* in MS medium supplemented with 2 to 4 mgl^{-1} BAP + 0.2 mgl^{-1} NAA. The sprouted shoots were successfully proliferated and rooted on half strength MS medium supplemented with 2 mgl^{-1} IBA and activated charcoal.

Chand *et al*. (2004) developed a protocol for callus induction, regeneration and micropropagation of *Ocimum basilicum* (lamiaceae). Two cytokinins were used, both BAP 2 mgl^{-1} and kin 2.5 mgl^{-1} were 100% responsive in shoot let formation. Length of the shoots were more in kin

supplemented media where as BAP supplemented media showed reduced shoot length. Multiple shots were also observed in some nodal explants in 1.5 mgl^{-1} kin and 2 mgl^{-1} BAP.

Gustavo *et al.* (2005) developed protocol for the *in-vitro* propagation of *Rubus geoides.* This proposed protocol allowed a multiplication rate of 1: 35 to obtain on MS medium supplemented with 1.10 µM BAP. Most (80%) of the shoots rooted on MS with half strength macronutrient salts and 2.46 µM IBA.

Ratna Rai and K .K. Mishra (2005) induced multiples shoots of *Carissa carandas* CV. Pant Sundrshen on MS basal medium supplemented with 3 mgl^{-1} BAP.

Bhosle *et al.* (2005) developed a protocol for multiple shoot regeneration and plant production through shoot tip culture of *Alysicarpus rugosus* D.C. var chinencis Baker on MS + 2.85 µM IAA plus 2.22 µM BAP after 4 weeks. Shoot elongation (3.0 – 3.5 cm) was achieved on M.S medium without any hormones. Rooting occurred in MS medium containing IAA (1.14-2.85 µM) alone or in combination with IBA (0.89 - 2.64) and or NAA (1.07-2.69 µM). Maximum rooting was established in M.S. medium supplemented with 2.85 µM IAA.

Gurung (2005) cultured shoot-tip explant of *in-vitro* grown *Aerides odorata* on MS medium supplemented with BAP 0.5-2.0 mgl^{-1} and NAA 0.5-2.0 mgl^{-1} singly and in combination. In the culture where BAP was used alone, best result (5.3 shoots / culture) was obtained at 1.5 mgl^{-1}. The number of Shoots decreased below and above this level of BAP. When NAA was supplied alone, highest number of shoots (5.7 shoots per culture) was obtained at 1.0 mgl^{-1}, while the number decreased above and below this concentration. NAA at 2 mgl^{-1} induced callus formation which failed to differentiate further. Among all the three rooting hormones (IAA, IBA and NAA), IAA was highly effective for rooting. IAA at 0.5 mgl^{-1} formed highest number of roots (4.7 roots / shoot).

The number of roots decreased with the increase in the concentration of IAA to 2 mgl^{-1}.

Salma *et al.* (2005) studied the effect of *Withania somnifera* (L.) Dunal root extract in preventing DNA damage (one of the mechanisms of action might involve scavenging of active oxygen radicals generated in reactions initiated by lead nitrate pollutant)

Owais *et al*. (2005) evaluated the antibacterial activity of ashwagandha against pathogenic bacteria. Both aqueous and alcoholic extract of the plant (root as well as leaves) were found to possess strong antibacterial activity against a range of bacteria including *Salmonela typhimurium.*

Das *et al*. (2005) formulated a successful protocol for *in-vitro* regeneration of *Vitex negundo* from nodal explants. Plantlets were directly regenerated from the nodal explants on M.S. medium supplemented with different concentrations of BAP, NAA and Kin. Multiple shoots were developed on MS medium supplemented with 0.1 mgl^{-1} IBA.

Sivanesaer and Murugesan (2005) were developed an efficient protocol for high frequency plant regeneration from leaf explants of *Withania somnifera* (L.) Dunal on MS medium supplemented with different concentration of auxins and cytokinins. Frequency of shoot bud regeneration varies with does of plant growth regulators in the medium. Highest frequencies of shoot buds were obtained at a concentration of 1.0 mgl^{-1} Kin. *In-vitro* rooting of micro shoots was obtained by growing them in ½ strength MS medium supplemented with 2.0 mgl^{-1} IBA. Rooted plantlets were successfully transferred to the field after acclimatization in the net house.

Faisal, M. and Anis (2005) developed callus from nodal explants of *Tylophora indica* (Burmf) on MSmedium supplemented with 10 µ M 2, 4, 5 tri-Chlorophenoxy acetic acid. Multiple shoots induction was achieved from the

surface of the callus on MS medium containing 5.0 μM Kin. These rooted plantlets were successfully acclimatized in green house condition.

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 and 1mgl^{-1} BAP + 1 mgl^{-1} NAA. Nodal explants were most suitable for shoot multiplication on MS + 1 mgl^{-1} BAP. For *in-vitro* rooting of shoots MS medium supplemented with 1 mgl^{-1} NAA was found most effective.

Misic *et al*. (2006) developed a protocol for *in-vitro* propagation of Balkan endemic plant *Salvia brachyodon* Vandas from nodal segments. They found that BAP was more effective in axillary bud promotion when compared to thidizuron. The rooting of regenerated shoots was induced by transferring them to the media supplemented with auxins. All tested auxins IAA, IBA and NAA stimulated rooting of *S.brachyodon* shoots. The acclimatization of *in-vitro* rooted shoots were successful.

Hepaksoy, S. and U. Aksoy. (2006) observed that MS medium complemented with 1.2 and 2.5 μM IBA or 1.0 μ M NAA were better in respect to rooting. Peat followed by volcanic tuff gave the best performance for acclimatization to out door condition.

Singh *et al*. (2006) has developed a protocol for plant regeneration from encapsulated shoot tips collected from *in-vitro* proliferated shoots of *Withania somnifera* (L.) Dunal. The best composition was achieved using 3% sodium alginate and 75 mm $CaCl_2.2H_2O$. The maximum percentage response (87%) for convention of encapsulated shoot tips into plantlets was achieved on MS medium supplemented with 0.5 mgl^{-1} IBA after 5 weeks of culture. The conversion of encapsulated shoot tips into plantlets also occurred when calcium alginate beds having entrapped propagules were directly shown in autoclaved soil moisture with ¼ MS salt.

Senthilnathan *et al*. (2006) studies the immunomodulatory effect of *Withania somnifera* (L). Dunal root powder extract on animals. They reported that Benzo pyrene induced cancer in animals was treated with 400 mg/kg body weight of *W. somnifera* extract for 30 days significantly alters the level of immuno component cells. Based on the data, the carcinogens as well as the paclitaxel affects the immune system, the toxic effect on immune system is more reversible and more controllable by *W. somnifera* root extract.

Gururaj *et al*. (2007) developed on efficient and highly reproducible protocol for micro-propagation of bird eye chili *Capsicum frubescens.* They found that shoot tip explants 100% grew well on MS medium supplemented with 1 mgl^{-1} kin. The regenerated shoots with 4-7 nodes had further growth upon sub culturing on to kin 1 mgl^{-1} + IBA 1mgl^{-1} and rooted simultaneously. The rooted plants were transferred to pots after hardening under controlled condition. The survival percentage in the pots was 80-90%.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Materials

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

The initiating material used for the present investigation was *Withania somnifera* (L.) Dunal, highly important medicinal plant. Its seeds were obtained from Koteshwar medicinal plant garden.

3.2 Methodology

Murashige and Skoog (1962) MS medium was used as the basal medium for the present experiment. Three types of phytohormones or plant growth regulators (PGRs) were used viz. Gibberellic acid (GA_3), Cytokinins and Auxins. Gibberellic acid was only used for the seed germination. Cytokinins and Auxins were supplemented with MS medium either alone or in combinations.

3.2.1 Sterilization of Glassware's and Metal Instruments

During the experiment, the necessary glassware's and metal instruments were subjected to dry heat sterilization before their use. Glassware's such as beakers, conical flasks, culture tubes, measuring cylinder, Petri dishes and metal instruments were dipped in detergent-water solution for 24 hours and rinsed with tap water, and final rinse was done with distilled water. Then the glassware's and metal instruments were sterilized in hot air oven at 150^{o} C for 30 minutes. Metal instruments were wrapped with aluminum foil before keeping inside the hot air oven.

3.2.2 Preparation of Stock Solution for MS Medium

- Stock solutions were prepared in the given concentration by first weighing the standard amount of each chemical ingredient one by one on electronic balance.
- These chemicals were dissolved in double distilled water one by one according to protocol with the help of magnetic stirrer.
- Due to light sensitivity each stock solution were kept in pre-sterilized brown bottles. These bottles were labeled by indicating the nature of stock and date. These were finally kept in refrigerator at 4ºC for latter use.

The chemical composition of MS medium is as follows.

A. Macro-nutrients

Chemical Components	**Components of MS (final Concentration in mg/l)**	**(10x) gm/l stock concentration**	**Volume to be taken for 1 liter medium**
Potassium nitrate (KNO_3)	1990	19.0	100ml
Ammonium nitrate (NH_4No_3)	1650	16.5	
Magnesium sulphate ($MgSo_4.7H_2O$)	370	3.7	
Calcium chloride ($CaCl_2.2H_2O$)	440	4.40	
Potassium dihydrogen phosphate (KH_2PO_4)	170	1.70	

B. Micronutrients

Chemical Components	Components of MS (final Concentration in mg/l)	(100x) gm/l stock concentration	Volume to be taken for 1 liter medium
Boric acid (H_3BO_3)	6.2	620	1 ml
Magnese sulphate ($MnSo_4.\ 4H_2O$)	22.3	2230	
Zinc sulphate ($ZnSo_4.\ 7H_2O$)	8.6	860	
Disodium molibdate ($Na_2MoO_4.2H_2O$)	0.25	25	
Copper sulphate ($CuSo_4.\ 5H_2O$)	0.025	2.5	
Cobalt chloride ($CoCl_2.6H_2O$)	0.025	2.5	
Potassium Iodide (KI)	0.83	83	

C. Iron Source (Fe, EDTA)

Chemical Components	Components of MS (final Concentration in mg/l)	(10x) gm/l stock concentration	Volume to be taken for 1 liter medium
di-Sodium ethylene diamine tetra acetatate (Na_2EDTA) $C_{10}H_{14}Na_2O_8.2H_2O$	3.73	373	10 ml
Ferrous sulphate ($FeSo_4.7H_2O$)	27.8	278	

D. Vitamins

Chemical Components	Components of MS (final Concentration in mg/l)	(10x) gm/l stock concentration	Volume to be taken for 1 liter medium
Glycine $H_2NCH_2.COOH$	2.0	200	1ml
Nicotinic acid CH:N.CH:CH.CH:C.COOH	0.5	50	
Pyridoxine HCl $C_8H_{11}NO_3.HCl$	0.5	50	
Thiamine HCl $C_{12}H_{17}N_4OSCl.HCl$	0.1	10	
Myo-inositol CH (CH). $(CH.OH)_4$ CH. CH.	100.0	10,000	

E. Carbon Source

Chemicals	g/liter
Sucrose ($C_{12}H_{22}O_{11}$)	30 g

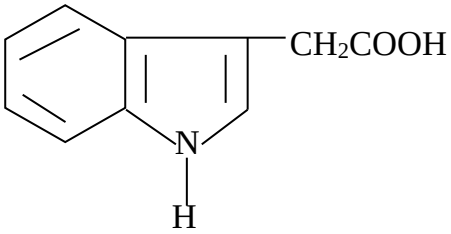
F. Carbon Source

Chemicals	g/liter
Difco Bacto agar	8 g

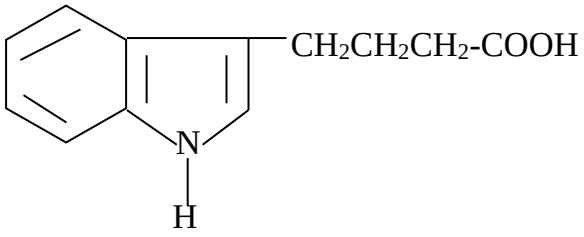
Hormones Used for the Experiments

(a) Auxins

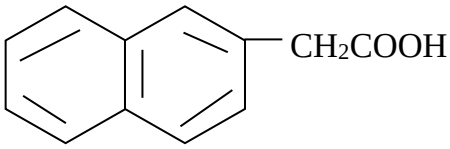
i. Indol - 3 - Acetic acid (IAA)
ii. Indol – 3- Butyric acid (IBA)
iii. Napthalene acetic acid (NAA)



Indol-3- acetic acid
(IAA)



Indol-3-butyric acid
(IBA)

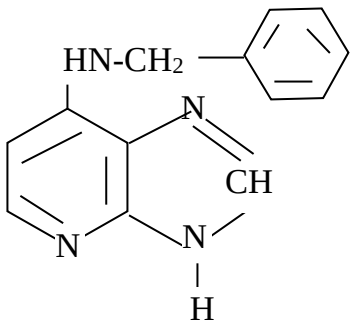


Napthalene acetic acid
(NAA)

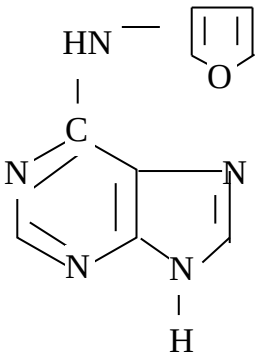
(b) Cytokinins

(i) Benzyl amino purine (BAP)

(ii) Kinetin (Kin)

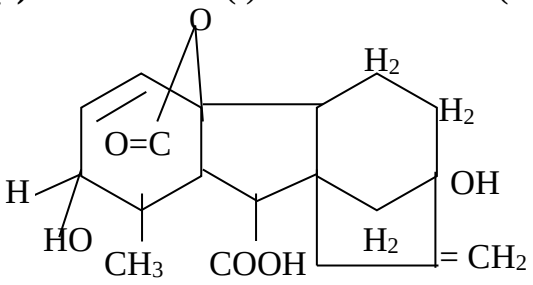


6-Benyl amino purine
(BAP)



Kinetin
(kin)

(c) Gibberilins: (i) Gibberellic acid (GA_3)



Gibberellic acid
(GA_3)

3.2.3 Preparation of Hormone Stock Solution

(i) Preparation of GA_3 (Gibberellic acid)

- 10 mg of GA_3 was dissolved in 2 ml of 70% ethyl alcohol.
- Then 98 ml of double distilled water was added to make its volume 100 ml.
- By using the formula any required concentration of hormone can be made.

$$\text{Wt. of substance in mg.} = \frac{\text{Vol. of water in ml}}{1000} \times \text{required ppm of hormone}$$

Therefore, 10 mg of GA_3 dissolved in 100 ml water became 100 ppm or 100 mg/l GA_3 stock solution.

(ii) Preparation of kin (Kinetin)

- 10 mg of kin was first dissolved in 2 ml of 95% ethyl alcohol.
- Next 48 ml. of double distilled water (dH_2O) was added and gently warmed till complete dissolution of hormone.
- Finally 50 ml of dH_2O was added to make total volume 100ml. Now it became 100 ppm or 100 mgl^{-1} kinetin stock.

3.2.4 Preparation of Media

For the preparation of 1 liter medium, following protocol was applied.

- 1 liter sterilized conical flask was taken.
- 400 ml of distilled water was taken in this flask.
- 100 ml of stock A, 1 ml of stock B, 10 ml of stock C and 1 ml of stock D were added one by one in the flask. [Myoinsitol was added in the fresh form by weighing its required amount i.e. 1 mg. for 1 liter MS medium]

- 30 gm. of sucrose was weighted and dissolved in stock solution.
- Distilled water was added to make final volume 1000 ml. The solution was stirred by magnetic stirrer in order to mix the stock and sugar solution more homogeneously.
- pH of the solution was adjusted (5.6 to 5.8) with 0.1 N NaOH or HCl.
- For the preparation of hormonal medium, hormone stocks were added according to the media requirement by using the volumetric analysis formula for concentration

$$[S_1V_1 = S_2 V_2]$$

Where:

S_1 = Concentration of hormone stock given in ppm or mgl^{-1}

V_1= Volume of hormone stock required in ml.

S_2= required concentration of hormone in the MS medium in ppm or mgl^{-1}

V_2= Volume of medium required for the investigation in ml

$$\text{Volume of hormone stock required in ml } (V_1) = \frac{\text{Required conc. of hormone in the MS medium in ppm } (S_2) \times \text{Volume of MS medium required for the investigation in (ml) } (V_2)}{\text{Concentration of hormone stock given in ppm } (S_1)}$$

- The medium was solidified by adding 8 gm of Agar in 1000 ml MS medium or 8% Agar in 100 ml. MS medium. It was heated with the help of heater up to boiling point to dissolve the agar. When the solution become clear, it was poured in culture tubes or beakers. Then each tubes or beakers were enclosed by aluminium foil or thermo stable plastic caps.

- The tubes/ beakers containing medium were sterilized in an autoclave at the temperature of 121.5ºC and pressure of 15-lb / sq inch for 15 minutes. After cooling down of autoclave tubes/ beakers were taken out and kept in culture room supplemented with air condition. (Tubes were kept in slanting position to increase the surface area of inner medium)

3.2.5 Preparation of Inoculation Chamber

- For inoculation, the laminar air flow chamber or clean bench was thoroughly cleaned by 70% ethanol or spirit.
- All the necessary materials except the plant materials were surface sterilized by ethanol and exposed under U.V. light for 1 hour.
- Then the air blower was kept on for 30 minutes prior to inoculation. In the mean time U.V. light turn off, gas lamp was burned; both hands were washed with detergent soap followed by antiseptic and spirit.
- Then the inoculation procedure was carried out, by dipping inoculating apparatus in spirit followed by flaming at each step of inoculation to check further infection.
- After the completion of inoculation, all the materials were taken off, chambers light, blower and gas lamp turned off. Laminar air flow chamber was again cleaned by using sprit or ethanol.

3.2.6 Sterilization and *In-vitro* Inoculation of Seeds

- Before going to inoculation, healthy pods were selected from the plant followed by removal of seed cover.
- The seeds were thoroughly cleaned by detergent Tween-20 solution and put under running tap water for about 2 hour, then treated with GA_3 for 24 hours. After that the seeds were washed with double distilled water and taken in laminar air flow hood.

- In the laminar air flow hood the seeds were again surface sterilized with 70% ethanol for 2 minutes followed by 1% sodium hypochlorite for 10 minutes and finally washed three times with double distilled water.
- Equal numbers of surface sterilized seeds were inoculated on MS basal medium containing culture tubes.
- Culture tubes were placed in the culture room at 25 $\pm$ 2^{o}C temperature and 12 to 15 hours photo period for obtaining sterile plants prerequisite for further experiments.

3.3 Culture of Explants

- The different explants i.e. shoot tips, nodes, leaves and roots were excised aseptically from eight to ten weeks old *In-vitro* grown seedlings in the laminar air flow hood.
- These explants were inoculated on MS basal medium supplemented with different concentrations of phytohormones.
- From this culture experiment, appropriate combinations of MS + Phytohormones were identified, in which multiplication was maximum.

3.3.1 Sub-culture of Explants

After identification of appropriate combination of MS + Phytohormone the explants i.e. shoot tips and nodes were subcultured for mass propagation.

3.3.2 Sub-culture of Callus

Callus obtained from shoot tip and node cultures were subcultured into different combinations of MS + phytohormones for multiplication.

3.3.3 Sub-culture of Shoots into Rooting Medium

Healthy shoots of 3 to 4 node stages were taken out from the multiplication medium and transferred aseptically into MS medium

supplemented with different concentration and combination of rooting hormones i.e. (IAA, IBA, NAA)

3.3.4 Acclimatization

- This stage involves the shift of micro-plants to out door environment or from heterotrophic to an autotrophic environment.
- The eight to twelve weeks old, well rooted *in-vitro* plantlets were taken off from the culture laboratory.
- Culture tubes caps were opened and plants were exposed for four to five days at room temperature.
- After that the plants were gently washed with distilled water followed by five minutes treatment with fungicide (Bavistine 1%).
- The plants were immediately transferred into four types of hardening media – coco-pit, sand, sand soil mixture and coco-pit + garden soil 50% each.
- Immediately upon transplanting, rooted micro-plants were kept in very high humidity followed by gradual exposure to outdoor condition for hardening.

3.4 Preparation of Bellings Iron-Acetocarmine (1926)

- 100 ml of 45% Acetic acid were prepared by mixing 45 ml of Glacial acetic acid and 55 ml of distilled water.
- Mixture was heated at boiling point and 1 gm of carmine is added. This mixture is boiled for couple of minutes by stirring with gloss rod and leaved it to cool down.
- After cooling down it was filtered with the help of filter paper, few drops of 45% Acetic acid with Ferric hydroxide was added and placed it at room temperature.

3.4.1 Study of Callus

- A small amount of callus was taken into Petri dish containing Acetocarmine solution.
- It was gently warmed, if callus were hard then few drops of 0.1N HCl was added to loose its parenchymatous wall.
- Small amount of callus were taken on slide and covered with cover slip, followed by gentle squash to observe its internal structure under the microscope.

3.5 Statistical Analysis

For statistical analysis, SPSS (Statistical Package for Social Science) version 11.5 was used for statistical analysis; means were based on 4 replicates for each treatment. Data were analyzed using t-test with a significant level of $\leq$ 0.05.

CHAPTER FOUR
RESULT

4.1 *In-vitro* Study of *Withania somnifera* (L.) Dunal

4.1.1 Seed Culture

The Seeds were pretreated with GA_3 100mgl^{-1} and 50 mgl^{-1} for 24 hours. These seeds were cultured on MS basal medium. Those seeds which were treated with 100 mgl^{-1} GA_3 germinated within six days as compare to those 50 mgl^{-1} GA_3 treated seeds which took ten days for its germination. The percentage of germination was same 80% in both conditions. Complete plantlets were developed after four weeks of culture, for further experiment (Fig 2).

4.1.2 Culture of Explants

After obtaining a good seedlings, different explants i.e. shoot-tips, nodes, leaves and roots were excised and cultured on MS basal medium supplemented with different concentrations of Auxins (IAA, IBA, NAA) and Cytokinins (Kin, BAP) either alone or in combination.

4.1.3 Culture of Shoot-tips

The shoot tips of length 3-4 mm were cultured on MS basal medium and MS medium supplemented with different concentrations of Kinetin, BAP, NAA + BAP and NAA for inducing multiple shoots. Following observations were made in successive two weeks of cultures, given below in table.

Table No. 1

Shoot-tip Culture on MS+BAP in Various Concentrations

S.N.	Concentration of BAP on MS media in [mgl^{-1}]	No. of replicates	Response (No. of shoots)					
			2 weeks	4 weeks	6 weeks	8 weeks	After 8 weeks	Callusing
1.	0.5	1	1	1	1	1	1	++
		1	1	1	1	1	1	+++
		1	1	1	1	1	1	+++
		1	1	1	1	1	1	+++
2.	1.0	1	1	2	2	2	2	+++
		1	1	1	2	2	2	+++
		1	2	3	3	3	3	+++
		1	1	-	-	-	-	+
3.	1.5	1	1	Callus	Callus	Callus	Callus	+++
		1	1	Callus	Callus	Callus	Callus	+++
		1	1	Callus	Callus	Callus	Callus	+++
		1	1	Callus	Callus	Callus	Callus	+++
4.	2.0	1	1	Callus	Callus	Callus	Callus	+++
		1	1	1	Callus	Callus	Callus	+++
		1	1	Callus	Callus	Callus	Callus	+++
		1	1	Callus	Callus	Callus	Callus	+++

Culture condition - MS solid medium, $25 \pm 2^{o}C$ temperature, 8 weeks, 12-15 hrs. photoperiods, 4 replicates were used in each combination.

Where: (+) - moderate callus

(++) - good callusing

(+++) - Profuse callusing

(-) - nil

Table No. 1.1

Effect of Different Concentrations BAP on Shoot-tip Culture of *W. somnifera* (L.) Dunal

S.N.	Concentration of BAP on MS media in [mgl^{-1}]	Response	No. of Shoots
1.	0.5	Single shoot with small brown callus at the base. The plantlets have 2-5 leaves, the brown coloured callus turned in dark brown colour in eight week (Fig.3).	Single shoot
2.	1.0	Two to three plantlets with light purple coloured callus at the base. Plantlets were shorter and thicker with many buds and thinner leaves. In tenth week whole callus along with plantlet turned into black brown colour (Fig. 4).	Three shoots
3.	1.5	Callus formed from the base of shoot tip in third week. In sixth week shoot disappear in light brown and light green coloured callus. Its vigorous growth was reported after eight week of culture (Fig.5).	No shoots
4.	2.0	Similar result were obtained like IIIrd condition but the callus were dark green and harder (Fig. 6).	No shoots

Table No. 2

Shoot-tip Culture on MS + Kin in Various Concentrations

S.N.	Concentration of Kin. On MS media in [mgl^{-1}]	No. of replicates	Response (No. of shoots)					
			2 weeks	4 weeks	6 weeks	8 weeks	After 8 weeks (mean ± SD)	Callusing
1.	0.25	1	1	3	5	5		+
		1	1	2	4	4		+
		1	1	2	3	4	**4.25 ± 0.5**	++
		1	1	1	2	3		+
2.	0.5	1	1	4	5	6		++
		1	1	4	6	6		+
		1	1	3	4	4	**5.5 ± 1.0**	++
		1	1	3	6	6		+
3.	1.0	1	1	6	8	8		++
		1	1	9	9	12		++
		1	1	10	12	16	**15.5 ± 4.272**	++
		1	1	8	12	14		++
4.	1.5	1	1	2	2	3		+++
		1	1	3	4	4		+++
		1	1	3	4	4	**3.75 ± 0.05**	+++
		1	2	4	4	4		+++
5.	2.0	1	1	1	2	2		+++
		1	1	1	2	2		+++
		1	1	1	3	4	**3.0 ± 1.12**	+++
		1	1	2	4	4		+++

Culture condition- MS solid medium, 25 ± 2°C temperature, 12-15 hrs photoperiods, 8 weeks, 4 replicates were used in each combination.

Where: (+) moderate callus, (++) good callusing, (+++) Profuse callusing, (-) nil

Table No. 2.1

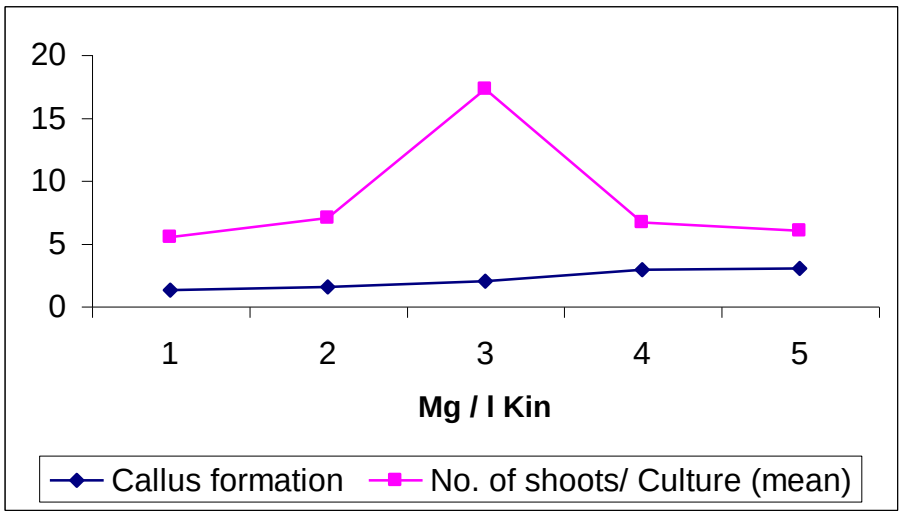
Result of t-test of Shoot-tip culture, Comparing Number of Shoots among Different Concentration of Kin, after 8 Weeks of Culture

S.N.	Treatments		t-value	Level of significant (p)
A.	MS + 0.25 mgl^{-1} Kin	MS + 0.5 mgl^{-1} Kin	-2.236	0.067
		MS + 1mgl^{-1} Kin	-5.115	**0.002**
		MS + 1.5 mgl^{-1} Kin	1.414	0.207
		MS + 2 mgl^{-1} Kin	1.987	0.094
B.	MS + 0.5 mgl^{-1} Kin	MS + 1mgl^{-1} Kin	-4.444	**0.004**
		MS + 1.5 mgl^{-1} Kin	3.130	**0.020**
		MS + 2 mgl^{-1} Kin	3.273	**0.017**
C.	MS + 1 mgl^{-1} Kin	MS + 1.5 mgl^{-1} Kin	5.347	**0.002**
		MS + 2 mgl^{-1} Kin	5.536	**0.001**
D.	MS + 1.5 mgl^{-1} Kin	MS + 2 mgl^{-1} Kin	1.192	0.278

For significance (P) value must be $\leq$ **0.05**

Graph No. 1

Statistical Data Showing Effect of different concentration of Kin on Shoot-tip Culture



Where, 1 = 0.25 mgl^{-1} Kin, 2 = 0.5 mgl^{-1} Kin, 3 = 1 mgl^{-1} Kin, 4 = 1.5 mgl^{-1} Kin, 5 = 2 mgl^{-1} kin

Table No. 2.2

Effect of Different Concentration of Kin on Shoot-tip

S.N.	Concentration of Kin on MS media in [mgl^{-1}]	Response	No. of Shoots
1.	0.25	Small dark green callus was formed, from the side and surface of the callus multiple shoots were emerged after three weeks of time. After eight weeks of culture the callus turned into black colour. The plants were distinct with nodes and internodes. The growths of shoots are very moderate (Fig. 7 and 8).	Multiple shoots (3 to 5)
2.	0.5	From the base of shoot tip green callus were formed. The surface of callus gave rise four to six thin shoots which were very distinct in node and internodes. These shoots were very much similar to seedlings of *W. somnifera* the growth of shoots were fast (Fig. 9, 10, 11, 12).	Multiple shoots (4 to 6)
3.	1.0	The shoot-tip transformed into callus, form the surface of callus multiple shoots (up to twenty shoots per culture) were developed, but the leaves were very thin and nodes and internodes were thick. The growths of shoots were slow (Fig. 13, 14, 15, 16,).	Multiple shoots (10 to 20)
4.	1.5	Soft, light brown callus in the form of patches were observed, the upper portion of callus gave rise two to three shoots which were short, fleshy with thin leaves. The shoots showed stunted growth. After 8 weeks plant shown necrotic symptoms (Fig. 17, 18).	Multiple shoots (3 to 4)
5.	2.0	Soft light green callus appeared after three weeks of culture. Short, fleshy shoots with longer petiole and thinner leaves were appeared after five weeks of culture. The growths of shoots were very slow (Fig. 19, 20).	Multiple shoots (2 to 4)

4.1.4 Culture of Nodes

The nodes of about five to eight mm were taken from *in-vitro* grown seedlings. The nodes were cultured on MS medium supplemented with BAP

and Kin separately. The inoculated nodes started to show visible differentiation and growth after seven days of culture. Above the contact point of media the swelling of nodes were started. The observation was recorded in every two-week intervals (up to ten weeks).

Table No. 3

Node Culture on MS + BAP in Various Concentrations

S.N.	Concentration of BAP on MS media in [mgl^{-1}]	No. of replicates	Response (No. of shoots)					
			2 weeks	4 weeks	6 weeks	8 weeks	After 8 weeks	Callusing
1.	0.5	1	1	1	1	1	1	++
		1	1	1	1	1	1	++
		1	1	1	1	1	1	++
		1	1	1	1	-	-	+
2.	1.0	1	1	1	1	1	1	+++
		1	1	1	callus	callus	callus	+++
		1	1	1	callus	callus	callus	+++
		1	1	1	1	1	1	+++
3.	1.5	1	1	1	callus	callus	callus	+++
		1	1	1	callus	callus	callus	+++
		1	1	1	1	callus	callus	+++
		1	1	1	1	1	1	+++
4.	2.0	1	1	callus	callus	callus	callus	+++
		1	1	callus	callus	callus	callus	+++
		1	1	callus	callus	callus	callus	+++
		1	1	callus	callus	callus	callus	+++

Culture condition- MS solid medium, 25 ± 2^0 C temperature ,12-15 hrs photoperiod, 8 weeks, 4 replicates were used in each combination.

Where: (+) moderate callus, (++) good callusing, (+++) Profuse callusing,

(-) nil

Table No. 3.1

Effect of Different Concentration of BAP on Node Culture

S.N.	Concentration of BAP on MS media in [mgl^{-1}]	Response	No. of Shoots
1.	0.5	Single plantlet with small light green callus surrounded by light brown patches. The plantlet has four to ten leaves. The light green callus turned dark brown colour in eight week (Fig. 21).	Single shoot
2.	1.0	Single shoot with multiple leaves. The shoots were thick and fleshy without any distinction between node and internodes. The shoots apical portion covered with dense thin leaves. The basal portion covered by black brown callus. The growth of shoot was very slow (Fig. 22).	Single Shoot
3.	1.5	The node swelled and became light brown callus like mass. Stunted growth of shoot. Shoot turned yellow colour at the end of eight week and completely died after ten week (Fig. 23).	Single shoot
4.	2.0	Hard and friable callus formed with dark green upper surface surrounded by white patches (Fig. 24).	No shoot

Table No. 4

Node culture on MS + Kin in Various Concentrations

S.N.	Concentration of Kin on MS media in [mgl^{-1}]	No. of replicates 4/4	Response (No. of shoots)					
			2 weeks	4 weeks	6 weeks	8 weeks	After 8 weeks (mean±SD)	Callusing
1.	0.25	1	1	2	2	2	**4.0 ± 1.414**	+
		1	1	3	5	5		++
		1	1	4	5	5		+
		1	2	3	3	4		+
2.	0.5	1	1	4	4	5	**7.75 ± 1.26**	+
		1	1	6	8	8		+
		1	2	8	8	8		++
		1	1	5	7	8		++
3.	1.0	1	1	8	14	16	**16.25 ± 5.91**	++
		1	1	5	10	12		+++
		1	2	6	12	18		+++
		1	2	4	6	9		+++
4.	1.5	1	1	1	2	3	**3.75 ± 0.58**	+++
		1	1	2	4	4		++
		1	2	3	4	4		+++
		1	1	1	2	3		+++
5.	2.0	1	1	2	3	4	**3.25 ± 0.25**	+++
		1	1	2	3	3		+++
		1	1	3	4	4		+++
		1	1	2	2	3		+++

Culture condition- MS solid medium, 25 ± 2°C temperature, 12-15 hrs photoperiods, 8 weeks, 4 replicates was used in each combination.

Where: (+) moderate callus, (++) good callusing, (+++) Profuse callusing, (-) nil

Table No. 4.1

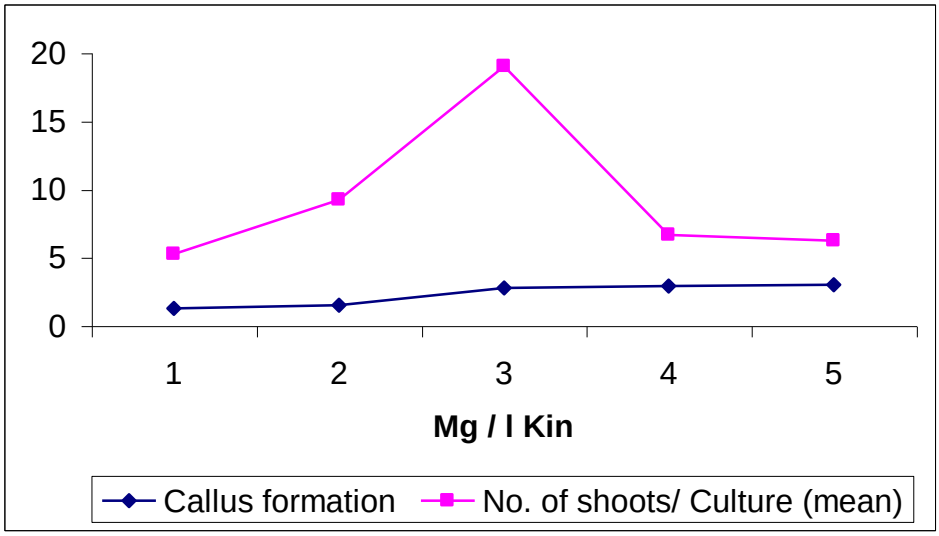
Result of t-test of Node culture, Comparing Number of Shoots Among Different Concentration of Kin, after 8 Weeks of Culture

S.N.	Treatments		t-value	Level of significant (p)
A.	MS + 0.25 mgl^{-1} Kin	MS + 0.5 mgl^{-1} Kin	-3.962	**0.007**
		MS + 1mgl^{-1} Kin	-4.032	**0.007**
		MS + 1.5 mgl^{-1} Kin	0.655	0.537
		MS + 2 mgl^{-1} Kin	0.333	0.750
B.	MS + 0.5 mgl^{-1} Kin	MS + 1mgl^{-1} Kin	-2.814	0.31
		MS + 1.5 mgl^{-1} Kin	6.140	**0.001**
		MS + 2 mgl^{-1} Kin	5.908	**0.001**
C.	MS + 1 mgl^{-1} Kin	MS + 1.5 mgl^{-1} Kin	4.295	**0.005**
		MS + 2 mgl^{-1} Kin	4.216	**0.006**
D.	MS + 1.5 mgl^{-1} Kin	MS + 2 mgl^{-1} Kin	-0.655	0.537

For significance (P) value must be $\leq$ **0.05**

Graph No. 2

Statistical Data Showing Effect of different concentration of Kin on Node culture



Where, 1 = 0.25 mgl^{-1} Kin, 2 = 0.5 mgl^{-1} Kin, 3 = 1 mgl^{-1} Kin, 4 = 1.5 mgl^{-1} Kin, 5 = 2 mgl^{-1} kin

Table No. 4.2

Effect of Different Concentration of Kin on Node Culture

S.N.	Concentration of Kin on MS media in [mgl^{-1}]	Response	No. of Shoots
1.	0.25	The node swelled into light green callus. From the surface of callus shoots were protruded out. After eight weeks multiple shoots were emerged. The callus became dark brown colour in tenth week. (Fig. 25, 26, 27).	Multiple shoots (2 to 5)
2.	0.5	Six to eight shoots were emerged from the upper cut edge of the node. The apical portion of shoot has dense foliage. The shoots were very normal in appearance like seedling plantlets and were also healthy and distinct in node and internodes. (Fig. 28, 29, 30).	Multiple shoots (6 to 8)
3.	1.0	The node turned into a green callus from which multiple shoots were emerged. The shoots were short fleshy and little distinct in node and internodes. The growth was slow. (Fig. 31, 32, 33)	Multiple shoots (9 to 22)
4.	1.5	The nodal portion transformed into white-brown callus from which multiple shoots were sprouted. The shoots were thick and indistinct with dense foliage at the apical portion. The growth was very slow. (Fig. 34)	Multiple shoots (3 to 4)
5	2.0	Patches of light green callus were formed, from which two to three short fleshy, indistinct shoots emerged out The growth of shoots were very slow. After ten week all shoots were turned in to yellow colour. (Fig. 35)	Multiple shoots (3 to 4)

The node and shoot-tip explants cultured in MS + 0.5 mgl^{-1} kin showed the most effective response for shoot multiplication in *in-vitro* condition. These inoculated explants gradually transformed into a small green callus. An average of 5.5 shoots per culture was emerged from the upper cut edge of the node. The apical portions of shoots were covered by dense foliage. The shoots also bear one to two branches. The node explants were selected for mass propagation

protocol because it lack physical injury during inoculation and also available in multiple number as compare to shoot-tip explants (single shoot tip explant per plant).

Table No. 5

Shoot-tip/Node Culture on MS + BAP and NAA in Various Concentrations

S.N.	Composition of media	No. of replicates	Response (No of shoots)				
		4/4	2 weeks	4 weeks	6 weeks	8 weeks	After 8 weeks
1.	MS	1	-	-	Callus	Callus	Callus
		1	-	-	Callus	Callus	Callus
		1	-	-	Callus	Callus	Callus
		1	-	-	Callus	Callus	Callus
		1	-	-	Callus	Callus	Callus
2	MS + 0.5 mgl^{-1} BAP	1	1	1	1	1	1
		1	1	1	1	1	1
		1	-	Callus	Callus	Callus	Callus
		1	-	-	Callus	Callus	Callus
3.	MS + 1.0 mgl^{-1} BAP	1	1	2	2	2	2
		1	1	4	4	5	5
		1	1	2	2	2	2
		1	1	1	1	2	2
4.	MS + 1.5 mgl^{-1} BAP	1	Callus	Callus	Callus	Callus	Callus
		1	-	-	Callus	Callus	Callus
		1	-	Callus	Callus	Callus	Callus
		1	1	1	Callus	Callus	Callus
5.	MS + 0.5 mgl^{-1} NAA	1	Callus	Callus	Callus	Callus	Callus
		1	-	-	Callus	Callus	Callus
		1	-	-	Callus	Callus	Callus
		1	-	Callus	Callus	Callus	Callus
6.	MS + 0.5 mgl^{-1} BAP + 0.5 mgl^{-1} BAA	1	1	1	1	1	1
		1	1	1	Callus	Callus	Callus
		1	1	Callus	Callus	Callus	Callus
		1	1	1	Callus	Callus	Callus
7.	MS + 1.0mgl^{-1} BAP + 0.5 mgl^{-1} NAA	1	1	5	6	6	6
		1	1	3	Callus	Callus	Callus
		1	1	Callus	Callus	Callus	Callus
		1					

8.	MS + 1.5mgl^{-1} BAP + 0.5 mgl^{-1} NAA	1	1	4	6	6	6
		1	2	5	6	8	8
		1	4	8	10	10	10
		1	-	-	-	-	-
9.	MS+1.0mgl^{-1} NAA	1	-	Callus	Callus	Callus	Callus
		1	-	Callus	Callus	Callus	Callus
		1	-	-	Callus	Callus	Callus
		1	-	-	-	Callus	Callus
10	MS + 0.5 mgl^{-1} BAP + 1.0 mgl^{-1} NAA	1	-	Callus	Callus	Callus	Callus
		1	-	-	Callus	Callus	Callus
		1	-	-	Callus	Callus	Callus
		1	-	-	-	Callus	Callus
11.	MS + 1 mgl^{-1} BAP + 1.0 mgl^{-1} NAA	1	-	Callus	Callus	Callus	Callus
		1	-	-	-	Callus	Callus
		1	-	Callus	Callus	Callus	Callus
		1	-	Callus	Callus	Callus	Callus
12.	MS + 1.5 Mgl^{-1} BAP + 1.0 mgl^{-1} NAA	1	-	-	Callus	Callus	Callus
		1	-	Callus	Callus	Callus	Callus
		1	-	Callus	Callus	Callus	Callus
		1	-	Callus	Callus	Callus	Callus
13.	MS + 1.5 mgl^{-1} NAA	1	-	-	-	-	-
		1	-	Callus	Callus	Callus	Callus
		1	-	Callus	Callus	Callus	Callus
		1	-	Callus	Callus	Callus	Callus
14.	MS + 0.5 mgl^{-1} BAP + 1.5 mgl^{-1} NAA	1	-	-	-	-	-
		1	-	Callus	Callus	Callus	Callus
		1	-	Callus	Callus	Callus	Callus
		1	-	-	Callus	Callus	Callus
15.	MS + 1.0mgl^{-1} BAP + 1.5 mgl^{-1} NAA	1	-	-	Callus	Callus	Callus
		1	-	-	-	Callus	Callus
		1	-	-	Callus	Callus	Callus
		1	-	-	-	-	-
16.	MS + 1.5 mgl^{-1} BAP + 1.5 mgl^{-1} NAA	1	-	Callus	Callus	Callus	Callus
		1	-	-	-	Callus	Callus
		1	-	-	-	Callus	Callus
		1	-	Callus	Callus	Callus	Callus

Culture condition: MS solid medium, 25 $\pm$ 2^{o}C temperature, 12-15 hrs photoperiod, 10 weeks, 4 replicates were used in each combination.

Table No. 5.1

Effect of Different Concentration of NAA + BAP on Shoot–tip and Node Explants Culture

S.N.	Composition of media	Response	No. of Shoots
1.	MS	Node / shoot-tip explants transformed into white callus. After five weeks of culture explants were turned into brown colour (Fig. 36).	_
2.	MS + 0.5 mgl^{-1} BAP	Single plant with small light green callus at the base was seen in third week. The light green callus turned into dark brown colour in tenth week (Fig. 37).	Single shoot
3.	MS + 1.0mgl^{-1} BAP	Four to five plantlets with light purple coloured callus at the base. Plantlets were shorter and thicker without any distinction in node and internodes (Fig. 38).	(four to five) shoots
4.	MS + 1.5 mgl^{-1} BAP	Callus formed at the base of the explants in third week in sixth week explants were transformed into light brown callus (Fig. 39).	_
5.	MS + 0.5mgl^{1} NAA	The basal portions of explants were swelled into light purple coloured callus. No any shoots and leaves were observed (Fig.40).	_
6.	MS + 0.5 mgl^{-1} BAP + 0.5 mgl^{-1} NAA	Soft, dark brown callus with dark green upper surface. A short pale and fleshy shoot appeared from upper part of the callus, Nodes and internodes are not distinct in the shoot (Fig. 41).	_
7.	MS + 1.0 mgl^{-1} BAP + 0.5 mgl^{-1} NAA	Soft, dark brown callus with dark green patches on it. The green portion of callus gave rise several short, yellow shoot buds (Fig. 42).	_
8.	MS + 1.5 mgl^{-1} BAP + 0.5 mgl^{-1} NAA	Multiple shoot buds were developed from the surface of the callus. After four week these buds turned into small shoots without any distinction between node and internodes (Fig. 43).	Six to ten shoots
9.	MS + 1.0mgl^{-1} NAA	Soft dark brown callus in the form of patches was observed. No any shoots and leaves were seen (Fig. 44).	_

10.	MS + 0.5 mgl^{-1} BAP + 1.0mgl^{-1} NAA	Soft dark brown callus with white patches around it were observed. No any shoots and leaves were developed.	_
11.	MS + 1.0mgl^{-1} BAP + 1.0mgl^{-1} NAA	Hard callus was formed with dark green lower surface and brown on upper surface. No any shoots and leaves were observed	_
12.	MS+ 1.5 mgl^{-1} BAP + 1.0mgl^{-1} NAA	Dark brown callus without any shoots were formed (Fig. 45).	_
13.	MS + 1.5mgl^{-1} NAA	No any growth taken place (Fig. 46).	_
14.	MS 0.5 mgl^{-1} BAP 1.5 mgl^{-1} NAA	Dark brown callus with very poor growth No any either root or shoot were observed	_
15.	MS + 1.0mgl^{-1} BAP + 1.5mgl^{-1} NAA	Small dark brown callus without any shoot.	_
16.	MS + 1.5mgl^{-1} BAP + 1.5mgl^{-1} NAA	Small dark brown callus without any shoot	_

4.1.5 Culture of Leaf Segment

The leaf explant obtained from *in-vitro* grown plantlets was cut into the section of about 3 mm each. These segments were treated with 100 mgl^{-1} concentration of IBA. The time of (dip) treatments were 15 and 30 minutes. The section of leaf was cultured on hormone free MS medium (full and half strength). The observation were taken periodically up to eight weeks

Table No. 6

Culture of Leaf Segment on MS medium after the Treatment with IBA

S.N.	Composition of media	Response			
		Two week	Four week	Six week	Eight week
1.	½ MS + 100 mgl^{-1} IBA (15 min)	-	3/3 Clb	3/3 Clb	3/3 Clb
2.	½ MS + 100 mgl^{-1} IBA (30 min)	-	3/3 Clb	3/3 Clb Cw	3/3 Clb Cw
3.	MS + 100 mgl^{-1} IBA (15 min0	-	3/3 Cb	3/3 Cb	3/3 Cb
4.	MS + 100 mgl^{-1} IBA (30 min)	-	3/3 Cw	3/3 Cw	3/3 Cw

Culture condition: MS solid medium, 25 ± 2 °C temperature, 12-15 hrs photoperiod, 8 weeks, 3 replicates were used in each combination.

Where: Clb = light brown callus
Cw = white patches around the callus
Cb = brown callus.

All the leaf explants were transformed into small white brown callus in fourth week and didn't shown any further change. Finally in eight week it turned into dark brown coloured callus (Fig. 47, 48, 49, 50, 51).

4.1.6 Culture of root segment

The root explant obtained from *in-vitro* grown seedling was inoculated on MS medium supplanted with or without PGRs.

The root explant when cultured on MS + 0.5 mgl^{-1} kin or MS alone, it swelled and remains as such without any response till fourth week. After four week some small green patches were seen on the upper portion of root in both medium (with hormone and hormone free).

4.1.7 Sub-culture of Callus on Different Concentration of BAP

Callus obtained after culture of shoot tip / node on 1 mgl^{-1} kin medium were sub-cultured on MS medium augmented with different concentration of BAP (Fig. 52 to 54).

Table No. 7

Sub-culture of Callus on Different Concentration of BAP

S.N.	Explant	Treatment	Callusing	Other response
1	Callus	MS + 0.5 mgl^{-1} BAP	+	-
			++	-
			++	-
			+++	-
2.	Callus	MS + 1.0 mgl^{-1} BAP	+++	-
			++	-
			+++	-
			+++	-

Culture condition: MS solid medium, 12-15 hrs photoperiod, 10 weeks, 3 replicates were used in each combination.

Where: (+) moderate callus, (++) good callusing, (+++) profuse, (-) nil

MS medium supplemented with 0.5 mgl^{-1} BAP formed light brown callus with green patches around it, in five weeks of time.

Very little change in the structure of callus had taken place till sixth week (Fig. 58). Cytological study reveals mass of elongated and some spherical cells (Fig. 56). After ten weeks the growth of callus was retarded.

In case of 1mgl^{-1} BAP supplemented MS medium callus started to show initiation of light brown protuberances with green patches after three weeks of culture. After that, callus slowly increases in mass till eight week. After ten week the growth of callus completely stopped without any considerable change. The callus was hard and friable in nature (Fig. 55).

Its cytological study reveals uninucleated elongated and spherical cells (Fig. 57).

4.1.8 Sub-culture of Callus on Different Concentration of Kin

Callus (from 1 mgl^{-1} kin medium) were sub-cultured on MS medium supplemented with different concentration of kin (Fig. 60).

Table No. 8

Sub-culture of callus on Different Concentration of Kin

S.N.	Explant	Treatment	Callusing	2 weeks (no. of shoots)	4 weeks (no. of shoots)	6 weeks (no. of shoots)	8 weeks (no. of shoots)	After8 week no. of shoots (mean ± SD)
1.	Callus	MS + 0.5 mgl^{-1} Kin	+	2	8	12	12	**14.5 ± 4.12**
		MS + 0.5 mgl^{-1} Kin	++	4	10	14	18	
		MS + 0.5 mgl^{-1} Kin	+	3	7	9	10	
		MS + 0.5 mgl^{-1} Kin	+	4	12	9	10	
2.	Callus	MS + 1 mgl^{-1} Kin	++	10	14	16	18	**14.25 ± 2.63**
		MS + 1 mgl^{-1} Kin	+++	8	10	12	12	
		MS + 1 mgl^{-1} Kin	++	3	7	12	14	
		MS + 1 mgl^{-1} Kin	++	6	8	10	13	

Culture condition: MS solid medium, 25 ± 2 °C temperature, 10 weeks of culture, 12-15 hrs photoperiod, 3 replicates were used in each combination.

Where: (+) moderate callus, (++) good callusing, (+++) profuse callusing, (-) nil.

Table No. 8.1

Result of t-test of Sub-culture of Callus, Comparing Number of Shoots among Different Concentration of Kin, after 8 Weeks of Culture

S.N.	Treatment		t-value	Level of Significance (P)
1.	MS+0.5 mgl^{-1} Kin	MS+1 mgl^{-1} Kin	0.102	0.922

For significance (P) value must be $\leq$ **0.05**

MS medium supplanted with 0.5 mgl^{-1} kin showed the initiation of green micro shoots (Fig. 57). After four weeks, micro shoots were transformed into multiple shoots with distinct node and internodes. After eight weeks whole culture tube was filled by healthy multiple shoots (Fig. 59). Cytological study reveals up to three celled stage (Fig. 63).

In case of MS + 1mgl^{-1} kin, small green multiple propagules of leafy shoots were emerged (Fig. 60). After four weeks of culture these small propagules became short, fleshy and little distinct multiple shoots (Fig. 61). After eight weeks, whole culture tube was filled by healthy little distinct multiple shoots (Fig. 62). Cytological study reveals multiple spherical clumps of elongated cells (Fig. 64).

4.1.9 Subculture of Shoot-tip and Node Explants

For mass propagation the shoot-tip and node explants (from *in-vitro*) grown plants) were cultured and re-cultured on multiple shoot forming medium.

First of all, all these explants were cultured on MS + 1 mgl^{-1} kin medium on which it multiplied up to 22 shoots per explant within four weeks of time (But these shoots were not distinct into node and internodes). Immediately after multiplication these shoots were transferred on MS + 0.5 kin supplemented

medium where healthy multiple shoots with distinct node and internodes were protruded out.

4.1.10 Rooting of Shoots

For root initiation, *in-vitro* regenerated shoots of length 1.5 to 2 cm (2 to 3 node stage) were used for rooting purpose (Fig.65). MS medium supplemented with different root initiating hormones (Auxins) viz. IAA, IBA and NAA, showed various responses of rooting could be tabulated below.

Table No. 9

Effect of Different Concentration of IAA on Rooting of Shoots, after six weeks of Culture

S.N.	Composition of media in mgl^{-1}	Number of roots per culture (mean ± SE)	Length of roots (cm) (mean ± SE)	Other responses
1.	MS + 0.5 mgl^{-1} IAA	50 ± 0.50	4.1 ± 0.19	Roots were normal in appearance (Fig. 66,67, 68)
2.	MS +1 mgl^{-1} IAA	25 ± 0.30	4.6 ± 0.77	Roots were comparatively thicker (Fig. 69,70)
3.	MS + 1.5 mgl^{-1} IAA	20 ± 0.50	4.2 ± 0.21	More thicker (Fig. 71,72)

Culture condition: MS solid medium, 25 ± 2 ^{0}C temperature, 8 weeks of culture, 12-15 hrs photoperiod, and 6 replicates were used in each combination.

Table No. 10

Effect of Different Concentration of IBA on Rooting of Shoots after six weeks of Culture

S.N.	Composition of media in mgl^{-1}	Number of roots per culture (mean ± SE)	Length of roots (cm) (mean ± SE)	Other responses
1.	MS + 0.5 mgl^{-1} IBA	13.5 ± 0.75	3.6 ± 0.5	Thicker roots, with many white hairy roots around the basal portion of shoot (Fig. 73, 74, 75, 76)
2.	MS+1mgl^{-1} IBA	4.2 ± 0.5	2.8 ± 0.35	Few roots, at the base some hairy roots were observed (Fig. 77, 78).
3.	MS + 1.5 mgl^{-1} IBA	4.2 ± 0.25	25 ± 0.80	Around the main root lots of white hairy roots were observed (Fig. 79, 80).

Culture conditions: MS solid medium, 25 ± 2 ^{o}C temperature, 8 weeks of culture, 12-15 hrs photoperiod, and 6 replicates were used in each combination.

Table No. 11

Effect of Different Concentration of NAA on Rooting of Shoots after Six Weeks of Culture

S.N.	Composition of Media in mgl^{-1}	Number of roots per culture (mean ± SE)	Length of roots (cm) (mean ± SE)	Other responses
1.	MS + 0.5 mgl^{-1} NAA	1.5 ± 0.50	1.5 ± 0.50	Roots emerged later in fourth week and were thread like in appearance (Fig. 81, 82, 83, 84).
2.	MS+1mgl^{-1} NAA	–	–	Only callus like structure were observed at the basal portion. No any roots were seen (Fig. 85, 86).
3.	MS + 1.5 mgl^{-1} NAA	–	–	Only basal portion swelled in callus (brown) like structure no any roots were observed (Fig. 87,88)

Culture conditions: MS solid medium, 25 ± 2 ^{o}C temperature, 8 weeks of culture, 12-15 hrs photoperiod, 6 replicates were used in each combination.

4.2 Acclimatization

Only few weeks of survival was noted. In coco-pit hardening medium plants were survived for 1 week, in case of sand and sand soil (50% each) mixture 8 weeks of survival were observed. In coco-pit + garden soil (50% each) 2 weeks of survival were observed. However, no any plantlets were survived in out door condition (Fig. 89, 90, 91, 92).

CHAPTER FIVE
DISCUSSION

The morphogenetic response of explants varies with genotype, age, nature and culture conditions. The result obtained from the culture and subculture of various plants parts of *Withania samnifera* (L.) Dunal has been discussed on the basis of relevant literatures.

5.1 Germination of Seeds

The seeds of were pretreated with GA_3 (100 mgl^{-1} and 50 mgl^{-1}) for 24 hours. 80% of seeds were germinated with in six to ten days. Only on MS (without treatment with GA_3) no any seeds germination were taken place. So, the use of GA_3 in this experiment initiated the germination of seeds by breaking the dormancy. Similarly, the work of Hartman and Kester (1972) also showed GA_3 was effective to increase the rate of germination. It also helps to reduce the time of dormancy of seed and also enhance seedlings growth.

Similarly Manandhar (2002) and Karanjit (2003) examined the seed germination of *Capsicum annum* on MS basal medium after GA_3 pretreatment, prior to inoculation. Shrestha and joshi (2004) on *Swertia chirata* and Bhadra *et al*. (2003) on *Bulbophyllum liliacinum* obtained least germination of seeds on MS without any hormone treatment.

5.2 Culture of Shoot-tips

In the present investigation, shoot-tips were inoculated in nine different combination of MS media (alone), MS supplemented with BAP (0.5 mgl^{-1} to 2 mgl^{-1}) and MS supplemented with kin (0.25 mgl^{-1} to 2 mgl^{-1}).

Shoot-tips in MS medium (hormone free) developed into white brown callus after five weeks of culture. Further development of callus stopped (Fig.36). It was probably due to lack of any particular growth substance. This

work seems to be similar with the work of Amatya and Joshi (1986), proliferated the callus from stem segments of *Brassica rapa* L. in MS medium without any hormone.

Shoot-tip cultured on MS with lower concentration of BAP (0.5 mgl^{-1} to 1mgl^{-1}), one to three plantlets with light brown callus at the base were formed. These plantlets were shorter and thicker with many buds in the basal portion and thinner leaves. The nodes and internodes were not distinct. In MS supplemented with higher concentration of BAP (1.5 to 2 mgl^{-1}), only callus were formed. These calluses were protruded out from the basal portion of shoot tip (Table no- 1 and 1.1). Various workers have also induced shoots and callus from the shoot-tip explant of different plants in different concentrations and combinations of BAP with MS. Jorder *et al.* (1988) developed a protocol for rapid multiplication of *Morus alba* L. through shoot-tip and lateral bud explant on MS + 1 mgl^{-1} BAP resulted in remarkable increase in plant. High concentration of BAP decreases the plant height but enhances more shoot production around the callus. Similarly Gurung (2005) obtained multiple shoots (5.3 shoots / culture) from shoot-tip explant of *Aeries odorata* on MS +1.5mgl^{-1} BAP. He also reported that the number of shoots decreased below and above this level of hormone concentration.

Gustavo *et al*. (2005) developed a protocol for the *in-vitro* propagation of *Rubus geoids* on MS + 1.10 μM BAP. Chrystiane *et al.* (2004) found the inclusion of BAP in MS medium induced callus formation *in Ficus carica* L. plant. Rai *et al.* (2005) found that shoot proliferation was higher on MS basal medium supplemented with 3 mgl^{-1} BAP in *Carssia carandas* L. plant. Like wise Zamir *et al*. (2003) on guava (*Pissum guajara* L.), Poudel (2003) on *Mentha spicata* L. Devkota (2004) on *Valeriana jatamansii* Jones and Chand (2006) on *Clinopodium umbrosum* obtained multiple shoots from shoots tip explant on MS media supplemented with 1 mgl^{-1} BAP. Prashad *et al*. (2003) observed single shoot at 80% efficiency of *Utleria salicifolia* on MS + 0.5mgl^{-1} BAP. Karki *et al*. (1992) observed plantlets from shoot-tip culture of *Musa*

spp. on MS medium supplemented with BAP. Ray and Jha (2001) produced multiple shoots of *Withania somnifera* (L.) Dunal on MS + 0.1 mgl^{-1} BAP. Das *et al.* (2005) obtained multiple shoots in *Vitex negundo* on MS + 1 mgl^{-1} BAP. Basnet (2004) observed soft pale yellow callus in *Citrus aurintifolia* on MS + 0.5 to 1.5 mgl^{-1} BAP after four weeks of culture. Dantu and Bhojwani (1988) initiated the shoot cultures of three commercial cultivars of *Gladiolus corns* on MS + 0.5 mgl^{-1} BAP as a most suitable combination for multiplication.

Shoot-tip cultured on MS with lower concentration of Kinetin (0.25 to 1 mgl^{-1}) formed multiple shoots. These multiple shoots were emerged from the surface of the green callus. While in higher concentration of kinetin (1.5 to 2 mgl^{-1}) callus formation was favored. (Table no- 1).

MS + 0.5 mgl^{-1} kin formed 5.5 shoots per culture (Table no- 2). This combination of medium was proved to be the most effective combination for shoot multiplication. The shoots proliferated in this combination has distinct node and internodes. These shoots were also very much similar to the seedling plantlets. However, in case of MS + 1 mgl^{-1} kin the shoot tips basal portion transformed into dark green callus. From the surface of the callus multiple shoots (15.25 shoots / culture) were developed (Table no- 2, 2.1 and Graph- 1). But these shoots were thick with narrow leaves and indistinct nodes and internodes. Sivanesaer and Murugesan (2005) have also obtained highest frequency of shoot bud regeneration of *Withania somnifera* (L.) Dunal on MS medium supplemented with 1mgl^{-1} kin in *in-vitro* condition. Similarly Gurung *et al*. (2004) also induced multiple shoots from shoot tip explant of *Capsicum frutescens* Mill on MS + 1 mgl^{-1} kin.

In case of MS + 1.5 and 2 mgl^{-1} Kin lesser number of shoots (3.75 and 3.0 shoots / culture) were formed respectively. These shoots were short, fleshly with thinner leaves and indistinct nodes and internodes. Pereira *et al.* (2002) induced multiple shoots of *Anenopaegmg orvense* (an endangered medicinal plant) by using shoot-tip explant on MS + 4.4 μM Kin. Similarly Agrawal and

Subham (2003) induced multiple shoots in *Centella asiatica* (Brahamee) from *in-vitro* raised shoots through callus phase on MS + 10μM Kin.

5.3 Culture of Nodal Segment

The node explants were inoculated on MS medium supplemented with nine different combinations of PGRs: MS medium plus four different concentrations of BAP (0.5 to 2 mgl^{-1}) and MS medium plus five different concentrations of Kin (0.25 to 2 mgl^{-1}).

In the present investigation, lower concentrations of BAP (0.5 to 1mgl^{-1}) induced callus formation in fourth week. These calluses were formed around the base of single shoot having multiple leaves. After eight weeks of culture these calluses were either died or turned into dark brown colour (Table no-3.1). Jorder *et al*. (1988) reported that low concentration of BAP induced the dense emergence of leaves in the *in-vitro* culture of *Morus alba* L. like wise Joshi *et al*. (2000) observed the good proliferation of micro shoots of *Eleocarpus sphararicus* (Gaerth) K. Schum on MS medium supplemented with 0.25 mgl^{-1} BAP. Manandhar (2002) obtained multiple shoots of *Daucus carota* L. on MS + 0.5 mgl^{-1} BAP. Similarly Krishnan and Seeni (1994) found that MS + 0.2 mgl^{-1} BAP concentration induced high frequency of shoot bud proliferation in *Woodfordia fruticosa* L. kurz (a rare medicinal plant). They also reported that shoot multiplication rate was further accelerated by re-culturing 4 to 6 mm nodal segment on 1mgl^{-1} BAP concentration. Batra *et al*. (2001) on *Azadirachta indica* (neem) noted that nodal segment on MS + 1 mgl^{-1} BAP showed slight swelling of the nodal region into callus like structure prior to emergence of shoot buds. Like wise Ranjit *et al.* (2000) on *Foeniculum vulagre*, Neeraja and Prashad (2003) on *W. somnifera*, Raju and Prashad (2003) on *Celastrus paniculatus*, Poudel (2003) on *Mentha spicata* L., Das *et al*. (2005) on *Vitex negundo* (Verbenacera) and Chand (2006) on *Clinopodium album* obtained multiple shoot from nodal explant, on MS medium supplemented with 1 mgl^{-1} BAP.

In case of higher concentrations of BAP (1.5 to 2 mgl^{-1}) nodal segment swelled into callus like structure only or sometimes few small indistinct shoots (in case of 1.5 mgl^{-1} BAP). Above this concentration only callus formation is favored (Table no- 3 and 3.1). Similar to those of the present findings, Jorder *et al*. (1993) observed that, with the increase in BAP concentration from 1 to 5 mgl^{-1}, the number of shoots produced per node culture was reduced considerably. Like wise Kitamura *et al.* (1984) reported that increase in concentration of BAP from 0.1 to 10 mgl^{-1} callus formation is favored in *Swertia pseudochinensis* Hara. Chand *et al*. (2004) on *Ocimum basilicum* (Lamiaceae), observed that higher concentration of BAP (2 mgl^{-1}) showed multiple shoot formation with the reduction in length. Bhatt *et al*. (2004) obtained multiple shoots of *Oroxylum indicum* L. Kurz on MS + 2mgl^{-1} BAP (reduced), on MS + 0.5 mgl^{-1} BAP smaller but elongated shoots were observed. Similarly Rao and Prasad (2003) obtained multiple shoots of *Strychnos potatorum* L. (an endangered medicinal plant) on MS medium supplemented with varying concentrations of BAP (0.5 to 2.5 mgl^{-1}).

Nodal segment cultured on MS medium supplemented with five different concentrations of Kin (0.25 to 2 mgl^{-1}). In case of MS with lower concentration of Kin (0.25 to 1 mgl^{-1}) light green callus along with healthy multiple (4.0 to 16.25) shoots were formed (Table no- 4 and 4.3). Varghese *et al.* (1992) obtained maximum shoot buds of *Vitex negundo* on medium supplemented with low concentration of Kin. Similarly Saeed *et al.* (2005), on *Gossypium hirsutum* L. produced maximum number of shoots (3.43 shoots / explant) on MS + 0.25 mgl^{-1} Kin. Faisal Mohammad and Anis (2005) on *Tylophora indica* (Burmf) merill (an endangered medicinal plant) obtained highest rate 80% of shoot multiplication on MS + 5.0 μM kinetin. These shoots were emerged from the surface of callus. Like wise Sivanesaer and Murugeson (2005) obtained highest frequency of shoot bud regeneration of *Withania somnifera* (L.) Dunal on MS + 1 mgl^{-1} Kin.

Pereira *et al*. (2003) also induced multiple shoots of *Anenopaegmg arvense* (vell) stellfeld ex de souza (an endangered medicinal plant) on MS + 4.4 μM Kin.

In case of MS supplemented with higher concentrations of Kin (1.5 to 2 mgl^{-1}) the node swelled into light green to light brown coloured callus. From the surface of the callus multiple shoots (3.5 to 3.75 shoots / culture) were sprouted out. These shoots were short, thick and densely covered by narrow leaves. The nodes and internodes portion were not distinct. Growths of shoots were also very slow and in tenth week all shoots were turned into yellow colour (Table no- 4, 4.1, and Graph- 2). Agrawall and Subham (2003) on *Centella asiatica* L. observed that 10 μM of kin in the basal medium was proved to be an optimum concentration for callus differentiation. Miyagawa *et al*. (1986) carried out shoot multiplication in *in-vitro* in *Stevia primordia* by using MS + 10 μ M Kin medium. Like wise Chand *et al*. (2004) on *Ocimum basilicum* (Lamiaceae) obtained multiple shoots from nodal explant on MS medium supplemented with 1.5 mgl^{-1} kin.

Among all these concentrations and combinations of Kinetin MS + 0.5 mgl^{-1} kin proved to be an effective combination for the multiple shoot formation in *in-vitro* condition. The inoculated nodes were gradually swelled and transformed into green callus. The upper edged of the callus gave rise (7.75 shoots / culture) multiple shoots. These shoots were healthy and distinct, with one to two branches. The apical portions of the shoots were covered by dense foliage of leaves.

5.4 Effect of Different Concentrations of NAA and BAP on Shoot-tip/ Node Culture

Shoot-tip and nod explants cultured on MS medium supplemented with 0.5 mgl^{-1} NAA did not form any plantlets. The explants were swelled and became light purple coloured callus. Equal concentration of BAP and NAA

formed only dark brown callus or sometimes gave raise a short pale and fleshy shoots without any distinction between nodes and internodes.

However, higher concentrations of BAP (1 to 2 mgl^{-1}) in combination with 0.5 mgl^{-1} NAA (Fig. 41, 42) on MS medium favored multiple shoot formation along with callus at the base. But these shoots were thicker, shorter without any distinction between nodes and internodes. MS medium supplemented with 1 mgl^{-1} NAA or above to this concentration were unfavorable for shoot proliferation. In these conditions only soft dark brown callus were observed. So it became clear that NAA exert inhibitory effect on shoot tip/ node explant culture in *W. somnifera* (Table no-5).

MS supplemented with lower concentrations of BAP along with higher concentrations of NAA favored the formation of dark brown callus without any shoot. However, higher concentrations of BAP (1 to 1.5 mgl^{-1}) along with higher concentrations of NAA (1 to 1.5 mgl^{-1}) also resulted the same, in the present investigation MS + 1.5 mgl^{-1} BAP and 0.5 mgl^{-1} NAA proved little bit effective for the multiple shoot formation. The shoot developed in this particular combination was short and not distinct into nodes and internodes (Table no- 5.1).

Many similar research works has been carried out on different plant species in different parts of the world. Results of some works were found to be similar to our result while the results of other were found to vary. Some research works that support the present work are summarized as under:

- Choi *et al.* (1991) obtained multiple shoots from the shoot apex of *Zingiber officinales* Rose on MS + 0.5 mgl^{-1}NAA and 5 mgl^{-1} BAP.
- Niraula (1994) obtained 2-4 shoots from eight week old Gebera plant on MS + 1 mgl^{-1} BAP and 0.01 mgl^{-1} NAA.
- Kharel and Karki (1994) obtained micro shoots of *Chrysanthemum morifolium* varieties (Giant fishtail violet) on MS + 1 mgl^{-1} BAP and 0.01 mgl^{-1} NAA.

- Rajkarnikar and Saiju (2000) showed the sprouting of micro shoots of *Rauvolfia serpentina* L. Benth on MS + 1 or 2 mgl^{-1} BAP and 0.1 mgl^{-1} NAA.
- Karki and Saiju (2000) obtained multiple shoots from the shoot tip explant of *Amomum subulatum* Roxb on MS + 1 mgl^{-1} BAP and 0.1mgl^{-1} NAA.
- Shahazal *et al*. (2001) obtained 10 to 12 shoots from the ten days old explant culture of *Mentha arvensi* on MS + 5 mgl^{-1} BAP and 0.2 mgl^{-1} NAA.
- Sarowar *et al*. (2003) developed on efficient *in-vitro* micropropagation protocol for direct shoot growth of inter specific hybrid on MS + BAP and NAA in different concentrations.
- Doe *et al.* (2003) developed a protocol for highest amount of shoot production in *Ocimum basilicum* L. on MS + 5 mgl^{-1} BAP and 2 mgl^{-1} NAA.
- Joshi *et al.* (2003) induced multiple shoots from node explant of *Foeniculum vulgare* mill on MS + 1 mgl^{-1} BAP and 0.5 mgl^{-1} NAA.
- Basnet (2004) investigated an effective medium for *in-vitro* micro-propagation of *Coffee arabica* L. on MS + 1 mgl^{-1} BAP and 0.5 mgl^{-1} NAA.
- Singh *et al*. (2004) reported that nodal explant of *Vitis vinifera* L. gave multiple shoots on MS + 2 to 4 mgl^{-1} BAP and 0.2 mgl^{-1} NAA.
- Rajkarnikar *et. al.* (2004) obtained multiple micro shoots of *Cephaelis ipecacuanta* nodal explant on MS + 2 mgl^{-1} BAP and 0.1 mgl^{-1} NAA.
- Liao *et al*. (2004) established rapid micro propagation protocol for *Aloe vera* L var chanensis (How) Berger, on MS + 2 mgl^{-1} BAP and 0.3 mgl^{-1} NAA on which Chinese aloe could multiply 15 times in four weeks of culture.
- Das *et al*. (2005) formulated a successful protocol for *in-vitro* regeneration of *Vitex negundo* (Verbenaceae) from nodal explants.

Plantlets were directly regenerated on MS medium supplemented with various concentrations of NAA and BAP.

5.5 Culture of Leaf Segment

In the present study, the leaf explants were cultured on full and half strength of MS medium after its treatment with 100 mgl^{-1} BAP. Only callus were formed in both of the conditions. The growth of callus was very slow and no organogenesis has taken place (Table no- 6). Shrestha (1999) has shown the induction of callus and organogenesis in *Guizotia abssinica* on MS medium alone.

Rani *et al.* (2003) observed callus induction from cotyledonary leaf explants of *Withania somnifera* (L.) Dunal on MS medium supplemented with various concentration of auxins and cytokinins. Similarly Govindaraju *et al.* (2003) obtained direct differentiation of multiple shoots of *Withania somnifera* (L.) Dunal on MS + BAP (0.5 to 3 mgl^{-1}) and IAA 0.5 mgl^{-1}. Next Sivanesar and Murugesan (2005) also obtained callus in *W. somnifera* (L.) Dunal at various concentrations of auxin and cytokinins.

5.6 Culture of Root Segment

The root explants obtained from *in-vitro* grown seedlings were inoculated on MS medium alone and MS medium supplemented with 0.5 mgl^{-1} of Kin. In both these conditions, it swelled and remained as such without any further change till fourth week. After four week some small green patches were seen on the upper cut edge of the root. No any further change has been observed up to tenth week. Amatya and Joshi (1986) proliferated the calli from stem and root segments of *Brassica rapa* L. in synthetic nutrient media with or without supplements of auxins and cytokinins.

5.7 Rooting of Shoots

In present work *in-vitro* grown shoot explants cultured on MS basal medium and MS medium supplemented with different root initiating hormones (IAA, IBA and NAA) showed various responses on rooting (Table no- 9,10 and 11).

Hormone free medium failed to induce roots in any shoot explants. Though the rooting on MS medium supplemented with 0.5 mgl^{-1} IAA was satisfactory, the rooting in the various concentrations of other two root initiating hormones i.e. IBA and NAA was found to be poor (Table no- 9).MS medium supplemented with lower concentrations of IAA i.e. (0.5 to 1 mgl^{-1}) were much better for rooting purpose. While higher concentrations of IAA (i.e. more than 1mgl^{-1}) leads to thicker and reduced number of root formation out of inoculated shoot explants. Similar to present result Govind Raju *et al*. (2003) also observed good rooting from the stem explants of *Withania somnifera* (L.) Dunal on MS + 0.5 mgl^{-1} IAA. Like wise Pandey (2003) on *Helianthus annus* and Shrestha (1991) on *Swertia chirayita* and Gurung (2005) on *Aerides odorata* obtained good rooting on MS + IAA in various concentrations.

Gustavo and Miriam (2005) reported 80% rooting of *Rubus geoides* on half strength MS + 2.46 μM IAA. Similar to that Bhosle *et al*. (2005) induced maximum rooting of *Alysicarpus rugosus* on MS + 2.85 μM IAA. Zamir *et al.* (2003) observed good rooting of *Psaidium gujava* on half strength MS + various concentration of IAA and IBA both. But Sarowar *et al*. (2003) on cucurbita , Faisal *et al*. (2005) on *Tylophera indica* and Park *et al*. (2005) on *Adonis amurensis* induced *in-vitro* rooting of shoots on MS + IBA at various concentrations. Next Misic *et al*. (2006) observed good rooting of *Salvia brachyodon* shoots on MS medium supplemented with all tested auxins (IAA, IBA and NAA).

CHAPTER SIX
CONCLUSION

The present study is focused on to develop a protocol for mass propagation and cultivation of *W. somnifera.* (L.) Dunal as a cash crop. On the basis of result, following conclusion can be made.

- For the seed germination in MS medium, 100 mgl^{-1} GA_3 treatment for 24 hours was found to be most effective condition.
- For mass propagation protocol node explants were selected because:
    - It formed multiple shoots (16.25 shoots / explant) as compare to any other explant.
    - Comparatively less physical risk of injury during inoculation.
    - Available in multiple numbers as compare to shoot-tip explant (available single in each branch).
- MS + 1 mgl^{-1} Kin and MS + 0.5 mgl^{-1} Kin both these mediums were found to be most effective for mass propagation.
- The various concentrations and combinations of BAP alone and NAA+ BAP were not found as effective as Kin supplemented MS medium for mass propagation protocol.
- For callus culture MS + 1 mgl^{-1} Kin and MS + 0.5 mgl^{-1} Kin were found to be the most effective concentrations for multiple shoot formation in comparison with BAP (0.5 to 2 mgl^{-1}) in which only callus along with few shoots were formed.
- Among all the three rooting hormones (IAA, IBA and NAA), IAA was found to be highly effective concentration for rooting. IAA at 0.5 mgl^{-1} formed highest number of roots (50 roots per shoot explant). The number of root formation decreased with the change in the concentration of IAA.
- For cultivation of *W. somnifera* (L.) Dunal, poor quality of soil (Sandy soil) with less irrigation was required. High watering leads to death of the plantlets.

CHAPTER SEVEN
RECOMMENDATIONS

Following recommendations have been outlined from the present investigation.

- This protocol can be applied for the direct mass propagation of *W. somnifera* (L.) Dunal, a highly exploited medicinal plant.
- Mass propagation of other valuable plant species should be promoted through the establishment of tissue culture laboratories at the regional levels which helps to develop effective protocol.
- Ecologically suitable regions for the cultivation of particular plant species should be surveyed and identified out in the map.
- Local communities should be encouraged through technical and financial support for medicinal plant farming on commercial scale.
- More liberal competitive and market oriented trade sectors should be established in the country to buy the crude raw materials supplied by the local farmers.
- High standard processing industries should be established by the government of Nepal.

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