CHAPTER-1

1. INTRODUCTION

1.1. Background:

Nepal has a wide range of climatic variation due to its geography and topography and almost all types of climate, season and soil from the world (Malla and Shakya, 1968). Due to its geographic and climatic diversity, the relatively small country of Nepal, occupying 0.1% of the world's total land mass is surprisingly rich in biological diversity. It is estimated that there are more than 7,000 flowering plants, 5,891of which have already been identified (Shrestha and Shrestha, 2008). The country ranks between 25th and 30th on the global scale and 11th on the continental scale for richness in floral diversity. Nepal's flora comprises 2.5% of the total global flora. Of the flowering species, 5% are endemic to Nepal and 30% are endemic to the Himalayan Mountains.

As such, Nepal is very rich in biological resources, including native medicinal plants. About 2,000 species have been found to have medicinal value out of which, 1,463 species are being locally used in curing different diseases. These plants are an integral part of traditional eastern medicine. The Ayurvedic (homeopathic) health care system depends solely on the use of these highly valued native medicinal plants. The southern part of the country mainly Terai, Siwalik hills and Dun valleys up to an elevation of 1000 m asl with warm climate have been recognized botanically as Tropical region. Similarly, from 1000 to 2000 m asl elevation with moderate climate is classified as Sub-tropical zone. An account of 49% and 54% of the total number of plant species growing in Tropical and Sub-tropical zones has medicinal properties respectively. Similarly, 18% and 7% of the Sub-alpine and Alpine regions respectively are medicinal (Malla and Shakya 1984-85). Some important medicinal plants of Tropical and Sub-tropical zones are: *Adathoda vasica, Cinnamomum tamala, Aegel marmelos, Asparagus racemosus, Acacia catacheu, Cassia fistula, Butea monosperma, Rauvolfia serpentina and Piper longum* (Maskay, 1996).

1.2. Medicinal Plants:

The herbal medicine is the oldest form of health care known to mankind. Herbs had been used by all cultures throughout history. It is estimated that around 70,000 plant species, from lichens to flowering plants, have been used at one time or another for medicinal purposes. Its history of use goes back to Vedic era, where 289 herbs having medicinal value (with their properties, mode of use, diseases in which they were used etc.) were described in Aatharva Veda and 67 in Rig Veda. Similarly, 81 medicinal herbs were described in Ajurveda, 31 in Upanisad and 129 in Braman Grantha. As being a part of Aatharva Veda, Ayurveda compiled the medicinal herbs systematically and used them to treat different diseases. The practical use of different herbs in the south East Asia in different tribes reveals the popularity of herbs as the integral part of Ayurveda. It has been mentioned that centuries ago philosopher Charak listed over 500 remedies and Sushruta, the father of Ayurvedic surgery, over 700 vegetable remedies. Charak divided the medicinal plants into 50 groups according to the physiological actions of the medicines that can be extracted from them. These fifty groups cover everything from curatives to preventatives. The plants decribed by Charak present a cross-section of medicinal herbs, shrubs and tress ranging from diuretics, cardiac tonics and plant extracts capable to increase fertility of both male and female. The traditional health care system based on Himalayan native plants has a long history. Pieces of literature written in the Nepali, Newari, and Sanskrit languages contain records of Nepali medicinal plants (www.ayurnepal.com, 2010). The original "Saushrut Nighantu," written on palm leaves in Newari script and Sanskrit verses during Mandeva Era 301 (879 AD), is said to be the oldest of these books. Today, western herbal medicine uses many thousand plant species of Europe, North Americas, Africa and Australia. In Ayurveda, about 2,000 plant species mostly from Himalayan forests and meadows are considered to have medicinal value while the Chinese Pharmacopoeia lists over traditional medicines, most of which are of plant origin (5100 plant species) (Chevallier, 1996). The Chinese and Tibetan medicine systems have borrowed many plants from Nepal (Maskay, 1996). About 5,000 herbs are still applied in within conventional medicine. World Health Organization notes that about 74 percent of the plant derived pharmaceuticals are used in modern medicine in the similar ways as used by the natives (traditional use). Major pharmaceutical companies of

the world are currently conducting extensive research on plant materials gathered from the different parts of the world for their potential medicinal value.

The herbs provide the starting material for the isolation or synthesis of conventional drugs. Digoxin, used for heart failure, was isolated from foxglove (*Digitalis purpurea*), and the contraceptive pill was synthesized from constituents found in wild yam *((Dioscorea villosa*) (Chevallier, 1996). Substances derived from the plants are the basis for a large number of commercial medicines used today for the treatment of various diseases. For example, ephedrine, the active ingredient in *Ephedra*, is used in the commercial pharmaceutical preparations for the relief of asthma and other respiratory problems.

The World Health Organization (WHO) estimates that 4 billion people, 80 percent of the world population, presently use herbal medicine for primary health care. Current estimates suggest that, in many developing countries, a large proportion of the population relies heavily on traditional practitioners and medicinal plants to meet primary health care needs. Although modern medicine may be available in these countries, herbal medicines have often maintained popularity for historical and cultural reasons (WHO, 1999). Sixty percent of the world population and 80% of the population in developing countries rely on traditional medicine, mostly plant derived drugs, for their primary health care needs (Shrestha and Dhillon 2003). An account of 70% of the population of India (Gadgil and Rao, 1998), 80% of Pakistan (Ahmad and Ghafoor, 2002) and 80% of Nepal are dependent on traditional plant based medicines (Kunwar *et al.,* 2006).

1.3. Trade and conservation status of the medicinal plants:

Herbs which have always been the principal form of medicine in the developing countries are becoming popular throughout the world. More and more people in Europe, North America and Australia are using the plant based medicines. The total sales of herbal medicines exceeded $3 billion in 1993 in Germany alone. In the same year, sales in the UK and Spain increased year on year by 10 and 35 percent respectively. Growth

figure elsewhere including the US also show a similar rise (Chevallier, 1996). These figures are increasing every year because of their safe mode of action in human health. The history of export of medicinal plants from Nepal seems to begin before 10th century to China. Nepal has been supplying more than 1,000 tons of medicinal plants every year to a number of countries and continues to be a leading supplier to the Indian sub-continent mainly to India (90%) as crude herbs and drugs (Duerbeck and Schaette, 1993). Other main export countries are Japan, Hong Kong, Singapore, Switzerland, France, Germany, Holland, USA and Canada (Maskay, 1996). The most common exported plants are *Lycopodium clavatum, Swertia chirata, Rubia cordifolia, Rauvolfia serpentina, Nardostachys jatamansi, Picrorhiza scrophularifolia, Rheum emodi, Orchis latifolia, Valeriana wallichii* (Maskay, 1996) and *Asparagus racemosus* (Khare, 2007). It is estimated that Nepal can make US$ 10,000,000 per year from the above mentioned plants (Dobremez, 1982). Since, medicinal plants are an open access resources in Nepal (Helle and Olsen, 2007), the two plants undertaken for this research are among the ones heavily collected from the wild. Local healers and local as well as cross border traders are rampantly collecting this species from the wild which is causing a severe threat to its existence in Nepal (Helle and Olsen, 2007). Due to above mentioned importance; medicinal plants are in threatened condition and are listed by as the plant of high economic importance by the government of Nepal.

It has been well pictured by Olsen and Bhattarai, (2005) and Helle and Olsen, (2007) that how medicinal plants are collected and used for trade from Nepal to India and other countries. Approximately, 470,000 house holds are involved in commercial collection of medicinal plants for their livelihood (Olsen, 1998). This figure shows the importance of medicinal plants in the daily life of many of the Nepalese. Most of the collectors are unaware of proper collection methods so that the plant can grow in successive years. A large number of plants are collected with root and rhizomes along with the fruiting part from the natural habitats without leaving any part of it for regeneration. Locals are very poor and unaware of the uses of such species, so they collect them and sell to the local traders for a nominal price (Helle and Olsen, 2007) which ultimately are exported legally or illegally mainly to the neighboring countries.

There are a number of acts and policies to conserve and use such species sustainably but due to lack of proper implementation the conservation part is in seriously critical condition. The continuously increasing demand of the medicinal plants offers new opportunities for the farmers who find that growing herbs is more beneficial than growing regular crops. Another reason for the decrease in medicinal plants' population is due to high population growth rate leading to heavy deforestation either for settlement or to meet the daily energy and other domestic demands. The rise in popularity of herbal medicines also directly threatens the survival of some wild species. Demand for American ginseng (*Panax quinquefolium*) has become so high that it costs around $1,100/Kg. It was a common plant in the forests of North America a couple of centuries ago, but is now an endangered species facing nearly extinction from the wild. This is not the only species facing such condition; many species are similarly threatened around the world. Today, if herbal medicine is to grow at its present rate, it is very important that manufacturers, suppliers, practitioners, and the public use only produce that has been cultivated or wildcrafted in an ecologically sensitive manner (Chevalliar, 1996).

Most of the species's industrial requirements for production of different commodities are met through their wild collections from forests. Very little effort has been made to conserve this species. Conservation of these species has become an immediate need of the country. An assessment of threats to endemic species suggests that 8 species may have become extinct, 3 appear endangered, 7 are vulnerable and 31 are rare (Shrestha, 1999).

If systems of trade are not modified, after few decades, a number of other species will be added in that list and ultimately many of the species will not be left to conserve. Hence, development of the simple, reproducible and cost effective techniques to multiply such species for cultivation and reforestation is necessary for a country like Nepal. Understanding the fact that a number of species are decreasing due to the ever increasing demand in the national as well as international markets, their population in the wild is decreasing. Hence, Ministry of Forest and Soil Conservation, Department of Plant Resources (Herbs and NTFP coordination committee) has identified 30 plant species as

the national priority herbs for thieir development, research and cultivation. Among those 30 species 12 species have been recommended for cultivation among which *Asparagus racemosus* and *Rauvolfia serpentina* are the important ones (MFSC, 2006, Nepal). *Rauvolfia serpentina* was assessed as "Critically Endangered" in Nepal during a 2001 CAMP workshop (Bhattarai et. al., 2002), where it has "already approached extinction in most areas of the country", according to (Bhattarai, 1997). Overharvesting, burning to create areas for livestock grazing, shifting cultivation and land encroachment for cultivation are the major threats (Amatya, 2005) for this species. Following consultation with representatives from the CITES Scientific Authority, Nepal's CITES Management Authority stated that they considered the species to be "threatened in the wild" in Nepal (Sharma, 2006).

1.4. Species selected for the present investigation:

In the present investigation two of the highly important medicinal plants of Nepal *Asparagus racemosus* and *Rauvolfia serpentina* have been selected.

1.4.a. *Asparagus racemosus*: *A. racemosus* Willd. locally known as "Kurilo" or "Shatavari" belongs to the family Liliaceae. It is an undershrub climber with extensively branched woody stems, growing up to 2m in height. The succulent tuberous roots are 30-100 cm long and 1-2 cm thick in bunch attached at the stem base. The leaves are reduced to small scales or needle-like spines called cladodes. The flowers are small, white, fragrant and in simple or branched racemes. When the plants are young, stems are very delicate, brittle and smooth. Its fruits are globular or obscurely 3-lobed, pulpy berries, that are purplish black when ripe; its seeds have hard and brittle testa. This plant can be found growing naturally in the tropical and sub-tropical forests throughout Nepal up to 1500 m above sea level and Nepal is the main source of this species (Khare, 2007). It is also distributed in India, Malaysia, Australia and Africa (Joshi and Joshi, 2001). All together eight species of this plant have been reported from Nepal. These are *Asparagus curillus* Buch.-Ham. ex Roxb., *A. filicinus* Buch.-Ham. ex D. Don, *A. filicinus* var. *brevipes* Baker, *A. officinalis* L., *A. penicillatus* H. Hara, *A. racemosus* Willd., *A.*

racemosus var. *racemosus* Willd. and *A. racemosus* var. *subacerosus* Baker (efloras.org., 2010).

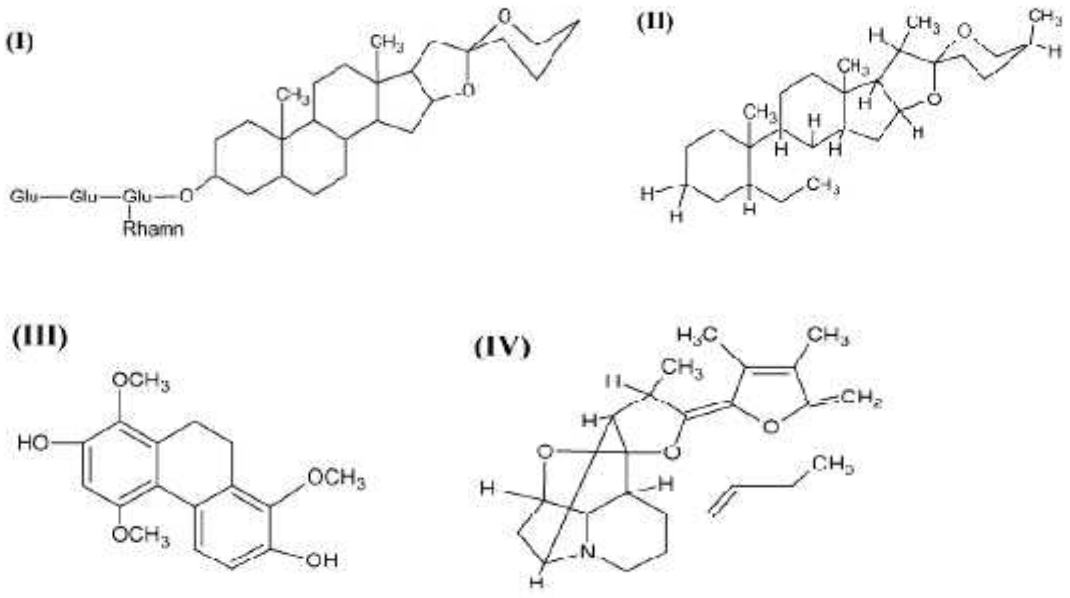
1.4.a.i. Uses and Phytochemical contents of *Asparagus racemosus*:

It is widely used for multiple purposes, and its medicinal importance has been recognized by Ayurveda for centuries. Although almost all parts of this plant have some medicinal properties, roots and young shoots are of higher significance. Young spears are consumed as vegetable or salad and are considered as a balanced health food with many essential nutrients. Traditionally the tuberous roots are used mainly to promote milk secretion and disorders of female genitourinary tract; as a styptic and ulcer healing agent, intestinal disinfectant, astringent in diarrhea, nervine tonic, sexual debility for spermatogenesis. Besides these, Ayurveda indicates the use of tuberous roots in gout, puerperal diseases, lactic disorders, haematuria, bleeding disorders and also recommends for hyperacidity, demulcent, diuretic, aphrodisiac, tonic, alterative, antiseptic, antidiarrheal, glalctogogue and antispasmodic (Goyal *et al.*, 2003). It is also used to treat debility, especially in women, and infertility, impotence, menopause, stomach ulcers, hyperacidity, dehydration, lung abscess, haematemesis, cough, herpes, leucorrhoea and chronic fevers, delay ageing process and form health food ingredients in several Ayurvedic formulations (Kumar *et al.,* 1997). According to (Krishna 2003), *A. racemosus* can be used to treat autoimmune disorder which is of common occurrence. Besides these the bitter, sweet and emollient roots are cooling, opthalimic, nutritive, mucilaginous, refrigerant, anobyne, stomachic and antidysenteric . They are useful in nervous disorders, dyspepsia, tumours, scalding of urine, throat infections, tuberclosis, cough bronchitis, muscle spasms, rheumatism, cancer - strengthens one from and for chemotherapy, convalescence, Immune system boost-good for AIDS, (Ayurnepal.com 2010).

Using the modern scientific tools many active compounds like several steroidal saponins—shatavarins I–IV (phytoestrogen compounds) (Hayes *et al.,* 2006 a and b and Hayes *et al.,* 2007), aglycones, alkaloids like *asparagin*- an anticancer agent (Joy *et al.*, 1998) and many other active pharmacologically important compounds have already been isolated from the roots of this species. Shatavarin IV is a glycoside of sarsasapogenin and

this species contains saponins >15%.(Khare, 2007). Leaves contain rutin, diosgenin and a flavonoid glycoside identified as quercetin - 3 - glucuronide. Flowers contain quercetin hyperoside and rutin. Fruits contain glycosides of quercetin, rutin and hyperoside and steroidal saponins (Mandal *et al.,* 2006) while fully ripe fruits contain cyanidin - 3 - galactoside and cyanidin - 3 - glucorhamnoside. This species especially its roots have been used as medicine since the Vedic time centuries ago (Ayurveda). Recent scientific investigations have confirmed and increased importance of this species in various medicines. Hence this species can be considered wholly a medicine having potential to cure different diseases. These studies have further strengthened the traditional medical knowledge with scientific bases.

This species is in high trade from the forests of Nepal to the international markets. Puri, (2003) have mentioned that the *Asparagus racemosus* from Nepal is highly traded in India because the ayurvedic physicians prefer the roots of *A. racemosus* from Nepal (pale brown slightly resinous) as it is more effective than the Indian ones. Khare, (2007) have also confirmed that the main source of *A. racemosus* in India is Nepal. To meet the demand, a large volume of this species is collected annually from different parts of the country and sold in both local as well as international markets. The exact data however are not available.



Velavan *et al.* 2007 (Figures)

Fig. 1.1 Active principles of *Asparagus racemosus*

(I) Chemical structure of Shatavarin, (II) Chemical structure of Sarsasapogenin, (III) Chemical structure of Racemosol and (IV) Chemical structure of Asparagamine.

1.4.b. ***Rauvolfia serpentina:*** *R. serpentina* (L.) Benth. ex Kurz. belongs to the family Apocynaceae . It is locally Known as "Sarpagandha" and the common names are Rauvolfia, Serpentina, Indian Snakeroot. It is an erect, perennial under shrub of about 73 cm to 1 m in height under very favorable conditions with 2n = 22. Its roots are prominently tuberous and usually branched (0.5 to 2.5 cm in diameter). The roots go up to 40 to 60 cm deep into soil. Its roots are the parts used in medicine because they possess high alkaloid concentration. Most of the alkaloids (approx. 40 to 50 %) are contained in the root skin and the skin (Anonymous, 2008). It is found and distributed in the foot hills of the Himalayan ranges, up to the elevation of 1300 - 1400 m and almost all over the country. Roots nearly verticle, tapering, as much as 15 cm thick at the crown and long giving a serpent-like appearance, occasionally branched or tortuous developing small fibrous roots. Roots are greenish-yellow externally and pale-yellow inside, extremely bitter in taste. Leaves borne in whorls of 3-4, deciduous, elliptic-lanceolate or obovate, acute or acuminate pointed, green on the upper surface, pale-green underneath, 7.5 x 20 cm in size. Flowers numerous, borne in terminal or axillary, long-stocked clusters, tubular, 5-lobed, 1-3 cm long, whitish pink in colour, peduncles 5.0-7.5 cm long, calyx red. Calyx lobes 2.5 mm long lanceolate. Corolla about 1-1.3 cm long, tube slender, inflated slightly above middle, lobes much shorter than tube, obtuse (WHO, 1999). At the onset of fruit/seed, calyx pedicel and flowering stock become bright red. Fruits in pairs, obliquely ovate, 7.5 mm in size, purple black and bluish when ripe.1 or 2 seeds. The chromosome number varies 2n = 20, 22, 24, 44 (Joshi and Joshi, 2001). The plant thrives well in deep fertile soils that are rich in organic matter. It prefers slightly acidic soils (pH 4 - 6.3). Although, the plant is native of tropical humid climate, it grows in tropical and subtropical areas that are free from frost. So far, two species of *Rauvolfia* have been recognized from Nepal viz. *R. serpentina* and *R. tetraphylla* (efloras.org, 2010).

Although *Rauvolfia serpentina* is listed in CITES Appendix II, it is believed that illegal collection and trade is prevalent in Nepal. Traders from Delhi markets stated in late 1990s that although the species was mainly supplied from Uttaranchal, substantial quantities were also imported from Bhutan, Nepal and Pakistan (Traffic India, 1998). Although trade data of this species are not easily available due to different ways of trades, in 2001-2002, Nepal had exported 6.0 metric tons of *R. serpentina* roots to India (Mulliken and Crofton, 2008). *R. serpentina* is traded under the names "Sarpagandha" and "Chandmaruwa" (Amatya, 2005).

1.4.b.i. Uses and chemical composition of *Rauvolfia serpentina*:

Rauvolfia serpentina is one of those highly used medicinal plants, which had been used in Indian sub continent for more than 4000 years. This plant is listed in earliest Ayurvedic medicinal text the Charaka Samhita (c. 700 B.C.) and has been used since that time to treat insomnia and certain forms of insanity (Kataria and Shekhawat, 2005). It was known in folk medicine even in pre-Vedic period. In Ayurvedic medicine, it was used for treatment of snake-bite, insect stings and mental disorders. Later on, it was also reported to be used for treatment of epilepsy, diarrhea, dysentery and as a uterine stimulant to facilitate childbirth in various parts of India. The total alkaloidal extract of the root induces bradycardia, hypotension and sedation. It is applied in hypochondria, neuropsychiatric disorders, psychosis and schizophrenia. According to German Commission E. this species is used in hypertension (borderline hypertension, especially with elevated tension of the sympathetic nervous system), for example, sinus tachycardia, anxiety, tension and psychomotor irritation, when dietetic measures alone are not sufficient (Khare, 2007). It has been popular with certain tribals in Bihar (India) as mad man's medicine for several thousand years. Cardiovascular disease is the number one killer in the world and hence *Rauvolfia serpentina* has been accepted for the trteatment of cardiac diseases in modern medicine (Ahmad *et al.*, 2006). *Rauvolfia serpentina* roots having alkaloids more than 8% have hypotensive effects and they are used either in the powder or paste forms (Khare, 2007). U.S. National Formulary, (1965) have mentioned that in western medicine, either tablets or finely powedered roots of *R. serpentina* have been used to treat hypertension and migraine (Wiart, 2006). In addition,

it is used in modern system of medicine for treatment of various disorders like sleeplessness, insomnia, mental disorders, fever etc (Anonymous, 2007a). *Rauvolfia serpentina* contains reserpine, an indole alkaloid which blocks the adrenergic transmission by depleting norepinephrine from sympathetic neurons (Wiart, 2006). Although, a number of species of *Rauvolfia* are reported to contain medicinally important alkaloids, only two species, viz., Serpent Wood (*Rauvolfia serpentina*) and African Serpentwood (*Rauvolfia vomitoria*) are commercially known to be important and used for isolation of therapeutically important indole alkaloids. The main hypotensive alkaloid - reserpine, which is part of oleoresin fraction, was isolated by Muller and co-workers in 1952, after which the plant was adopted in modern medicine as an effective hypotensive agent and tranquilizer (Anonymous, 2007b). *Rauvolfia serpentina* is indigenous to moist deciduous forests of South East Asia including Nepal, India, Bangladesh, Burma, Sri Lanka, Thailand, Myanmar and Indonesia. Most of the supply of this drug is obtained from wild sources of this region. Hence this species is listed in the Appendix II of CITES which means the species is not yet threatened with extinction, but which could become endangered if trade is not controlled. This species is in the IUCN threat status E and has been listed in the National list of plants banned for export.

Zaire is the largest producer and exporter of the drug to Europe (Anonymous, 2007b). The root and root bark are rich in alkaloids, the most important being reserpine, others are, ajmaline, ajmalicine (raubasine), ajmalicine, yohimbine, coryanthine, iso-ajmaline, neo-ajmaline, papaverine, raubasine, rauwolscine, sarpagine, serpentine, serpentinine, serpinine and deserpidine. Reserpine is hypotensive and tranquilizer, used for certain forms ofmental disorders. Ajmalicine (raubasine) and rescinnamine are also hypotensive and tranquilizer (Fabricant and Farnsworth, 2001). Deserpidine is sedative, as well as hypotensive. Ajmaline exhibits antiarrhythmic activity (Yarnell, 2004)

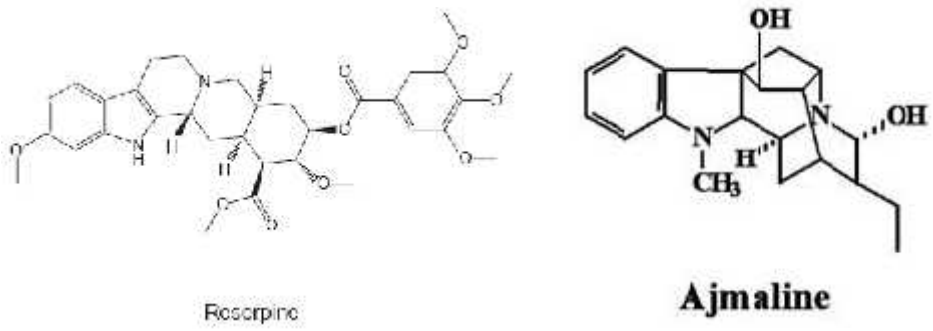


Fig. 1.2- Active principles of *Rauvolfia serpentina.* (I) Chemical streucture of Reserpine and (II) Chemical streucture of Ajmaline.

1.5. Conservation and awareness for sustainable use:

As Nepal is the main source of *Asparagus racemosus* required in the Ayurvedic formulations both in Nepal and India. A large volume of this species is collected annually from different parts of the country and sold in both local as well as international markets. The exact data however are not available. Similarly, *R. serpentina* got its value due to a large number of medicinally active alkaloids in its roots. This importance is causing its excessive collection from the forests of Nepal though a few efforts have been made to conserve them in the community forests. Its indiscriminate use and poor methods of conventional propagation and conservation have led this species to be included in the list of endangered plants. *R serpentina* merit special attention for their economic value and present critical condition in the ecosystem due to unsustainable extraction (Mishra, 2008). Understanding this fact, the government of Nepal, Ministry of Forest and Soil Conservation has identified these species for conservation and to study for the identification of methods to propagate and cultivate. Some efforts have been made in different parts of the country to cultivate these species specially in the community forests of the terai and government farms. Local awarness regarding these species is very little but in a growing trend. The locals are very poor and unaware of the importance and sustainable harvesting techniques of these species. Hence, they are attracted towards collection of maximum amounts they can and sell to the local traders to meet their daily monetary demands. Beside these the seed germination ability of the seeds and the seedling reaching maturity of these species vary greatly ranging from 5-26 % (Baksha *et al.*, 2007) in *R. serpentina* and 17-60 % in *A.racemosus* (Gupta *et al.*,

2002). All these reasons are leading to the depletion of the number of these species in nature. *R. serpentina* is already in the CITES Appendix II and if preventive measures are not taken in time *A. racemosus* also is running rapidly towards the list.

1.6. Plant Tissue Culture (Micropropagation)

Micropropagation can be defined as the cultivation of plant parts: cells, tissues and organs under aseptic and environmentally supportive conditions in/on a synthetic media that is nutritionally supportive *in vitro.* This method is based on the principle of "**Totipotency**". Totipotency literally means the ability of an isolated undifferentiated plant cell including somatic cell to develop in to a whole plant (Cassells and Gahan, 2006). Steward, (1970) showed that individual cells isolated from carrot-derived callus could be cultured to produce individual carrot plants.

1.6.1. History of Plant Tissue Culture:

Some 70 years ago, Phillip White discovered the science of plant tissue culture when he successfully maintained cut tomato roots *in vitro* (Hopkins, 2007). Mainly developed in the early 1960s, plant tissue culture has turned into a standard procedure for modern biotechnology and today one can recognize five major areas where *in vitro* cell cultures are currently applied: large-scale and rapid propagation of elite materials (Ahuja, 1992 and Jain *et al.,* 1995) , multiplication of genetic modified fertile individuals, as a model system for fundamental plant cell physiology aspects, preservation of endangered species (Loyola-Vargas and Vázquez-Flota, 2006), and metabolic engineering of fine chemicals (Singh, 2004). Today, micropropagation is a money making biotechnology business. Many common houseplants, fruit trees, and forest trees began life in a test tube or petri dish. By the early 1980s, growers in the Netherlands alone were producing over 21 million plants annually by micropropagation (Hopkins, 2007).

1.6.2. Importance of Plant Tissue Culture:

Number of advantages of micropropagation over traditional methods of plant propagation has been recognised. One is that exceptionally large numbers of seedlings can be produced more rapidly in a very limited space (Ahuja, 1992; Jain *et al*., 1995). In

the floral industry, it is commonly used to produce clones of cultivars (varieties under cultivation) that are particularly popular because of flower color or other characteristics. The technique is used extensively in the production of forest tree species for plantations and reforestation because it avoids the tedious process of collection and germinating of seeds. Moreover, trees with superior characteristics can be cloned for higher productivity. Micropropagation is also an effective way to eliminate viruses and other pathogens. Potato is another plant that is often troubled with virus infections. The most effective way to eliminate potato viruses is by micropropagation of virus free lines through shoot-tip cultures (Kartha, 1981). It may be possible *in vitro* to multiply plants that are very difficult to propagate by cuttings or other traditional methods. Large numbers of genetically identical clones may be produced (Murashige, 1990). Seeds can be germinated with no risk of damping off/predation. Under certain conditions, plant material can be stored *in vitro* for considerable periods of time with little or no maintenance. Tissue culture techniques are used for genetic manipulation, somatic hybridization and other procedures that benefit propagation, plant improvement, and basic research. Tissue culture is an essential part of many genetic transformation protocols.

Micropropagation is not always perfect. In spite of the fact that clones are supposed to be identical, somaclonal variations can arise sometimes. Most somaclonal variants are discarded, but occasionally one exhibits a particularly useful trait relating to field crops or an interesting flower color, so all is not lost (Hopkins, 2006). Such variation is rarely detectable with DNA markers. Thus, somatic embryogenesis induced in callus tissues of *Panax notoginseng* resulted in plantlets that could not be differentiated even with a large set of RAPD primers (Shoyama *et al.*, 1997). Similarly, no variation in RAPD banding patterns was encountered among first-regeneration cycle somaclones of *Hypericum perforatum*, whereas three out of 51 somaclones obtained from the second cycle differed by a single band (Haluskova and Kosuth, 2003).

1.6.3. Culture types and their applications:

Cultures are generally initiated from sterile pieces of a plant's part. These pieces are termed 'explants', and may consist of pieces of organs, such as leaves or roots, or may be specific cell types, such as pollen or endosperm. Many features of the explant are known to affect the efficiency of culture initiation. Generally, younger, more rapidly growing tissue (or tissue at an early stage of development) is most effective. Several different culture types most commonly used in plant micropropagation studies are described in more detail below.

1.6.3.1. Callus cultures:

Explants, when cultured on the appropriate medium, usually with both an auxin and a cytokinin, can give rise to an unorganised, growing and dividing mass of cells from any part of the plant. This proliferation can be maintained indefinitely if the callus is subcultured on to fresh medium periodically. The callus is usually composed of unspecialised parenchyma cells. Callus culture is generally performed in the dark as light can initiate differentiation of the callus. During long-term culture, the culture may lose the requirement for auxin and/or cytokinin. This process is known as 'habituation'. Manipulation of the auxin to cytokinin ratio in the medium can lead to the development of shoots, roots or somatic embryoids from which whole plants can subsequently be produced. Callus cultures are extremely important in plant biotechnology. Callus cultures can also be used to initiate cell suspensions, which are used in a variety of ways in plant transformation studies. Callus can also be used as a source of protoplasts in protoplast cultures. Callus cultures fall into one of two categories: compact or friable. In compact callus the cells are densely aggregated, whereas in friable callus the cells are only loosely associated with each other and the callus become soft and breaks apart easily. Calli are the good material for *in vitro* production of secondary metabolites.

1.6.3.2. Cell-suspension cultures

Friable callus provides the inoculum to form cell-suspension cultures. When friable callus is placed into a liquid medium and then agitated, single cells and/or small clumps of cells are released into the medium. Under the correct conditions, these

released cells continue to grow and divide, eventually producing a cell-suspension culture. Cell suspension culture has been considered to be the most ideal method for the production of secondary metabolites (Nunes *et al.,* 2007)

1.6.3.3. Protoplasts

Protoplasts are plant cells withiout the cell walls. Protoplasts are most commonly isolated from either leaf mesophyll cells or cell suspensions. The walls are generally removed in two ways viz. mechanical or enzymatic isolation. Mechanical isolation often results in low yields, poor quality and poor performance due to substances released from damaged cells. Enzymatic isolation is usually carried out in a simple salt solution with a high osmoticum and the cell wall degrading enzymes like cellulase and pectinase. Protoplasts can be plated on the solid medium forncallus induction. Whole plants can be regenerated by organogenesis or somatic embryogenesis from this callus. Protoplasts are ideal targets for genetic transformation by a variety of means.

1.6.3.4. Root cultures

Root cultures can be established *in vitro* from explants of the root tip of either primary or lateral roots and can be cultured on simple media. The growth of roots *in vitro* is potentially unlimited, as roots are indeterminate organs. Although the establishment of root cultures was one of the first achievements of modern plant tissue culture, they are not widely used in plant transformation studies. Agrobacterium induced hairy root cultures usually in the bio reactors are used to induce secondary metabolites.

1.6.3.5. Shoot tip and meristem culture

The tips of shoots can be cultured *in vitro*, producing clumps of shoots from either axillary or adventitious buds. This method can be used for clonal propagation. Shoot apical meristem culture is a potential technique of producing virus free germplasm (Kartha, 1981). Meristems are also good materials for cryopreservation which can offer a great possibility for future research (Fay, 1992) or *in vitro* conservation of germplasm.

1.6.3.6. Embryo culture

Both mature and immature embryos can be used as explants to generate callus cultures or somatic embryoids. Immature, embryo-derived embryogenic callus is the most popular method of monocot plant regeneration. This method is also used in overcoming embryo abortion due to incompatibility barriers, overcoming seed dormancy and self-sterility of seeds, embryo rescue in distant (interspecific or intergeneric) hybridization where endosperm development is poor and shortening of breeding cycle.

1.6.3.7. Microspore culture

Haploid tissue can be cultured *in vitro* by using pollen or ovule as an explant. Both callus and embryoids can be produced from pollen. Anthers can be cultured on solid and liquid medium ti induce embryoids. Regeneration from microspore explants can be obtained by direct embryogenesis, or via a callus stage and subsequent embryogenesis. The ploidy of the plants obtained from haploid cultures is a consequence of chromosome doubling (generally induced by treatment with chemicals such as colchicine) during the culture. may be an advantage. Such plants are often referred to as ‘di haploids’.

1.6.3.8. Somatic embryogenesis:

This is one of the major paths of regeneration and is very effective in large sclale clonal propagation (Timmis, 1998). This method is commonly used in mass multiplication through production of artificial seeds. The somatic embryoids are formed from somatic cells without going through sexual cycles and these are similar to zygotic embryos. These Somatic embryoids serve as a source of of embryogenic protoplasts in protoplast cultures. This method was first described in *Daucus carota* by Steward *et al.* 1958, after which many researchers worked in different plant species.

1.6.3.9. Seed culture:

The importance of the seed culture can be listed as increasing efficiency of germination of seeds that are difficult to germinate *in vivo*, rapid germination by application of plant growth regulators and production of clean seedlings for explants or

meristem culture. This method is also equally suitable for the infertile plant varieties such as hybrids and polyploids.

1.6.3.10. Node culture:

A shoot explant containing at least a node is cultured on a solid medium containing Auxin and or Cytokinin to induce adventitious multiple shoots for mass multiplication.

1.6.3.11. *In vitro* mutagenesis:

The importance of *in vitro* mutagenesis can be listed as induction of polyploidy, genetic variability, protoplast isolation, culture, fusion, combining genomes to produce somatic hybrids, asymmetric hybrids or cybrids, production of organelle recombinants and transfer of cytoplasmic male sterility.

1.6.3.12. Micrografting:

This method can be used to overcome graft incompatibility, rapid mass propagation of elite scions by grafting onto rootstocks that have desirable traits like resistance to soilborne pathogens and diseases, to allow survival of difficult to root shoots and the development of virus free plants.

1.6.3.13. Genetic transformation:

In genetic transformation, many different explants can be used, depending on the plant species and its favored method of regeneration as well as the method of transformation. This method is also used to introduce foreign DNA to generate novel genetic combinations and to study the function of genes. This method has been widely used to produce Genetically Modified Organisms (GMOs).

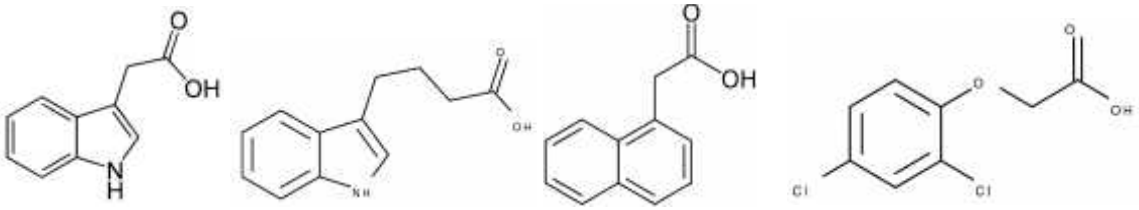
1.7. Plant Growth Regulators (PGRs)

Some chemicals occurring naturally within plant tissues (*i.e.* endogenously), have a regulatory, rather than a nutritional role in growth and development. These compounds, which are generally active at very low concentrations, are known as *plant hormones* (or

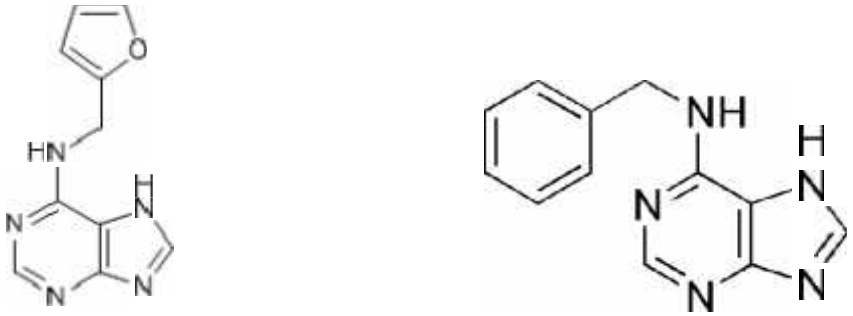
plant growth substances). Synthetic chemicals with similar physiological activities to plant growth substances, or compounds having an ability to modify plant growth by some other means are usually termed *plant growth regulators*. The auxins like IAA, IBA, NAA and 2,4-D etc. and the cytokinins like Kinetin, BAP, Zeatin, (2-isopentyl) adenine, thidiazuron etc. are commonly used in tissue culture experiments.

1.7.1. Auxins and cytokinins used for this investigation:

Auxins and cytokinins are by far the most important for regulating growth and morphogenesis in plant tissue and organ cultures. IAA and IBA are natural auxins, IBA, NAA and 2,4-D are synthetic auxins. Together with cytokinins, 2,4-D is used primarily for callus induction and the formation and maintenance of suspension cultures, being replaced by NAA and IBA when morphogenesis is required. Synthetic cytokinins most commonly used in micropropagation work are the compounds kinetin (Kn) and benzylaminopurine (BAP).



1. IAA 2. IBA 3. NAA 4. 2,4-D



5. Kinetin 6. BAP

Fig. 1.3- Plant Growth Regulators used in the Experiment. (I) Chemical streucture of IAA; (II)- Chemical streucture of IBA; (III)- Chemical streucture of NAA; (IV)- Chemical streucture of 2,4-D; (V)- Chemical streucture of Kinetin and (VI)- Chemical streucture of BAP.

1.8. Justification:

As mentioned in the earlier paragraphs, the species undertaken for this study (*A. racemosus* and *R. serpentina*) are highly potent medicinal plant resources of Nepal. Both of them are widely used in various ayurvedic, conventional as well as other traditional systems of medicine formulations since the vedic period. Both of them are prioritized by the government of Nepal for their development, research and cultivation. These species mainly grow in the tropical and sub-tropical regions throughout the country. *R. serpentina* is listed in the CITES Appendix II and is banned for the trade in crude form. A latest report by the Ministry of Forests and Soil Conservation, Government of Nepal (2009) have identified both the species undertaken for research as the ones highly harvested unsustainabally from the forests of Nepal. The report also highlights the urgent need of their conservation through regulations or by reducing the unsustainable harvest. *A. racemosus* is now considered to be "endangered" in its natural habitat (Bopana and Saxena, 2007). This species in the wild is decreasing due to various facors like overharvesting, burning to create areas for livestock grazing, shifting cultivation and land encroachment for cultivation (Amatya, 2005). Although, some efforts to cultivate them for commercial purpose have been made both by the government as well as local levels, still the wild collection has not stopped. *A. racemosus* from the wilds of Nepal are in high demand in Indian as well as in local markets. Similarly, *R. serpentina* is also collected from the wild and sold either in the local markets or illegally exported to India. Hence, from the reports of Puri, (2003); Khare, (2007); and Mulliken and Crofton, (2008), it is obvious that both of these species are collected rampantly and sold in these markets. Olsen and Bhattarai, (2005); Helle and Olsen, (2007); have reported how the medicinal plants are traded from Nepal and how medicinal plants are an open access resources in Nepal. Understanding these facts, there is an urgent need of an efficient way out to conserve these valuable species. If immediate actions are not taken, in few decades, there will be no species left to conserve.

For the conservation of such species, rapid multiplication and rehabilitation in their natural habitats and their cultivation is necessary. To conserve these species an attempt has been made using an *ex situ* way of conservation using tissue culture

technique as a tool. This technique can be a break throughcan in multiplying rare and endangered plant species with very little or no damage to the mother stock (Bhatt *et al.*, 2008). Various workers (Paul and Basu 2008; Baksha *et al.*, 2007 etc.) have reported very low rate of seed germination of *R. serpentina* (5-26%) and Gupta *et al.*, (2002) reported 17 to 60 % *A. racemosus*.. In 1955, Nair observed 15-20% seed germination, but only 10-13% plant could be developed from the germinated seeds of *R. serpentina*. According to Hedayatullah (1959), seed germination of *R. serpentina* was quite erratic, ranged from 8-48% with an average of 19%. Even the latest internet sources report the seed germination percentage to vary from 5 to 30 even when only heavy seeds are chosen for sowing purpose. Seeds older than one year have very low viability (almost not viable) in the wild as well as in cultures. Other conventional vegetative propagation tools are very slow and laborious.

Thus a need arises to generate efficient protocols for rapid cloning of *A. racemosus* and *R. serpentina* in order to regenerate propagules to replenish depleting forests and meeting the demand of commercial cultivation (Kataria and Shekhawat, 2005). To overcome these problems, a reliable method of quick multiplication like tissue culture and methods of *in situ* as well as *ex situ* conservation could well provide a viable solution to the problem (Bopana and Saxena, 2007). *In-vitro* micro propagation or tissue culture however offers a solution in which several thousands of plants can be produced from the same amount of material that is required to produce a single new plant by traditional methods. This would enable us to gradually replenish the population of a threatened species to where it is entirely removed from the list. It would also take an endangered population and move it only to a threatened status within a short period of time. Some tissue culture works on these species have been previously done and mainly with species of other countries growing under completely different climatic conditions. A very few literatures are available on the micropropagation/ tissue culture and almost no literatures on somatic embryogenesis of both the species from Nepal. *A. racemosus* being less known to the western world, very little work has been published. Hence, it becomes essential to work on different aspects of plant tissue culture of these species from a completely different ecological zones of Nepal. In the present study, we are trying

to work on the wild Nepalese species. The studies on the somatic embryoids of these two species are very rare. In somatic embryogenesis there is always a high chance of getting genetic variation but in germination through seeds and shoot multiplication through nodes as in our experiments have a very high chance of getting normal and true copy of the parents. Hence, this technique might be more appropriate especially for the medicinal plants where importance of the plant' is evaluated from its active compounds content.

Since, the plants undertaken for the present study have high medicinal values and their importance are due to their chemical compositions; if they are propagated by seeds the successive generations have high risk of genetic variations and eventually they might lose their medicinal property (Annonymous, 1950). Hence it is necessary to keep the purity of the plant. As tissue cultured plants (especially node and shoot tip cultures) are homogenous, this technique may also provide an effective solution to this problem of genetic alteration. The technique of acclimatization and hardening of the micropropagated seedling could be beneficial in taking the plant from the lab to land. So, we believe that the result of this study will be a step forward in meeting the national conservation goal.

1.9. Hypothesis

- Plant Growth Regulators (PGRs) of the same group will have similar effects in the morphogenesis of different plant parts under *in vitro* culture conditions.
- *In vitro* multiplication method is a reliable tool for the rapid multiplication of endangered plant species in a short time and space without damaging the mother stock.
- It helps to maintain genetic stability of plant species.

1.10. Objectives:

The medicinal plants *Asparagus racemosus* and *Rauvolfia serpentina* are collected in high amounts each year from the wild without any consideration for the conservation of the natural stock. As already stated earlier, Nepal is the major supplier of *A. racemosus* in the Indian sub-continent and *R. serpentina* is rapidly depleting in the wild and is listed in the IUCN endangered category as well as in the CITES Appendix II. Domestication of these plants and large scale production/cultivation can fulfill the ever increasing demand of the market as well as lower the pressure in the wild by the supply of good quality planting material to the farmers engaged in medicinal plant cultivation. At the same time, rehabilitation of these species in the wild after acclematization in the lab can further strengthen the biodiversity conservation programme of the country.

The main objectives of this research are to find out a reliable and cost effective method of rapid propagation of these two plants viz. *A. racemosus* and *R serpentina* as well as to understand their behavior under different phytohormone conditions. In the present study, the general morphogenesis of these two plants including somatic embryogenesis *in vitro* using various Auxins and Cytokinins have been conducted. This research was conducted to understand the behavior of these two Nepalese species *in vitro* and to develop a rapid multiplication protocol which will be useful to propagate sufficient number of these species in a short period of time for conservation as well as mass cultivation.

Specifically, the objectives of the study are:

- to find out the appropriate explant for rapid multiplication.
- to develop protocol for micropropagation of the plants undertaken.
- to understand the effects of different phytohormones singly or in combinations at different concentration levels.
- to study the callus induction, caulogenesis and somatic embryogenesis
- to acclimatize the micro propagated plants *in vivo*.

CHAPTER-2

LITERATURE REVIEW

2.1. Importance of the plants selected for the present study:

Kataria and Sekhawat, (2005) have mentioned the importance of *Rauvolfia serpentina* giving references to the earliest literatures like Ayurvedic medicinal text the *Charaka Samhita* (c. 700 B.C.) and its uses since that time to treat mental illness and insomnia. They have also mentioned about the numerous alkaloids it contains due to which indiscriminate use and poor method of conventional propagation led this species to be included in the list of endangered plants. Thus, they pointed out a need to generate an efficient protocol for clonal propagation of *R. serpentina* in order to regenerate propagules to replenish depleting forests and meeting the demand of commercial cultivation.

Bopana and Saxena, (2007) have pointed out the importance of *Asparagus racemosus* Willd. as an important medicinal plant of tropical and subtropical India. They have listed the medicinal usage of this species like treatment of neurodegenerative disorders and in alcohol abstinence-induced withdrawal symptoms. In Ayurveda, *A. racemosus* has been described as a rasayana herb and has been used extensively as an adaptogen to increase the non-specific resistance of organisms against a variety of stresses. Besides use in the treatment of diarrhoea and dysentery, the plant also has potent antioxidant, immunostimulant, anti-dyspepsia and antitussive effects. The use of this species has been reported in the Indian and British Pharmacopoeias and in traditional systems of medicine such as Ayurveda, Unani and Siddha. They have also given emphasis on its phytoestrogenic properties and have linked with an increasing realization that synthetic oestrogens is neither as safe nor as effective as plant-derived oestrogens which is making *A. racemosus* particularly important. Due to its multiple uses, the demand for *Asparagus racemosus* is constantly on the rise; however, the supply is rather inconsistent and inadequate. They have also pointed out the common causes of its

decreasing population sue to destructive harvesting, habitat loss due to deforestation etc. The plant is now considered 'endangered' in its natural habitat. Hence they pointed the urgent need for conservation of this species. They have evaluated the biological activities, pharmacological applications and clinical studies of *A. racemosus* in an attempt to provide a direction for further research. They have also mentioned the importance of various biotechnological tools like chemoprofiling, tissue culture and use of molecular markers and proper agro-techniques and adequate marketing opportunities would encourage cultivation of *Asparagus racemosus* and thereby contribute to its conservation. They have also shown the gaps in the existing literature with regard to the pharmacological actions of this species such as an incomplete understanding about the interaction/synergy between *A. racemosus* and other plant constituents in polyherbal formulations; lack of information on the mode of action of the various constituents of *A. racemosus*, etc. At the end, they have suggested a 'systems biology' approach that includes metabolite profiling, metabolic fingerprinting, metabolite target analysis and metabonomics to enable further research.

Bopana and Saxena, (2008) mentioned that due to destructive harvesting, the natural population of *A. racemosus* is rapidly disappearing, and it is recognized as ‘vulnerable’. They have pointed out an urgent need for the development of an efficient micropropagation protocol which will play a significant role in meeting the requirements for commercial cultivation, thereby conserving the species in its natural habitat. In their experiment on this species they observed shoot proliferation from the single node segments of *A racemosus* on MS medium supplemented with 2-iP 3.69 µM with a multiplication rate of 3.5 shoots per node. They obtained 85% rooting within 20 days when they cultured the micro shoot clusters on ½ MS medium with NAA 1.61 µM + K 0.46 µM + adenine sulfate 98.91 µM + malt extract 500 mg/l + phloroglucinol 198.25 µM. Finally following a simple hardening procedure involving sequential transfer of plants to a greenhouse, polyhouse, and shade net, they obtained 100% survival of the tissue-cultured plants in the field.

2.2. Seed germination:

Gupta *et al.,* (2002) tested seed germination of *Asparagus racemosus* Willd. They observed a wide variation in germination percentages of the seeds ranging from from 17 to 60 in different collection samples. Although all seeds exhibit dormancy, they found that when the seeds were treated with a 20 percent sulfuric acid solution greatly increased the seed germination up to 86 percent. They also concluded that Gibberellic acid also stimulates seed germination in *Asparagus racemosus,* but to a lesser extent than sulfuric acid treatment.

Cermano *et al.,* (2002) tested the effects of various pregermination and germination conditions to improve the germination rate of *Asparagus officinalis* L. Using environmental control chambers, they used various pregermination treatments (7 days at 5°C, 24 hours at 25°C, 0.2% KNO_3, gibberellic acid -GA_3- 100 p.p.m. and 500 p.p.m.), different temperatures (5, 10, 15, 20, 25, 30, 35°C and temperature cycles between 20°C (12 hours) and 30°C (12 hours), different substrates (between paper (BP), osmosis water, top paper (TP), top sand (TS) and top peat (TPT)) and different maturity state of seeds (green fruit, 25% red, 50% red and 100% red). Finally, they found that the most favorable temperature conditions overall were the 20-30°C (12h-12h) cycle, and 30°C (constant temperature conditions). The best substrate they found was sand. They also observed that the maturity of the seed also influenced in germination potential of the seeds i.e. as the green fruit changes colour from green to red, the seeds acquire the capacity to germinate.

Bittencoure *et al.,* (2005) conducted seed germination tests on four distinct lots of 'Mary Washington'asparagus (*Asparagus officinalis* L.) with 7.5-8.0 % moisture in the seeds. They pre-treated these seeds with 20 ml of polyethylene glycol at 1.0 or 1.2 MPa of sea water 3.3 MPa soaked in the paper towels and kept in germination plastic boxes for 7 or 14 days. They pretreated some seeds with distilled water for three days as control. Then they washed and dried the seeds and finally put for germination experiments in the paper towels moistened with distilled water. Here among all the pretreatments, PEG at 1.0 MPa for 14 days showed the best result in germination

percentage of the seeds with 40-94% germination after 13 days. They also observed that the priming of the seeds not only increased the percentage germination but also speed up the germination time, and improved the vigour of the seedlings.

Baksha *et al.,* (2007) highlited the drawback of the propagation by direct sowing of the seeds of *Rauvolfia serpentina*. They mentioned that the direct sowing of seeds in the field has not been found successful. They also highlited that the propagation of this species by means of seeds might prove ultimately even unwise, since variation in alkaloidal yield is apparently genetically controlled and might get reduced in successive progenies through adverse gene recombination. They also mentioned that the sun-dried and stored seeds generally gave a low rate of germination and seeds stored for more than 7-8 months practically did not germinate. The germination percentage of seed is very poor and variable (25-50 %) and is often as low as 10%.

Paul and Basu, (2008) subjected the freshly collected seeds of *R. serpentina* with various pre treatments like mechanical scarification with sand paper or grind stone or nicked with a needle. Hot water soaking at 80±20C for 5, 10, 15 and 20 minutes; conc hydrochloric and sulphuric acid treatments for 3, 5, 10, 15, 30, 40, 60 and 90 minutes; dry heating at 70, 80 and 90^0 C for 16, 24, 48, 72 and 96 hours duration in an oven; soaking for 24 hours in 1% boric acid, 1% calcium hydroxide, 1% sodium dihydrogen phosphate, 1% potassium nitrate, 0.5% thiourea, 100 ppm GA_3 and 100 ppm NAA. They also studied the interactive effects of KNO_3 with GA_3 and NAA. All the pre-soaked seeds were re-dried for 24 hours in a stream of air and the untreated seeds were used as control. They sowed the seeds on two layers of blotting paper in petri dishes. The germination data they presented were 26% in control and a maximum of 48% with sand paper scarification. They in their final comments mentioned that all the pretreatments tested could not significantly increase the germination potential of the seeds possibly because of the some germination inhibitors in the seed and they also mentioned the necessity of further research to understand its complex behavior.

Mishra *et al.,* (2010) studied the germination potentials of the seeds produced by the mature fruits of *in vitro* raised plants of *Rauvolfia serpentina*. They scarified the seeds with sand paper and treated overnight with 300 mg/l gibberelic acid. They inoculated the seeds in the sand and found an average of 65.1% germination after six weeks.

Pant and Joshi, (2009) induced multiple shoots of wild *Asparagus racemosus* directly from the seeds after pre-soaking in water overnight. An average of 13 shoots from a single seed has been formed all together after successive cultures on the hormone free MS medium. The multiple shoots obtained in this experiment were morphologically similar to the wild ones in nature.

2.3. Callus induction and general multiplication:

2.3.1. *Asparagus:*

Schröder and Eimert, (2000) have studied the temporary immersion of *in vitro* grown internodal pieces of *A. officinalis* and their incubation in the dark in a liquid embryo induction medium which resulted in the formation of numerous somatic embryoids using temporary immersion system. They observed the callus after two weeks and globular-shaped embryoids after 4 weeks. They observed that during the following two weeks the embryoids elongated, and formed bipolar structures with cotyledons, the radicle, and the lateral shoot apex. The histological analyses showed that the development of the somatic embryoids is similar to nucellar embryos. The somatic embryoids germinated into normal plantlets with shoots and a well developed root system in the same embryo induction medium.

Grivet and Jullien, (2000) studied somatic embryogenesis in *Asparagus officinalis* L. they observed emergence of either a few somatic embryoids or an embryogenic callus from a restricted area of a primary callus. In the first case, somatic embryoids emerged from 1 % of calli induced with NAA and transferred to a medium without auxin. On isolation and subculturing on hormone free medium, the embryoids developed habituated embryogenic lines (H lines) growing by adventive embryogenesis.

In the second case, 3 % of primary calli developed then subcultured on 2,4-D produced a new type of friable and yellowish-white callus, constituted of clusters of globular somatic embryoids which can be continuously maintained on 2,4-D (2,4-D lines). Among 2,4-D lines, two types were identified by subculturing them on hormone–free medium. Half of the 2,4-D lines were habituated and half were 2,4-D dependent. Most plants regenerated from H lines exhibited a strong increase in embryogenic capacity compared to control plants, unlike plants regenerated from the 2,4-D dependent lines. This increased embryogenic capacity was transmitted to the progeny as a monogenic dominant trait. H lines would therefore be issued from mutation(s) occurring *in vitro*, conferring both the embryogenic and habituated phenotypes. On the contrary, in the 2,4-D dependent lines, the embryogenic processes appeared to remain under exogenous auxin control and no evidence of a mutational origin could be inferred from the behaviour of regenerated plants.

Saensouk and Suddee, (2004) used young leaves and nodes of *A. racemosus* as explants and MS as the basal medium and NAA and BA as the PGRs in their experiment. The percentage of callus on medium combination with 0.5 mg/l NAA and 2 mg/l BA were 90 percent and 80 percent from leaves and nodes, respectively. Shooting was 100 percent from leaves cultured on medium added with 1 and 2 mg/l BA. Shoot was 1.01 cm in height from leaves cultured on BA free medium. Nodes yielded 90 percent shoot formation in medium with the addition of 1 mg/l BA and produced shoot with 1.03 cm in height on 4 mg/l BA. Roots were not induced on any medium supplemented with IBA.

Pontaroli and Camadro, (2005) studied the callus growth and plant regeneration from long-term callus cultures in two clones of *Asparagus officinalis* cv. Argenteuil, to establish a suitable protocol for a prospective *in vitro* selection program. They used the MS medium with 3% sucrose, 0.9% agar + K 1.0 mg/l and three levels of 2,4-D for the callus initiation and growth. The highest callus relative growth was obtained on medium with 2,4-D 1.5 mg.l + K 1.0 mg/l. They induced shoots from >18-months-old calluses from various media but the highest of 89% and average number of 8.6 were obtained with clone ‘265’ on MS medium with 2iP 5.0 mg/l + IAA 1.0 mg/l. they observed the

highest percentage of root induction (100%) on clone ‘265’ on MS medium with K 0.1 mg/l + NAA 0.1 mg/l + ancymidol 1.32 mg/l with 7% glucose and 0.8% agar in the medium.

Mehta and Subramanian, (2005) observed shoot induction from the nodal explants of *A. adscendens* after 4 weeks of incubation when they used MS medium supplemented with NAA 0.27 M + K 0.46 M, NAA 0.54 M + K 0.46 M, NAA 1.07 M + K 0.46 M, NAA 0.54 M + K 0.93 M and NAA 0.54 M + K 1.39 M. The first three concentration combination showed a little amount of callus induction. When they used either IAA or IBA in place of NAA all the explants died. 2,4-D either alone or in combinations was ineffective in shoot induction but induced creamy pale yellow, friable callus. They have pointed out the difficulty in root induction with all the strengths of media incorporated with different auxins. Finally they achieved success when they used ancimyol at different concentrations along with IBA 0.49 M to 1.48 M in the MS medium.

Kumar and Vijay, (2008) used MS medium and BA and K as cytokinin (0.5, 1.0, 2.0, 3.0 and 4.0 mg/l) and IAA, IBA and NAA as Auxins (0.1, 0.5 and 1.0 mg/l) in combinations to see the various effects in *A. racemosus* nodes. They very little callus with IAA 3.0 mg/l whereas maximum callus as well as shoot proliferation at 2.0 mg/l NAA. They observed that BA up to 2.0 mg/l with any concentration of IAA either a very little no callus at all. They observed that BA up to 3.0 mg/l elongated the shoot tip explants whereas above this shoot an average of 23.6 buds at the nodes were observed. K at lower and very high concentrations (0.1 - 0.5 and 4-5 mg/l) were not good for shoot induction but at 2-3 mg/l induced 11 shoots per explants. They have highlited that BA is much more effective than K in case of shoot proliferation. BA+ NAA = callus at all concentration, Low NAA + High cytokinin is the key factor for shoot production however higher auxin suppressed shoot induction. Higher NAA + low BA = callus. Maximum shoot elongation MS+ BA 3.0 + Kn 3.0 + NAA 0.1 mg/l. No rooting etal with IAA and IBA but 30-40% with NAA. Full strength (63%)- thin fibrous short white roots ½ strength (87%)- white thick and vigorous. 75% survival.

Hurgoiu and Blidar, (2008) studied the callus growth and organogenesis from the previously induced callii in *Asparagus officinalis* (Jersey Knight F1). They observed that until 12 weeks culture the MS medium supplemented with BA 1.0 mg/l and IBA 1.0 mg/l as the best combination for both callus growth as well as shoot regeneration. They also observed that after 12 weeks of culture in the same medium the shoot started to vitrify.

Pant and Joshi, (2009) have successfully multiplied *Asparagus racemosus* using tissue culture technique. For the multiplication, all the explants including callus have been used and the callus and shoot explants (shoot tips and nodes) played significant role. Mainly four parameters: callus, buds, shoots and roots inductions were studied. NAA singly played a good role in all parameters except bud induction. Similarly, BAP played good roles in shoot and bud inductions, whereas combinations of NAA and BAP at various levels were found to be effective in almost all cases. The multiplied adventitious shoots were successfully rooted (*in vitro* and *in vivo*) using NAA, acclimatized and transferred to natural conditions.

Kohmura *et al.,* (1996) have developed an effective micropropagating system involving induction of multiple bud clusters and somatic embryogenesisin *Asparagus officinalis* L. cv "Hiroshimagreen", and other 14 genotypes. They induced the bud clusters from shoot apices in MS liquid medium supplemented with 10 mg/l ancymidol using gyrating drum culture. From all of the tested genotypes, they induced bud clusters and subcultured in the same medium at monthly intervals. During each subculture the bud clusters rapidly increased to between 9 and 21 fold of their initial fresh weight without calli formation. They also tested the induction of embryogenic calli from bud clusters using MS solid medium supplemented with 10^{-5} M 2,4-D. They found it easy to initiate with some genotypes but for those with high ability for root formation, emberogenic calli were very difficult to induce. They also observed direct formation of globular embryoids frequently on bud clusters of all genotypes, and embryogenic calli were formed from embryos after transfer to MS solid medium supplemented with 10^{-5} M

2,4-D. These calli when subcultured, stable embryogenic cell lines without nonembryogenic calli were obtained in ten genotypes. Somatic embryoids were formed on MS medium without growth regulators, from which plants were regenerated.

2.3.2. *Rauvolfia:*

Sudha and Seeni, (1996) cultured shoot tip and single node explants from young shoots of 1-year old flowering plants of *Rauwolfia micrantha* on MS + NAA + BA. They found that MS + NAA 2.68 µM + BA 13.2 µM A induced a frequency of 77% formation of up to 3 shoots from each node in 8 weeks. They also observed that the regeneration of shoot tips from the field-grown plants and *in vitro* shoots placed horizontally differed. Repeated subculturing of the shoot tips and single nodes at 6-week intervals for over a year in combination of MS + NAA 0.27 + BA 4.4 µM enabled mass multiplication of shoots without any evidence of decline. Rooting of the excised shoots on MS medium containing NAA 2.6 µM was preceded by callus formation whch were removed and the plants were hardened with 80% success in pots. They also observed that the micropropagated plants showed uniform morphology, growth, flowering, fruiting and seed germination characteristics.

Sarker *et al.,* (1996) induced multiple shoots from nodal segments and shoot apices of *Rauvolfia serpentina* on + NAA 0.1 + BA 1.0 mg/l and mentioned it as best combination for shoot proliferation. They also obtained adventitious shoots from the callus subcultured on media containing low concentration of BA (0.5 or 0.1 mg/l) + NAA (0.1 mg/l).

Ahmad *et al.,* (2002) used shoot tips from the field grown *Rauvolfia serpentina* as explants and cultured on MS + NAA 0.1 + BA 2.5 mg/l to induce multiple shoots with 93.33 % rate, 3.9 cm long 5.9 shoots/ explant. They also observed that MS + NAA 0.2 + BA 0.5 mg/l was the best combination for callus induction and development of shoot buds but failed to grow further. These tiny shoots were transferred to MS + NAA 0.05 + BA 2.0 mg/l for elongation. The rooting was done on MS + NAA 0.2 + IBA 0.2 mg/l.

Finally after acclimatization they achieved 95% success in making the plants survive in the field with good growth.

Alamgir and Ahmad, (2005) propagated *Rauvolfia serpentina* Benth. from the cuttings of root, stem and root-stem junction of 15 cm length of 1.5-2 years old plants. They obtained root formation and successful propagule development from cuttings of root, stem and root-stem junction as 62, 42 and 78%, respectively. 2,4-D at 5 ppm had the highest positive impact on root formation and propagule development (100%), followed by IBA 50 ppm (83%) and NAA 10 ppm (66%). IBA and NAA in combination showed more than 50% positive effect on rootings. They also observed that this plant performs better growth activities under double or triple doses of NPK, especially under increased N nutrition. Crude alkaloid contents of the roots increased significantly up to 466.12 mg/ plant root under the increased N level (1PK + 3N).

Kataria and Sekhawat, (2005) have mentioned the importance of *Rauvolfia serpentina* giving references to the earliest literatures like Ayurvedic medicinal text the *Charaka Samhita* (c. 700 B.C.) and its uses since that time to treat mental illness and insomnia. They have also mentioned about the numerous alkaloids it contains due to which indiscriminate use and poor method of conventional propagation led this species to be included in the list of endangered plants. Thus, they pointed out a need to generate an efficient protocol for cloning of *R. serpentina* in order to regenerate propagules to replenish depleting forests and meeting the demand of commercial cultivation. They used nodal shoot explants which produced 3-5 multiple shoots/ node by axillary bud proliferation on MS+ IAA 0.5 + BAP 10 µM. They further multiplied on MS + BAP 5.0 + IAA 0.5 µM. After 3-4 subculture cycles (each cycle of 15-20 days) shoot multiplication rate increased up to 20-25 fold. Later the shoots were multiplied on MS + BAP 4.0 µM to prevent hyperhydration. They used pulse treatment of shoots in NAA 50 + IBA 50 to induce roots which gave about 98% result. They hardened the plantlets on soilrite moistened with half-strength MS macrosalts and achieved 80% success. Finally they they transferred these plants to soil and observed normal growth responses in the nursery.

Faisal *et al.,* (2005) studied the effect of TDZ on *in vitro* shoot proliferation from nodal explants of *Rauvolfia tetraphylla*. They observed MS + TDZ (0.5–10 µM to be effective in inducing shoot buds and maintaining high rates of shoot multiplication on hormone free medium. The highest shoot regeneration frequency of 90% and mean number of 18.50 shoots per explant were achieved on MS + 5 µM TDZ for 4 weeks prior to transfer to MS medium without TDZ for 8 weeks. The regenerated shoots rooted best on MS medium containing 0.5 µM IBA. Micropropagated plantlets were hardened in the pots containing vermicultite and covered with plastic bags. After one months the plants were transferred to pots containing garden soil.

Sudha and Seeni, (2006) achieved plant regeneration through direct somatic embryogenesis from root segments derived from *in vitro* shoots of *Rauvolfia micrantha* Hook. f. grown for 6 wk in half-strength MS medium with 3% sucrose, 100 mg/l myo-inositol, and 0.5 mg/l NAA. They also studied the effects of photoperiod and plant growth regulators (PGRs) in half-strength MS medium for the rapid and maximum induction of somatic embryoids. They did not observe globular or heart-shaped somatic embryoids but observed cotyledonary stage embryoids occasionally in total darkness and 16-h photoperiod. Root segments cultured in the medium containing 0.1 mg/l NAA and 0.2 mg/l BA under 16-h photoperiod showed the maximum frequency (39%) of embryogenesis. The frequency of embryo formation was increased to 63% when they were cultured in medium with 0.1 mg/l NAA and 0.2 mg/l BA in the dark for 4 wk, then grown under the 16-h photoperiod. Explants with developing embryoids developed into plants after transfer to half-strength MS medium supplemented with 0.1 mg/l BA and 0.05 mg/l NAA. The well-developed plants were hardened and most plants (80%) survived and were phenotypically similar to the mother plants.

Anitha and Kumari, (2006a and b) highlited the importance of *Rauvolfia tetraphylla* L. due to the reserpine in them and is an endangered plant. They cultured the leaves on IAA, IBA NAA and 2,4-D among which they found MS+ 2,4-D 9 µM or MS+ 2,4-D 9 µM + 25, 50, 75 and 100 mg/l tryptophan as the best combination and induced

calli with 95% response. They cultured the found MS+ 2,4-D 9 µM induced calli on MS containing (0, 25, 50, 75 and 100 µM NaCl). They found normal callus growth up to NaCl 50 µM but reduction and cessation at 75 and 100 µM respectively. They observed an increase in the reserpine content at 50 mg/l tryptophan and 75 µM NaCl than in other concentrations. They used tryptophan because this is the starting material in the biosynthesis of reserpine.

Anitha and Kumari, (2007), cultured nodal segments and shoot tips of *R. trtraphylla* and observed maximum number of multiple shoots (4.56)at 4.44 µM of BAP after 2 weeks of culture. They observed *in vitro* flowering after using GA_3 in the same medium. The best combination was MS + BAP 4.44 + GA_3 4.3 µM but this concentration reduced the shoot number.

Baksha *et al.,* (2007) studied the direct regeneration from the shoot tips of *Rauvolfia serpentina* under *in vitro* conditions. They obtained a maximum of up to 7.5 shoots from + NAA 0.5 + BAP 4.0 mg/l within 10-15 days with an average height of 4.0 cm. They used half strength MS + NAA 0.5 mg/l and achieved 100 result with 3.5 roots/ shoot. The higher concentrations of both NAA and IBA yielded low percentages as well as low number of roots. The *in vitro* raised plantlets were acclimatized in glass house in the poly bags containing soil and sand (2:1) and successfully transplanted to field condition with 80 % survival.

Bhatt *et al.,* (2008) used the internodes and leaves as explants for the callus induction and shoot tips and nodes of *Rauvolfia serpentina* for direct regeneration on MS media supplemented with either 2,4-D + BAP or IBA + BAP. They found that the combination of IBA 0.125 + BAP 1.0 mg/l. as the best among all the combinations for both callus induction and direct regeneration.

Salma *et al.,* (2008) used nodal segments with buds from 3-4 years old *Rauvolfia serpentina* as explants and observed maximum of 4 shoots/ explants with 90 % induction on the MS + NAA 0.2 + BA 1.5 mg/l. They found a maximum of 4.08 cm long shoots from the same medium after 6 weeks of culture. They rooted these shoots on ½ MS +

IAA 1.0 + IBA 1.0 mg/l with 5.01 roots of 4.38 cm length. They also acclimatized these plant in the glass house and finally in the field condition with 95% success.

Salma *et al.,* (2008) studied the influence of different hormone concentrations on callus induction and plant regeneration of *Rauvolfia serpentina*. From the nodal explants they obtained 96.43% response in yellowish green callus induction on MS + NAA 2.0 + BA 0.5 mg/l however NAA more than 1.5 mg/l induced heavy callusing irrespective of BA's concentration. They also reported that the adventitious shoots were induced on all the concentration combinations but a maximum of 80% response with 3.1 shoots per culture on MS + NAA 0.2 + BA 2.0 mg/l. Here also they rooted these shoots on ½ MS + IAA 1.0 + IBA 1.0 mg/l.

Pant and Joshi, (2008) have identified the way for rapid *in vitro* multiplication of *Rauvolfia serpentina.* They have induced high callus from the leaf and node explants on MS containing NAA 1.0, 2,4-D 2.0, NAA 1.0 + BAP 0.1 and NAA 1.0 + BAP 0.5 mg/l. The best media for shoot multiplication from the node and callus cultures have been identified as MS + NAA 0.5 + BAP 0.5 and MS + BAP 2.0 mg/l respectively. For rooting *in vitro* MS + NAA 0.1-1.0 mg/l and for *ex vitro* NAA 100 mg/l (pulse treatment) has been found to be the best.

Singh *et al.,* (2009) cultured the juvenile leaf explants of *Rauvolfia serpentina* and observed that the intensity of callus induction was highest on MS + IAA 2.0 + BAP 2.5 mg/l and MS + IAA 0.5 + BAP 1.0 mg/l with 77.77% frequency. They also observed that the light green, fragile calli responded well for the induction of shoots. They found 75% response in shoot regeneration on MS + IAA 0.4 + BAP 2.5 mg/l. they used 1mg/l GA3 in the media for shoot elongation. They regenerated the roots from the shoots on MS containing BAP 2.5 + IAA 0.3-0.5 + NAA 0.3-0.5 mg/l. The obtained a 100% root regeneration on MS + IAA 0.5 + NAA 0.5 + BAP 2.5 mg/l. After rooting the plantlets were shifted to sterile soil field pots for acclimatization and got 67% hardened.

Mishra *et al.,* (2010) studied on the protocol development for *in vitro* clonal propagation of *Rauvolfia serpentina* (L.) Benth. var. CIM-Sheel through direct

regeneration from nodal segment explants. They found the maximum response of 98.33% of bud out-growth on MS + BA 1 M + NAA 1 M with highest axillary shoot number of 7.66 per explant. Single shoots cultured on MS + BA 10 M maximum number of multiple shoots of 9.16 per explant within 20-25 days. They used ex- vitro technique for the induction of roots from the micro shoots by pulse-treatment with NAA 50 M for 24 h. They achieved the rooted plantlets successfully within 30 days. They maintained the plantlets in polythene bags for 2 months in shadehouse with regular irrigation and successfully transplanted to field with 90% survival rate after six
months of transfer. After one year they observed normal flowering, fruiting, seed germination, morphological growth characteristics and root yield.

Jocelyne and Chenieux, (1991) *Rauvolfia vomitoria* mesophyll protoplasts have been isolated from axenic shoot cultures cultured in Murashige and Tucker liquid medium containing various concentration mixtures of 2,4-D, NAA, BA and K. Within 6-8 weeks, a mixed population of calli and proembryoids were obtained and transferred on solid media. Calli produced shoots; however, different patterns of development. In particular, whole plantlets have been obtained either directly through germination of primary embryoids or via embryogenic calli.

2.3.3. Other plants:

Leshem *et al*., (1987) observed vitrification in melon shoot tips cultured on solid and liquid media which contained growth factors in various concentrations. The found that the cytokinin as the primary inducer, without which vitrification did not occur in spite of the presence in the culture of other accepted inducers. Spontaneous vitrification appeared on non-inductive media but in a low percentage. On solid medium vitrification gradually increased with time, whereas on liquid medium it was an ‘all-or-nothing’ effect. On solid medium, all the vitrified plantlets turned into callus after two or three subcultures. It is suggested that vitrification is the bud’s reaction to an excess of cytokinin.

Jain and Bashir, (2010). Used the nodal segments of *Portulaca grandiflora* Hook. for multiplication. They observed the best shoot proliferation (56.55 per explants with 98% induction) on MS + 4.0mg/l BAP. For rooting of the microshoots, ½ MS + 0.75mg/l NAA was found to be best with 9.2 roots per shoot at an average root length of 6.0 cm with average rooting response of 95%. They achieved 100% survival rate after acclimatization.

Wala and Jasrai, (2003) induced multiple shoots from the meristem tips of *Curculigo orchioides* Gaertn., an endangered perennial medicinal herb of the family Hypoxidaceae. They obtained multiple shoots on MS medium supplemented with BA 2.21 µM. They rooted the shoots either on half strength of MS basal medium or on the one supplemented with NAA 0.53 µM. They acclimatized the in vitro plantlets in a mixture of vermiculite and soil (1:1) for two – three weeks. Finally in the period of three-month they obtained an average of 125 plants from a single meristem.

Pandeya *et al.,* (2010) achieved multiple shoot formation from shoot tip, node and cotyledonary node explants of a rare medicinal plant *Clitoria ternatea* L. on MS + 2.0 mg/l BAP. They found that the nodal explants as the best with 11.1 shoots followed by cotyledonary node and shoot tip. MS + 0.5 mg/l GA3 was found suitable for shoot elongation. They successfully rooted the plantlets *Ex vitro by* dipping the cut ends in 250 mg/l IBA solution for half an hour followed by transplantation in plastic cups containing sterilized soilrite which gave 90% survival rate and without any phenotypic aberrations.

Kambaska and Santilata, (2009) developed a micropropagation method for *Zingiber officinale* Rosc.) cv, Suprava and Suruchi using fresh rhizome sprouting bud in semisolid culture media. They used MS medium supplemented with different concentrations and combinations of BAP and NAA for shoot and root induction. They observed the best shoot multiplication rate on MS supplemented with 2.0 mg/l BAP + 0.5gm/l NAA. *In vitro* plantlets rooted on half strength MS + 2.0 mg/l NAA. They acclimatized the rooted shoots in the green house and achieved 95% survival rate in field condition.

Saher *et al.,* (2005) Micropropagated the Carnation through shoot cultures using two different agar concentrations (0.58 and 0.85%) and placed in a bottom cooling system. In 28 days of culture, they observed that relative humidity, hyperhydricity, dry weight, multiplication rate, and the activity of the antioxidant enzymatic system changed in relation to the agar concentration used and the application of bottom cooling. The percentage of hyperhydric shoots also showed a significant decrease under bottom cooling conditions for both agar concentrations. Lipid peroxidation was always lower in shoots cultured with bottom cooling. All the antioxidant enzymatic activities were lower in bottom cooling treatments compared to controls.

Tang and Newton, (2007) observed callus induction after 6 weeks only when the media were incorporated with either NAA 12 M + 2,4-D 15 M + 2 ip 6.0 M in *Pinus elliotii* Engelm. Embryo cultures. The calli then differentiated to give out adventitious shoots in 6-12 weeks when IBA 2.0 M + BA 3.0 M + TDZ 9.0 M were added in the media. Shoot elongation was observed in 6 weeks when on IAA 2.0 M + BA 1.0 M. Rooting of the microshoots were observed after 6 weeks on 0.01M each of IAA and IBA.

Prasad *et al.,* (2007) cultured Microshoots along with a part of the stem disc of *Chlorophytum borivilianum* a lilliaceae on MS + 2,4-D (0.25, 0.5, 0.75 and 1.0 mg/l) and in combination with different concentrations of BAP and K. They observed 100% callus induction in 29.8 days. The number of embryoids was 67. 2 and diameter of embryogenic callus was 2.88 cm at all low concentrations of 2,4-D in 29.8 days. The diameter of callus, the average fresh (1.073 g) and dry (0.100 g) weights of callus were more when 1.0 mg/l when 2,4-D was used. The callii resulting from these treatments were friable, glossy and creamish-yellow in appearance. After 60 days of culture, 100% maturation was achieved on MS or MS + ABA 0.1 mg/l. Embryoids derived from treatments with 2,4-D did not show 100% embryo maturation. A 62% of matured embryoids formed shoots on MS basal medium in 9 days unlike matured embryoids treated with 0.5 mg/l GA3 which germinated in only 5 days. Shoot induction formed in 60% of embryoids treated with 0.5 mg/l TDZ and 0.5 mg/l K but most shoots showed malformation during growth.

Kemat *et al.,* (2010) used young shoot buds as explants for rapid multiplication of *Chlorophytum borivilianum* on MS incorporated with various concentrations of BAP and K for shoot induction. MS + BAP 3.0 mg/l produced the highest mean number of shoots per explants (18.90) and a mean length of shoots (6.0 cm) after 28 days of culture. Regenerated shoots were successfully rooted on MS medium supplemented with 1.0 mg/l indole-3-butyric acid (IBA). For ex vitro establishment, well-rooted plantlets were transferred in potting medium containing vermiculite : organic matters (1:1).

Wani *et al.,* (2010) studied the callus induction in *Tridax procumbens* from various explants like leaf, internodes and shoot apical buds. They inoculated in MS media containing various combination of 2, 4-D, NAA, K and BAP. They found that the leaf and apical bud explants showed early and profuse callus induction whereas internpdal explants showed comparatively delayed but profuse callus induction. Leaf and apical bud explants showed maximum response in callus induction by using MS media along with 2, 4-D 0.5 + BAP 0.5 mg/lit followed by 2, 4-D 0.5 + K 0.5 mg/lit, 2, 4 –D 0.5mg/lit and BAP+KIN 0.5 mg/lit, NAA 0.5mg/lit and BAP 0.5mg/lit, NAA 2mg/lit and BAP 0.5mg/lit respectively. The internodal explants showed maximum callus induction on MS + 2,4, D 2 + BAP 0.5 mg/lit. They have also pointed out the potential use of the callus thus obtained as a source for the isolation of secondary metabolites.

Roy *et al.,* (2008) have studied the callus induction and the influence of plant hormones like 2,4-D, IAA, kinetin and BAP in *Gymnema sylvestris,* a medicinal plant. They have found that the intermodal explants proved to be the best for this species. They also used MS as the basal medium for the experiment and found the highest efficiency of callus formation on the medium containing different concentration of 2,4-D and kinetin.

Anu *et al.,* (2001)cultured rhizome buds of *Acorus calamus* L (sweet flag) in MS supplemented with BAP (8.87µM) and NAA (5.37pM) at light intensity of 30trE m. 2 s’ and photoperiod of 12 h and achieved up to 10 shoots after eight week of inoculation . They rooted the microshoots on hormone free MS medium, transplanted in sand and kept in shade for 4 to 5 weeks and achieved up to 95% success in establishing the plants in the field after one month.

Beena and Martin, (2003) worked on *in vitro* propagation of *Ceropegia candelabrum* L. (Asclepidaceae) through somatic embryogenesis. They induced friable callus from leaf and internode explants on MS + 2,4-D 4.52µM which developed somatic embryoids. Compared to solid media they found suspension culture to be superior and obtained higher number of somatic embryoids. Transfer of the friable callus to suspension cultures of ½ or ¼ strength MS with lower levels of 2,4-D (0.23 or 0.45µM) yielded the highest number of somatic embryoids up to the torpedo stage. Somatic embryogenesis was asynchronous with the dominance of globular embryoids. They obtained more than 500 embryoids from 100 mg of callus. Upon transfer to ¼ MS agar medium without growth regulators, 50% of the somatic embryoids underwent maturation and developed into plantlets. Plantlets acclimatized under field conditions with 90% survival.

Our selection of node as the explants was from the response of different explants in our previous experiences and trial experiments at the beginning of this research. Many other researchers like Gangaprasad *et al.*, (2003) in *Uteleria salicifolia*, Ajithkumar and Seeni, (1998) in *Aegel marmelos* etc. have also proven this part of the plant as the suitable (best) explants for the induction of multiple shoots which probably is the main goal of most tissue culture researchers.

CHAPTER-3

MATERIALS AND METHODS

3.1. Plant material:

The seeds of *A. racemosus* were collected from a local garden of Sim Gaun, Kirtipur, Kathmandu (approx. 1400 m asl). Similarly, the seeds of *R. serpentiana* were collected from Sauraha, Chitwan and Botanical Garden, Godavari, Kathmandu Nepal. All the seeds were stored in zip lock plastic bags inside the refrigerator at 16^0C. The nodal parts were of both the plants were selected for the whole experiment as the main explants. However, in case of callus induction of *R. serpentiana* the leaves were also used as explants.

3.2. Sterilization:

3.2.1. Surface sterilization of seeds:

To produce sterile explants for the experiment, the healthy seeds that sank in the water were selected and treated with liquid detergent for 15 minutes and was washed under running tap water for 45 minutes. After this the seeds were treated with 90% ethyl alcohol for 5 minutes and washed with distilled water to remove the alcohol. Finally, the seeds were treated (surface sterilized) earlier with 1.0% Sodium hypochlorite solutions (NaHOCl). Depending upon the occurrence of contamination the seeds were treated with this disinfectant for 5-20 minutes and washed well with sterile water four times under the laminar air flow hood chamber before inoculation in the hormone free Murashige and Skoog, (1962) medium (MS) to remove all the traces of NaHOCl.

3.2.2. Sterilization of glasswares:

All the glasswares (petridishes, culture tubes, beakers, conical flasks etc.) were dipped in the detergent water for overnight. They were washed thoroughly with tap water using bottle brush. They were then rinsed with sterile water and dried in the oven at 120^0C for one hour just before using.

3.2.3. Sterilization of Media and equipments:

All the media, utensils (forceps, blade holders, blades if reused, needles, brush etc) and sterile water used inside the laminar hood were sterilized at 15 lb/sq. inch pressure and 121^{o}C for 20 minutes in a portable autoclave.

3.3. Preparation of explants:

For the seed explants, after above mentioned surface sterilization process they were used as the explants for *in vitro* as well as *in vivo* germination experiments. In case of nodal, shoot tip, leaf and root explants, approximately 0.5-1.0 cm. long parts of these organs were excised from the the *in vitro* germinated seedlings inside the laminar hood using a sterile blade. The calli were sub cultured by separating (approx. 2.0 mm. in diameter) from the bigger mass (old cultures) in fresh media. The whole process was done under the laminar hood with blower on.

3.4. Media:

A medium is the formulation of different inorganic salts and organic compounds necessary for the nutrition of plant or plant part under *in vitro* culture condition. There are various types of culture media recommended for various types of cultures and plants. This is necessary to identify a suitable media for better results (outcomes). This can be done either by a better understanding of the nutritional requirements of cultured cells and tissues or by the results of previous similar experiments conducted by various workers on similar plants or plant parts. In general, the tissue culture medium must contain the 16 essential elements for plant growth.

3.4.1. Culture Media:

The Murashige and Skoog's (1962) medium was used for the entire research. Composition of the medium is given below.

Table 3.1. Murashige and Skoog (1962) Media Composition

Macroelements (Stock A)	**mg/L**	**10x (gm/l)**
NH_4NO_3	1650.00	16.50
KNO_3	1900.00	19.00
$CaCl_2 \cdot 2H_2O$	440.00	4.40
$MgSO_4 \cdot 7H_2O$	370.00	3.70
KH_2PO_4	170.00	1.70
Iron Source (Stock C)	**mg/L**	**10x (mg/100 ml)**
Na_2EDTA	37.30	373.00
$FeSO_4 \cdot 7H_2O$	27.80	278.00
Microelements (Stock B)	**mg/L**	**100x (mg/100 ml)**
H_3BO_3	6.20	620.00
$MnSO_4 \cdot 4H_2O$	22.30	2230.00
$ZnSO_4 \cdot 7H_2O$	8.60	860.00
$Na2MoO_4 \cdot 2H_2O$	0.25	25.00
$CuSO_4 \cdot 5H_2O$	0.025	2.5
$CoCl_2 \cdot 6H_2O$	0.025	2.5
KI Solution (separately prepared)	0.83	
Vitamins (Stock D)	**mg/L**	**100x (mg/100 ml)**
Nicotinic acid	0.5	50.00
Pyridoxine·HCl	0.5	50.00
Thiamine·HCl	0.1	10.00
Glycine	2.0	200.00
Myo-Inositol	100.00	Added fresh
Sucrose	30,000.00	
Agar	8,000.00	

3.4.2. Preparation of stock solutions:

Different stock solutions containing macro elements (A), micro elements (B), iron source (C), vitamins (D) and iodine source (KI) were prepared using the prescribed compounds in appropriate amounts (Table 3.1).

The strength of “Stock A” and “Stock C” containing macro elements were raised to 10x while making the stock solutions for convenience. All the compounds of macrosalts were dissolved in distilled water and stored in the brown bottle inside the refrigerator. The iron source compounds needed slight warming to dissolve completely in the distilled water.

Similarly, the strengths of the micronutrients and vitamins were raised to 100x in the stock solutions. The KI solution was prepared separately.

For the preparation of stock solutions, all the chemicals were weighed separately using a digital electronic balance. The individual chemicals were dissolved one by one and a required final volume (500 ml) was made by adding required amount of distilled water. All the stocks were stored in clean brown bottles inside the refrigerator.

The myoinositol was added freshly during the media preparation time.

3.4.3. Preparation of MS medium:

For the preparation of one liter of MS medium, following procedure have been followed:

By using either a measuring cylinder or a pipette 100.00 ml of Stock A (X10), 1.00 ml of stock B (X100), 1.00 ml of KI (X100) solution, 10.00 ml of stock C (X10) and 1.00 ml of stock D (X100) were mixed in a conical flask of capacity 1000.00 ml. An appropriate amount of myoinositol (0.1 gm) was weighed and added freshly. Similarly, 30.00 gm of sucrose was added and stirred with the help of a glass rod. When the sucrose was completely dissolved the pH was measured using a pH meter. The pH of the medium was adjusted to 5.8 ±0.1 with 0.1 M HCl or NaOH. After adjusting the pH, the medium was heated over an electric heater. When the media was nearly boiling, it was

taken out of the heater and added pre-weighed 8.00 gm of bacto agar (gelling agent) with continuous stirring.

Now, the media was again heated, boiled and poured in either glass culture tubes (150 mm × 25 mm) or in glass jam bottles. One litre of medium was poured either in 80 culture tubes or in 40 jam bottles. The mouths (openings) of the tubes and bottles were covered with two layers of aluminum foil and tightened with the help of rubber bands. All the media were sterilized at 15 lb/sq. inch pressure and 121^{o}C for 20 minutes in a portable autoclave.

The required amounts of PGR/s in the media were added before autoclaving from their stocks using sterile pippettes.

3.5. Plant Growth Regulators (PGRs)

Some chemicals occurring naturally within plant tissues (*i.e.* endogenously), have a regulatory, rather than a nutritional role in growth and development. These compounds, which are generally active at very low concentrations, are known as *plant hormones* (or *plant growth substances*). Synthetic chemicals with similar physiological activities to plant growth substances, or compounds having an ability to modify plant growth by some other means are usually termed *plant growth regulators*. Some of the natural growth substances are prepared synthetically or through fermentation processes and can be purchased from chemical suppliers. When these chemicals have been added to plant tissue culture media, they are termed plant growth regulators, to indicate the fact that they have been applied from outside the tissues (*i.e.* exogenously). It is believed that two or more hormones can interact synergistically or antagonistically in many circumstances. At the same time, any given hormone may affect the biosynthesis or metabolism of another, thus affecting endogenous levels. It is difficult to predict how any hormone (or growth regulator or inhibitor) will affect any given plant system.

There are several recognised classes of plant growth substance. Until relatively recently only five groups were recognised namely: auxins, cytokinins, gibberellins, ethylene and abscisic acid.

Auxins and cytokinins are by far the most important for regulating growth and morphogenesis in plant tissue and organ cultures. IAA and IBA are natural auxins, IBA, NAA and 2,4-D are synthetic auxins. Together with cytokinins, 2,4-D is used primarily for callus induction and the formation and maintenance of suspension cultures, being replaced by NAA and IBA when morphogenesis is required. NAA and IBA are favoured auxins for shoot culture. Synthetic cytokinins most commonly used in micropropagation work are the compounds kinetin (Kn) and benzylaminopurine (BAP).

Table 3.2. Plant growth regulators (PGRs) used to supplement the basic MS medium.

Auxins	**Mol. Wt.**	**Solvent**
Indole-3-acetic acid (IAA)	175.2	Ethanol
Indole butyric acid (IBA)	203.2	Ethanol
Napthalene acetic acid (NAA)	186.2	KOH
2,4-dichlorophenoxy acetic acid (2,4-D)	221.04	H_2O
Cytokinins		
Benzylaminopurine (BAP)	225.3	KOH
6-furfuryl aminopurine, Kinetin (Kn)	241.46	NaOH

3.5.1. Preparation of PGRs stock solutions:

(A) For the preparation of hormone solutions of all the auxins and cytokinins, 10 mg of each of the PGRs are dissolved in few drops of their respective solvents (Ethanol, KOH or NaOH) in separate test tube. These solutions are made 100ml. by adding distilled water. These are taken as the stock solution of 100 mg/l and are preserved in brown bottles in the freezer. The amount in ml of the stock added to one liter of the final medium makes the same amount of PGR in mg/l (i.e. 1.0 ml of IAA stock if added to one liter of medium makes 1.0mg/l IAA).

(B) For the preparation of different concentrations of different hormones from the stock solutions for various purposes, the following formula is used;

$S_1V_1 = S_2V_2$. Where,

S_1= strength of stock i.e., 1000 ppm., V_1 = volume of the stock to be taken

S_2 = strength of the hormone required (ppm) V_2 = Total volume required.

3.6. Inoculation of explants:

All the media and necessary utensils were again sterilized under the UV light for 45 minutes before using. The laminar air flow hood was washed/ sprayed with alcohol before turning the UV light on to minimize the risk of contamination. The seeds after a series of surface sterilization process mentioned earlier and different plant parts like nodes, internodes, cladodes, leaves, shoot tips, roots and calli were excised from the *in vitro* grown seedlings using a sterile blade. The explants of about 0.5-1 cm were prepared and inoculated in the medium under the laminar air flow hood. The transfer was done close to a burner with a pair of sterile forceps. Similar method was used in all cultures and sub cultures.

3.7. Culture conditions:

All the culture tubes and bottles containing specific media after inoculation of the explants were kept in a culture room. The temperature of the culture room was maintained at $25^0 \pm 1^0$C by running an air conditioner. The room was illuminated for 16-hours (everyday photoperiod) using cool white fluorescent tubes.

3.8. Sub-cultures:

Most of the responses in the first trials were sub cultured in the same but fresh media after 10 weeks depending on the urgency. Mostly the callii induced by the auxins were subcultured in the different hormone media for various responses like somatic embryogenesis, organogenesis and callogenesis.

3.9. Agar manipulation in the media (for *A. racemosus*):

To test the effects of agar and its concentration especially in the induction of storage roots from the *in vitro* grown shoot explants of *A. racemosus*, the MS medium

was supplemented with usual concentrations of NAA (0.1, 0.5, 1.0 and 2.0 mg/l) but varied concentrations of agar (0.6, 0.8 and 1.0 %).

3.10. Cytological study of callus:

The callii were dipped in 1% acetocarmine for 12 hours and heated with it in the test tube until boil. The callii then were squashed and analyzed under the various magnifications of a compound microscope for the study of different stages of somatic embryoids.

3.11. Rooting:

Micropropagated shoots were rooted *in vitro* using various auxins (IAA, IBA and NAA) at various concentrations (0.1, 0.5, 1.0 and 2.0 mg/l). The multiple shoots induced from various media were separated and incubated on the auxin media for root induction. The shoots were placed gently on the media on upright position. The rooting response data were recorded regularly after the first week of incubation.

Similarly, *in vivo* rooting experiments were also conducted by pulse treatment of cut ends of the shoots (usually with two nodes) in various auxin solutions (previously mentioned) of strength 100 mg/l. Before the pulse treatment (just dip) of cuttings, all the shoots removed from the media were thoroughly washed with water to remove the media and callus attached to them. After pulse treatment the cuttings were planted on pure sand moistened with ½ Stock A for the first time. Later the sand was moistened with water regularly without disturbing the cuttings. All the cuttings were covered gently with transparent plastic to maintain the humidity. *In vivo* rooting responses were recorded. After formation of roots on the cuttings, they were transferred to the plastic cups containing coco peat. All the substrates (sand-soil and coco peat) were pre treated with a weak (0.1%) solution of bavistin (fungicide).

3.12. Acclimatisation:

The i*n vitro* rooted plantlets were removed, washed carefully and planted in the coco peat. All the rooted plantlets (both *in vitro* and *in vivo*) were transferred to sand soil

mixture (1:1) after 10 weeks from the coco peat. All the plantings were regularly observed and watered at an interval of 2-5 days depending upon the moisture on the bed. The humidity inside the dome was maintained up to 90% for the first week. Gradually the humidity was decreased by allowing more air to circulate in the successive weeks. Finally, well established plants were either transferred to the garden or distributed to the interested growers.

3.13. Data recording and analysis:

The callus induction period, callus growth, number of shoots and roots/explants (shoot tip, nodes and callus mass), length of shoots/roots, presence and absence of somatic embryogenesis and acclimatization status were recorded. The callus mass induced from the explants were coded as 0 (no induction), 1(low induction), 2(high induction) and 3(very high induction) points according to their approximate volume. The Data were calculated and analyzed using analysis of variance (ANOVA) with statistical software (SPSS 11.5 for Windows) and the mean comparison was by a least significant difference (Duncan's Multiple Range Test) at 5% level of probability.

CHAPTER-4

RESULTS

4.A. *Asparagus racemosus*

4.A.1. Seed germination:

The seeds sown on hormone free MS medium germinated after two weeks of culture. After eight weeks of culture an average of 52.25 per cent seeds germinated *in vitro*. Similarly 22% *in vivo* germination response was achieved. The seeds presoaked in water overnight performed much better than those not pre soaked ones under both the conditions. The time for the germination process to complete was also relatively faster in *in vitro* condition (Table 4.1).

Table 4.1. **Variation in seed germination**

Germination method		% germination	Time for germination	% Response				
				1 shoot	2 shoots	3 shoots	4 shoots	5 shoots
In vitro	Pre-soaked	62.5	2-3 weeks	12	10.5	18.5	45.0*	14
	Not soaked	42						
In vivo	Pre-soaked	30	3-4 weeks	81	19	0	0	0
	Not soaked	14						

*Indicate very high significance at $p < 0.05$.

Multiple shoot formation has been observed in the germination process on *in vitro* condition. The multiple shoots obtained were morphologically similar to that of *in vivo* grown plants. The number of shoots raised from each seed ranged from 0-5 (Fig.1-6). The maximum number observed was 5 and all the shoots emerged from the same point (Fig. 7). In most of the cases the number of shoots per seed was 4-5. In a few cases either root or shoot only have also been observed (Fig. 1 and 2). On the MS medium shoot bases either detached from the seeds or intact with the seed showed both root and shoot

initiation after four days of culture (Fig. 8 and.9). The number of shoots gradually decreased from 5 in every successive detachment of the shoots and sub-culture in the same fresh medium. Multiple shoot buds have been observed at the bases of shoots which later give rise to new shoots. Altogether up to 13 shoots have been achieved from a single seed. All these shoots when cultured on MS medium containing different concentrations of NAA (0.1-2.0 mg/l) gave normal roots. The best rooting was observed at NAA 0.1 mg/l. Presence of IAA and IBA at any concentration did not induce good roots.

The roots did not respond to any of the media with or without hormone. Similarly, internodes and cladodes produced slight amount of callus in some of the media which were not found to be significant. All the shoot tips elongated to give out a single shoot in most of the media. Nodes responded the best in all respects (i.e. callus induction and multiple shoot induction). Hence, nodes were selected for the sub cultures and data recording in all the media.

4.A.2. Effect of different hormones either alone or in combinations on nodes.

Various auxins like IAA, IBA, NAA and 2,4-D and cytokinins like BAP and Kinetin were supplemented in the MS medium either alone or in combinations at different concentration levels. The concentration of all hormones ranged between 0.1and 2.0 mg/l when used singally in some cases it has been raised up to 3.0 mg/l (mentioned in the text and tables). In combination the concentration of auxins was limited up to 1.0 mg/l whereas cytokinins were raised up to 2.0 mg/l. The node explants and shoot induced calli when sub cultured on these media showed various effects on different aspects of morphogenesis like callus induction, multiple shoot formation, root differentiation, root and shoot elongation, caulogenesis, bud induction and somatic embryogenesis. The initial response to the media of culture was observed from as early as third week but for some of the cultures it was much longer. However, during summer, the risk of fungal contamination was observed to be much higher. The effects of these media on node and callus explants are described separately.

4.A.2.1. Effects of auxins on nodal explants:

Callus induction:

The explants obtained from the *in vitro* germinated seedlings were inoculated on different auxin containing media. The data given in Table 4.2 shows that the auxins play a significant role in callus induction from the shoot explants of *A. racemosus*. Among all the auxins tested NAA was found to be the best for callus induction. The highest amount of callus induction was observed when the shoots were inoculated in the MS media supplemented with either 0.5 or 2.0 mg/l of NAA (Fig. 10 and 11). The lowest effect i.e. no callus formation was observed when the shoots were subjected to IBA 0.1 mg/l. For the induction of callus from the nodal explants IAA and IBA at low concentrations were either ineffective or were insignificant as compared to the control however, their higher concentrations were found to be significant. Another auxin, 2,4-D was observed as the most effective hormone for the induction of callus at all levels with very high significance and frequency of 100% (0.5 and 1.0 mg/l) A regular trend in callus induction was observed while using IAA, IBA and 2,4-D but it was somewhat irregular in case of NAA.

Shoot multiplication and their lengths:

The shoot explants when cultured on MS + 2,4-D (all concentrations) did not show any effect on shoot multiplication. All other auxins induced multiple shoots. The highest number of shoots (7.83) was initiated on MS supplemented with 0.1 mg/l NAA with 100% success rate (Fig. 12). The lowest frequency and number of shoots with only 50% occurance and 0.66 shoots per explant was observed on MS + 1.0 mg/l NAA. Here also NAA and IBA do not follow the regularity or a general trend whereas IAA follows the regular trend. The highest mean shoot length recorded was 5.96 cm in the medium containing 0.5 mg/l IBA which was highly significant among all the auxins (Fig. 13). It was followed by NAA 0.1 mg/l with 5.73 cm long shoots. The shortest shoots (0.66 cm) were seen with IAA 2.0 mg/l (Fig. 14). Here also NAA did not follow the rule whereas; IAA and IBA do follow the general trend of rise up to a limit and fall.

Table- 4.2: Effects of various Auxins on nodal explants at different concentrations.

	Callus growth	Shoot number	Root number	Shoot length	Root length
PGR	Mean±Std. Error	Mean±Std. Error	Mean±Std. Error	Mean±Std. Error	Mean±Std. Error
Control	0.166±0.166^{a}	2.000±0.258^{ab}	0.000±0.000^{a}	3.016±0.588^{bc}	0.000±0.000 ^{a}
NAA 0.1	1.500±0.341^{*c}	7.833±3.646^{*c}	4.333±1.308^{*c}	5.733±1.238^{*c}	3.300±0.273 ^{*d}
0.5	2.166±0.166^{*d}	2.833±0.542^{ab}	2.000±0.258^{b}	4.816±0.367^{c}	1.000±0.339 ^{*b}
1.0	1.666±0.210^{*cd}	0.666±0.333^{a}	2.166±1.077^{*b}	1.800±0.782^{b}	1.083±0.083 ^{*b}
2.0	2.166±0.307^{*d}	2.333±0.714^{ab}	3.833±1.376^{*c}	3.916±1.085^{bc}	1.783±0.681 ^{*c}
IBA 0.1	0.000±0.000^{a}	2.500±0.428^{ab}	0.000±0.000^{a}	0.983±0.200^{ab}	0.000±0.000 ^{a}
0.5	0.500±0.223^{ab}	2.333±0.557^{ab}	0.000±0.000^{a}	5.966±1.318^{*c}	0.000±0.000 ^{a}
1.0	1.166±0.307^{*b}	0.833±0.307^{a}	0.000±0.000^{a}	1.433±0.360^{b}	0.000±0.000 ^{a}
2.0	0.666±0.210^{ab}	1.500±0.341^{a}	0.000±0.000^{a}	1.250±0.266^{ab}	0.000±0.000 ^{a}
IAA 0.1	0.166^{a}±0.166^{a}	1.666±0.421^{a}	0.000±0.000^{a}	0.983±0.144^{ab}	0.000±0.000 ^{a}
0.5	1.333±0.494^{*bc}	4.833±1.077^{b}	0.000±0.000^{a}	3.966±1.406^{bc}	0.000±0.000 ^{a}
1.0	1.833±0.307^{*cd}	1.833±0.307^{a}	0.000±0.000^{a}	0.950±0.140^{ab}	0.000±0.000 ^{a}
2.0	1.166±0.307^{*b}	1.333±0.333^{a}	0.000±0.000^{a}	0.666±0.095^{ab}	0.000±0.000 ^{a}
2,4-D 0.1	1.166±0.307^{*b}	0.000±0.000^{a}	0.000±0.000^{a}	0.000±0.000^{+*a}	0.000±0.000 ^{a}
0.5	1.500±0.223^{*c}	0.000±0.000^{a}	0.000±0.000^{a}	0.000±0.000^{+*a}	0.000±0.000 ^{a}
1.0	1.500±0.223^{*c}	0.000±0.000^{a}	0.000±0.000^{a}	0.000±0.000^{+*a}	0.000±0.000 ^{a}
2.0	1.333±0.333^{*bc}	0.000±0.000^{a}	0.000±0.000^{a}	0+.000±0.000^{+*a}	0.000±0.000 ^{a}

Root differentiation and their lengths:

Except NAA none of the auxins could induce roots when they were added alone in the medium. In most of the cases they gave callus. In root induction as well as in root length NAA did not follow any trend. The maximum average number of roots induced was 4.33 per explant or per shoot by NAA 0.1mg/l with 83.33% occurance however, the frequency was 100% at NAA 0.5 and 2.0 mg/l. The longest roots (3.30 cm) were observed on NAA 0.1 mg/l (Fig. 15) whereas the shortest ones of 1.0 cm were recorded from MS containing 0.5 mg/l.

4.A.2.2 Effects of cytokinins on nodal explants:

Callus induction:

All the treatments of cytokinins were significant at 1% level and showed similarities in callus induction except Kn. 0.1 mg/l (0.00). The highest amount of callus induction with 100% frequency was marked when the shoots were cultured on medium with Kn. 2.0 mg/l after 12 weeks of culture although other concentrations of Kn. except 0.1 mg/l were also highly significant (Table 4.3 and Fig. 16).

Table- 4.3: Effects of various Cytokinins on nodal explants at different concentrations.

	Callus growth	Shoot number	Root number	Shoot length	Root length
PGR	Mean±Std. Error	Mean±Std. Error	Mean±Std. Error	Mean±Std. Error	Mean±Std. Error
Control	0.166±0.166^{a}	2.000±0.258^{ab}	0.000±0.000^{a}	3.016±0.588^{b}	0.000±0.000^{a}
BAP 0.1	1.500±0.223*^{b}	7.500±2.232*^{c}	0.000±0.000^{a}	3.983±0.965^{c}	0.000±0.000^{a}
0.5	1.500±0.223*^{b}	5.666±1.605^{cd}	0.000±0.000^{a}	4.733±0.780^{cd}	0.000±0.000^{a}
1.0	1.333±0.210*^{b}	4.833±1.137^{c}	0.666±0.666^{a}	5.483±0.790*^{d}	0.766±0.501^{b}
1.5	1.666±0.210*^{b}	2.166±0.749^{ab}	0.000±0.000^{a}	1.400±0.372^{ab}	0.000±0.000^{a}
2.0	1.333±0.333*^{b}	1.666±0.557^{ab}	0.000±0.000^{a}	2.533±0.736^{ab}	0.000±0.000^{a}
Kn 0.1	0.000±0.000^{a}	1.333±0.210^{a}	0.000±0.000^{a}	0.666±0.138^{a}	0.000±0.000^{a}
0.5	1.333±0.210*^{b}	2.166±0.477^{ab}	2.666±0.988*^{b}	1.100±0.198^{a}	1.300±0.178*^{c}
1.0	1.833±0.307*^{b}	4.666±1.429^{b}	0.833±0.307^{a}	5.366±1.081^{d}	0.450±0.117^{ab}
2.0	1.833±0.307*^{b}	3.500±0.763^{ab}	2.000±0.577^{b}	2.150±0.578^{ab}	0.983±0.192*^{bc}

Shoot multiplication and their lengths:

From the data recorded, BAP 0.1 mg/l was found to be the best among all the concentrations of cytokinins tested with 100% occurance and an average shoot number of 7.50 per explant which was also found to be highly significant at 1% level of significance (Fig 17). The lowest response was shown by Kn 0.1 mg/l with an average shoot number of 1.33 but with high response of 100%. Here in case of shoot multiplication, BAP and Kn showed almost the opposite response. In case of shoot length, BAP 1.0 mg/l showed the best result with an average shoot length of 5.48 cm (Fig. 18), but the shortest shoots

(0.66 cm) were recorded with Kn 0.1 mg/l. High BAP (2.0 mg/l) increased vitrification of shoots (Fig. 19, Table 4.3).

Root differentiation and their lengths:

Almost all the BAP concentrations except 1.0 mg/l failed to induce roots either from shoot or callus. Reverse to BAP almost all the Kn concentrations except 0.1 mg/l induced roots from callus. The highest root number (2.66) as well as longest roots (1.30 cm) was recorded in the medium containing Kn 0.5 mg/l with a frequency of 83.33% (Fig. 20). Similarly, lowest number of roots with only 16.66% response was observed on the medium containing BAP 1.0 mg/l whereas the shortest roots were measured when the medium was supplemented with Kn 1.0 mg/l (Table 4.3).

4.A.2.3. Effects of NAA and BAP in combinations on nodal explants:

Callus induction:

All the treatments tested except NAA 0.5 + BAP 0.1 mg/l were found to be significantly inducing the callus fron the shoot explants. The maximum callus formation was found in the medium containing combination of NAA 1.0 mg/l and BAP 2.0 mg/l (Fig. 21). The lowest amount of callus formation was recorded from the medium containing NAA 0.5 + BAP 0.1 mg/l with 66.66% occurance (Table 4.4). The callus mass were hard, smear preparation of these callishowed the presence of embryoids.

Shoot multiplication and their lengths:

None of the media tested in this group were found to be significant at 1% level of significance in case of shoot multiplication. However, the highest number of shoots i.e. 5.33 per explant was obtained in MS supplemented with low auxin and high cytokinin levels (NAA 0.1 + BAP 2.0 mg/l) with 100% success rate (Fig. 22). In case of shoot length, a highly significant shoot elongation was observed on the medium containing NAA 1.0 + BAP 0.1 mg/l, whereas no shoots were observed on the medium containing NAA 1.0 + BAP 0.5 (Table 4.4 and Fig. 23). Presence of BAP above 1.0 mg/l in the medium induced vitrified shoots (Fig. 24)

Root differentiation and their lengths:

The root induction was observed in some treatments but most of the treatments produced no roots. The average highest number of roots induced by NAA 1.0 + BAP 0.1 mg/l was 4 per explant or per shoot with 83.33% response whereas the least number was 0 in most of the cases (Fig. 25). The longest roots (2.90 cm) induced in the medium with NAA 1.0 + BAP 0.1 mg/l while the shortest ones (0.20 cm) were recorded from medium with NAA 0.1 + BAP 0.1 mg/l/ was 0.16 (Table 4.4).

Table 4.4: Effects of various concentration combinations of NAA and BAP on nodal explants.

	Callus growth	Shoot number	Root number	Shoot length	Root length
PGR	Mean±Std. Error	Mean±Std. Error	Mean±Std. Error	Mean±Std. Error	Mean±Std. Error
Control	0.166±0.166^{a}	2.000±0.258^{ab}	0.000±0.000^{a}	3.016±0.588^{b}	0.000±0.000^{a}
NAA0.1+BAP0.1	2.333±0.210*^{bd}	3.333±1.201^{ab}	0.166±0.166^{a}	4.800±1.553^{c}	0.200±0.200^{a}
NAA0.1+BAP0.5	1.666±0.210*^{ab}	2.666±0.881^{ab}	0.000±0.000^{a}	1.733±0.495^{a}	0.000±0.000^{a}
NAA0.1+BAP1.0	1.666±0.210*^{ab}	2.833±0.945^{ab}	0.000±0.000^{a}	0.366±0.049+*^{a}	0.000±0.000^{a}
NAA0.1+BAP2.0	1.666±0.210*^{ab}	5.333±1.115^{c}	0.000±0.000^{a}	4.983±1.083^{c}	0.000±0.000^{a}
NAA0.5+BAP0.1	0.666±0.210^{a}	1.666±0.557^{ab}	0.000±0.000^{a}	1.216±0.291^{a}	0.000±0.000^{a}
NAA0.5+BAP0.5	1.166±0.166*^{ab}	3.500±0.763^{b}	1.833±0.909^{b}	3.783±1.209^{bc}	1.466±0.245*^{b}
NAA0.5+BAP1.0	1.333±0.210*^{ab}	2.000±0.774^{ab}	0.666±0.666^{a}	0.650±0.105^{a}	1.050±0.547*^{b}
NAA0.5+BAP2.0	1.833±0.166*^{b}	2.666±0.714^{ab}	0.666±0.333^{a}	0.466±0.055+*^{a}	0.866±0.326^{ab}
NAA1.0+BAP0.1	1.500±0.223*^{ab}	2.500±0.763^{ab}	4.000±1.712*^{c}	5.816±0.993*^{d}	2.900±0.676*^{c}
NAA1.0+BAP0.5	2.333±0.333*^{bc}	0.000±0.000^{a}	0.000±0.000^{a}	0.000±0.000+*^{a}	0.000±0.000^{a}
NAA1.0+BAP1.0	1.833±0.307*^{b}	2.166±1.973^{ab}	0.000±0.000^{a}	2.083±0.713^{ab}	0.000±0.000^{a}
NAA1.0+BAP2.0	2.500±0.223*^{c}	0.666±0.666^{ab}	0.000±0.000^{a}	1.500±0.152^{a}	0.000±0.000^{a}

4.A.2.4. Effects of NAA and Kinetin in combinations on nodal explants:

Callus induction:

NAA at lower concentration i.e. 0.1 mg/l was found to be insignifantly inducing callus when incorporated with any concentration of Kn but above 0.1 mg/l of NAA showed significant result with all the concentrations of Kn. The maximum callus inductions were recorded when the shoot explants were inoculated in NAA 0.5 + Kn 0.5

(Fig. 26), NAA 0.5 + Kn 1.0, NAA 0.5 + Kn 2.0 and NAA 1.0 + Kn 2.0 mg/l all at a frequency of 100% whereas the lowest induction was recorded on the MS medium containing NAA 0.1 + Kn 0.1 mg/l with only 33.33% frequency (Table 4.5).

Table 4.5: Effects of various concentration combinations of NAA and Kinetin on nodal explants.

	Callus growth	Shoot number	Root number	Shoot length	Root length
PGR	Mean±Std. Error	Mean±Std. Error	Mean±Std. Error	Mean±Std. Error	Mean±Std.Error
Control	0.166±0.166^{a}	2.000±0.258^{ab}	0.000±0.000^{a}	3.016±0.588^{bc}	0.000±0.000^{a}
NAA0.1+Kn 0.1	0.333±0.210^{a}	0.833±0.307^{a}	0.000±0.000^{a}	0.383±0.040+*^{a}	0.000±0.000^{a}
NAA0.1+Kn 0.5	1.000±0.258^{ab}	1.166±0.307^{ab}	0.166±0.166^{a}	1.150±0.402^{ab}	0.066±0.066^{ab}
NAA0.1+Kn 1.0	1.000±0.365^{ab}	8.166±2.358*^{c}	0.000±0.000^{a}	3.766±0.423^{c}	0.000±0.000^{a}
NAA0.1+Kn 2.0	0.833±0.307^{a}	8.833±3.525*^{c}	0.000±0.000^{a}	2.350±0.408^{b}	0.000±0.000^{a}
NAA0.5+Kn 0.1	1.500±0.223*^{b}	5.166±0.872^{b}	0.500±0.500^{a}	1.550±0.236^{ab}	0.166±0.076^{ab}
NAA0.5+Kn 0.5	1.833±0.401*^{c}	3.000±0.730^{ab}	4.000±1.712*^{b}	3.516±0.559^{c}	2.550±0.536*^{e}
NAA0.5+Kn 1.0	1.833±0.166*^{c}	1.166±0.307^{ab}	0.333±0.333^{a}	1.900±0.403^{b}	0.683±0.436^{ab}
NAA 0.5+Kn 2.0	1.833±0.166*^{c}	4.166±1.249^{b}	0.833±0.542^{a}	2.383±0.644^{b}	1.400±0.462*^{d}
NAA 1.0+Kn 0.1	1.500±0.223*^{b}	4.333±1.054^{b}	1.000±0.516^{a}	1.600±0.570^{ab}	1.016±0.261*^{c}
NAA 1.0+Kn 0.5	1.666±0.210*^{bc}	4.500±1.087^{b}	0.333±0.210^{a}	1.950±0.556^{b}	0.883±0.702^{b}
NAA 1.0+Kn 1.0	1.666±0.210*^{bc}	3.833±1.400^{b}	0.000±0.000^{a}	1.333±0.443^{ab}	0.000±0.000^{a}
NAA 1.0+Kn 2.0	1.833±0.166*^{c}	5.833±2.242^{bc}	0.000±0.000^{a}	3.716±0.732^{c}	0.000±0.000^{a}

Shoot multiplication and their lengths:

From the data, it has been found that NAA 0.1 + Kn 1.0 and NAA 0.1 + Kn 2.0 mg/l were the only combinations of hormone concentrations that induced multiple shoots at 1% level of significance. The maximum average number of 8.83 shoots was induced on the medium containing NAA 0.1 + Kn 2.0 mg/l with 100% response (Fig. 27). The 66.66% of the explants responsed and induced a minimum average shoot number of 0.83 per explant with NAA 0.1+kn 0.1mg/l. In case of shoot length all the concentration combinations were found to be insignificant. The longest shoots were of average length 3.76 cm from the medium containing NAA 0.1 + Kn 1.0 mg/l (Fig. 28) but the shortest ones of 0.38 cm were found with the medium containing NAA 0.1 + Kn 0.1 mg/l (Table

4.5). The higher NAA (1.0 mg/l) with low Kn (0.1-0.5 mg/l) after 20 weeks of culture gave healthy multiple shoots (Fig. 29 and 30).

Root differentiation and their lengths:

NAA 0.5 + Kn 0.5 mg/l was the only medium which induced the root number as well as root length from the shoot induced calli at a significant level of 1% and at a frequency of 100%. At this concentration combination the average root number was 4 and the length was 2.55 cm (Table 4.5 and Fig. 31).

4.A.2.5. Effects of IBA and BAP in combinations on nodal explants:

Callus induction:

Callus induction was achieved maximum when IBA 0.5 + BAP 2.0, IBA 1.0 + BAP 0.5 and IBA 1.0 + BAP 2.0 mg/l were incorporated in the media with a success rate of 100% and all these combinations were also found to be significant at 1% level of significance (Fig. 32). The minimum callus formation was observed from the various combinations like IBA 0.1 + BAP 0.1and IBA 0.1 + BAP 0.5 with 50% responses and IBA 0.5 + BAP 0.5 and IBA 1.0 + BAP 1.0 mg/l with 66.66% response (Table 4.6).

Shoot multiplication and their lengths:

The maximum average number of shoot induction per explant was obt erpentirom the 100% explants cultured on the medium containing IBA 1.0 +BAP 1.0 mg/l with 10.83 shoots/explant (Fig. 33) whereas 66.66% explants produced the minimum number of shoots (1.83). The maximum shoot length (3.78) was recorded on the medium containing lowest amounts of both IBA and BAP (IBA 0.1 + BAP 0.1 mg/l) (Fig. 34). None of the combinations were found to be effective in increasing shoot length significantly but IBA 0.1 + BAP 2.0 and IBA 0.5 + BAP 1.0 mg/l were found to be negatively significant i.e. retarding the shoot elongation. IBA 0.5 +BAP 0.5 mg/l was another good shoot inducer (Fig. 35, Table 4.6).

Root differentiation and their lengths:

Almost all the hormone concentration combinations except IBA 0.5 + BAP 1.0 and IBA 1.0 + BAP 1.0 mg/l failed to induce any root (Fig. 36). The later one induced 1.00 root/per explant with 66.66% response and the former induced 0.16 root/explant with 16.66% response (Table 4.6).

Table 4.6: Effects of various concentration combinations of IBA and BAP on nodal explants.

	Callus growth	Shoot number	Root number	Shoot length	Root length
PGR	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error
Control	0.166±0.166^{a}	2.000±0.258^{ab}	0.000±0.000^{a}	3.016±0.588^{c}	0.000±0.000^{a}
IBA 0.1+BAP 0.1	0.666±0.333^{ab}	8.833±1.701*^{cd}	0.000±0.000^{a}	3.783±0.701^{c}	0.000±0.000^{a}
IBA 0.1+BAP 0.5	0.666±0.333^{ab}	2.5±0.885^{ab}	0.000±0.000^{a}	1.25±0.262^{ab}	0.000±0.000^{a}
IBA 0.1+BAP 1.0	1.000±0.365^{b}	2.833±0.542^{ab}	0.000±0.000^{a}	1.716±0.272^{ab}	0.000±0.000^{a}
IBA 0.1+BAP 2.0	1.166±0.307*^{bc}	2.833±0.703^{ab}	0.000±0.000^{a}	0.900±0.177-*^{a}	0.000±0.000^{a}
IBA 0.5+BAP 0.1	1.333±0.210*^{bc}	5.666±0.714^{bc}	0.000±0.000^{a}	3.516±0.321^{c}	0.000±0.000^{a}
IBA 0.5+BAP 0.5	0.666±0.210^{ab}	8.666±1.282*^{c}	0.000±0.000^{a}	6.366±0.542*^{d}	0.000±0.000^{a}
IBA 0.5+BAP 1.0	0.833±0.307^{ab}	5.833±0.542^{bc}	0.166±0.166^{a}	1.083±0.303-*^{ab}	0.216±0.216^{ab}
IBA 0.5+BAP 2.0	1.833±0.166*^{c}	5.000±1.064^{b}	0.000±0.000^{a}	2.550±0.309^{b}	0.000±0.000^{a}
IBA 1.0+BAP 0.1	1.333±0.210*^{bc}	1.833±0.792^{a}	0.000±0.000^{a}	1.933±0.631^{ab}	0.000±0.000^{a}
IBA 1.0+BAP 0.5	1.833±0.166*^{c}	3.5±1.408^{ab}	0.000±0.000^{a}	2.333±0.434^{b}	0.000±0.000^{a}
IBA 1.0+BAP 1.0	0.666±0.210^{ab}	10.83±1.815*^{d}	1.000±0.365*^{b}	1.500±0.273^{ab}	0.400±0.139^{b}
IBA 1.0+BAP 2.0	1.833±0.166*^{c}	5.666±1.686^{bc}	0.000±0.000^{a}	2.266±0.749^{b}	0.000±0.000^{a}

4.A.2.6. Effects of IBA and Kinetin in combinations on nodal explants:

Callus induction:

Only two of the combinations IBA 1.0 + Kn 0.5 and IBA 1.0 + BAP 2.0 mg/l in this series were significant in inducing callus from the shoots of *A. racemosus* with a high frequency of 83.33% (Fig. 37). The minimum induction was observed when the shoots were cultured on IBA 0.5 + Kn 0.1, IBA 0.5 + Kn 0.5 mg/l with 33.33% frequency (Table 4.7).

Table 4.7: Effects of various concentration combinations of IBA and Kinetin on nodal explants.

	Callus growth	Shoot number	Root number	Shoot length	Root length
PGR	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error
Control	0.166±0.166^{a}	2.000±0.258^{ab}	0.000±0.000^{a}	3.016±0.588^{ab}	0.000±0.000^{a}
IBA 0.1+Kn 0.1	0.500±0.223^{ab}	3.500±1.477^{ab}	0.000±0.000^{a}	2.633±0.696^{ab}	0.000±0.000^{a}
IBA 0.1+Kn 0.5	0.666±0.333^{ab}	3.000±1.316^{ab}	0.000±0.000^{a}	3.033±0.535^{ab}	0.000±0.000^{a}
IBA 0.1+Kn 1.0	1.000±0.258^{ab}	6.666±0.843*^{d}	0.000±0.000^{a}	2.616±0.678^{ab}	0.000±0.000^{a}
IBA 0.1+Kn 2.0	0.500±0.223^{ab}	5.500±0.500^{c}	0.000±0.000^{a}	3.400±1.104^{ab}	0.000±0.000^{a}
IBA 0.5+Kn 0.1	0.333±0.210^{ab}	1.333±0.421^{ab}	0.500±0.500^{a}	2.533±0.154^{ab}	1.183±0.565*^{b}
IBA 0.5+Kn 0.5	0.333±0.210^{ab}	1.166±0.307^{a}	0.500±0.500^{a}	2.366±0.298^{ab}	0.333±0.154^{a}
IBA 0.5+Kn 1.0	0.666±0.210^{ab}	4.500±1.979^{ab}	0.000±0.000^{a}	3.883±0.910^{b}	0.000±0.000^{a}
IBA 0.5+Kn 2.0	0.833±0.166^{ab}	8.33±1.308*^{e}	0.000±0.000^{a}	5.600±0.975*^{c}	0.000±0.000^{a}
IBA 1.0+Kn 0.1	1.000±0.258^{ab}	3.500±0.991^{ab}	0.000±0.000^{a}	2.033±0.635^{ab}	0.000±0.000^{a}
IBA 1.0+Kn 0.5	1.166±0.307*^{b}	2.166±0.477^{ab}	0.166±0.166^{a}	1.466±0.509^{a}	0.133±0.133^{a}
IBA 1.0+Kn 1.0	0.833±0.166^{ab}	4.333±1.115^{ab}	0.000±0.000^{a}	1.400±0.180^{a}	0.000±0.000^{a}
IBA 1.0+Kn 2.0	1.166±0.307*^{b}	2.166±0.872^{ab}	0.833±0.654^{a}	1.766±0.480^{ab}	1.333±0.358*^{b}

Shoot multiplication and their lengths:

A 100% of the explants produced the maximum number of multiple shoots on the medium containing IBA 0.5 + Kn 2.0 mg/l with 8.33 shoots per explant, which was highly significant at 1% level of significance (Fig. 38). The same concentrations combination also induced significantly longest shoots of 5.6 cm. The least number of shoots (1.16 shoots/explant) was produced on medium with IBA 0.5 + Kn 0.5 mg/l whereas the shortest shoots (1.40 cm) were recorded in MS medium containing IBA 1.0 + Kn 1.0 (Table 4.7). The explants on higher concentration of Kn (2.0 mg/l) with lower IBA concentration (0.1mg/l) produced highly vitrified shoots (Fig. 39).

Root differentiation and their lengths:

A 33.33% of the explants cultured on the medium containing IBA 1.0 + Kn 2.0 mg/l induced a maximum number of 0.83 roots/explant with an average of 1.33 cm length

(Fig. 40). The minimum number of 0.16 roots per explant with only16.66% response and an average length of 0.13 cm were observed on MS + IBA 1.0 + Kn 0.5 mg/l (Table 4.7).

4.A.2.7. Effects of IAA and BAP in combinations on nodal explants:

Callus induction:

None of the concentration combinations in this series induced callus significantly. The maximum callus formation was observed on the medium containing IAA 1.0 + BAP 1.0 mg/l with 100% success rate (Fig. 41). The least amount of callus formation was recorded on the MS medium containing IAA 0.1 + BAP 0.1 mg/l with only 33.33% success (Table 4.8).

Table 4.8: Effects of various concentration combinations of IAA and BAP on nodal explants.

	Callus growth	Shoot number	Root number	Shoot length	Root length
PGR	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error
Control	0.166[a]±0.166	2.000±0.258[a]	0.000±0.000[a]	3.016±0.588[b]	0.000±0.000[a]
IAA 0.1+BAP 0.1	0.333±0.210[a]	5.166±2.625[a]	0.000±0.000[a]	3.316±0.825[b]	0.000±0.000[a]
IAA 0.1+BAP 0.5	0.500±0.223[a]	3.500±1.727[a]	0.000±0.000[a]	1.000±0.152+*[a]	0.000±0.000[a]
IAA 0.1+BAP 1.0	0.833±0.307[a]	3.000±0.966[a]	0.000±0.000[a]	1.016±0.245+*[a]	0.000±0.000[a]
IAA 0.1+BAP 2.0	0.500±0.223[a]	2.000±0.577[a]	0.000±0.000[a]	0.866±0.248+*[a]	0.000±0.000[a]
IAA 0.5+BAP 0.1	0.500±0.223[a]	1.166±0.401[a]	0.000±0.000[a]	0.900±0.429+*[a]	0.000±0.000[a]
IAA 0.5+BAP 0.5	0.666±0.210[a]	1.000±0.365[a]	0.000±0.000[a]	0.500±0.068+*[a]	0.000±0.000[a]
IAA 0.5+BAP 1.0	0.666±0.210[a]	4.500±1.668[a]	0.000±0.000[a]	0.650±0.117+*[a]	0.000±0.000[a]
IAA 0.5+BAP 2.0	0.500±0.223[a]	1.833±0.703[a]	0.000±0.000[a]	0.750±0.133+*[a]	0.000±0.000[a]
IAA 1.0+BAP 0.1	0.833±0.166[a]	2.166±0.833[a]	0.000±0.000[a]	0.650±0.099+*[a]	0.000±0.000[a]
IAA 1.0+BAP 0.5	0.666±0.210[a]	2.333±0.557[a]	0.000±0.000[a]	0.966±0.076+*[a]	0.000±0.000[a]
IAA 1.0+BAP 1.0	1.000±0.000[a]	3.000±1.064[a]	1.666±1.308*[b]	1.333±0.277[a]	1.083±0.350*[b]
IAA 1.0+BAP 2.0	0.833±0.166[a]	1.833±0.401[a]	0.000±0.000[a]	0.766±0.196+*[a]	0.000±0.000[a]

Shoot multiplication and their lengths:

In this experiment significant amount of shoot formation was not observed in any of the combinations. The maximum number of shoots per explant was 5.16 on medium with IAA 0.1 + BAP 0.1 mg/l but only by 66.66% explants (Fig. 42). The same medium gave the longest shoots of 3.31 cm long. Minimum shoot number of 1.0 per explant was observed on IAA 0.5 + BAP 0.5 mg/l containing medium also at 66.66% rate. The shortest shoots were also observed in the same medium with an average length of 0.5 cm (Table 4.8). BAP above 1.0 mg/l induced vitrification of shoots (Fig. 43).

Root differentiation and their lengths:

Among all the concentration combinations, only 33.33% of the total explants cultured on the medium containing IAA 1.0 + BAP 1.0 mg/l were able to induce an average number of 1.66 roots per explant with an average length of 1.08 cm (Table 4.8 and Fig. 45).

4.A.2.8. Effects of IAA and Kinetin in combinations on nodal explants:

Callus induction:

The maximum callus formations were found from the 83.33% of the explants each when they were cultured on IAA 0.5 + Kn 0.1, IAA 0.5 + Kn 1.0, IAA 0.5 + Kn 2.0 and IAA 1.0 + Kn 1.0 mg/l which were significant at 1% level of significance (Fig. 46). Only 66.66% of the explants cultured on MS containing IAA 1.0 + Kn 0.5 mg/l produced the minimum amount of callus (Table 4.9).

Shoot multiplication and their lengths:

None of the treatments of this series induced shoots significantly. The maximum shoot proliferation of 5.83/ explant was achieved when the shoot explants were cultured on MS medium containing IAA 0.1 + Kn 2.0 mg/l with 83.33% response (Fig. 47). The longest shoots of 2.98 cm were recorded from the medium containing IAA 1.0 + Kn 1.0 mg/l; however, none of the shoots in this series of experiment were longer than that of hormone free MS medium i.e. 3.01 cm. Similarly, 83.33% of the explants cultured on the media containing IAA 0.1 + Kn 0.1 and IAA 0.5 + Kn 1.0 mg/l produced only 1.5 shoots

per explant. The shsortest shoots were measured to be of 0.55 cm long on the medium containing IAA 0.1 + Kn 1.0 mg/l (Table 4.9).

Table 4.9: Effects of various concentration combinations of IAA and Kinetin on nodal explants.

	Callus growth	Shoot number	Root number	Shoot length	Root length
PGR	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error
Control	0.166±0.166^{a}	2.000±0.258^{a}	0.000±0.000^{a}	3.016±0.588^{d}	0.000±0.000^{a}
IAA 0.1+Kn 0.1	0.833±0.166^{ab}	1.500±0.428^{a}	0.333±0.333^{a}	1.733±0.576^{ab}	0.966±0.620*^{b}
IAA 0.1+Kn 0.5	0.833±0.166^{ab}	1.666±0.333^{a}	0.000±0.000^{a}	0.850±0.168+*^{ab}	0.000±0.000^{a}
IAA 0.1+Kn 1.0	1.333±0.210*^{b}	2.000±0.577^{a}	0.000±0.000^{a}	0.550±0.05+*^{a}	0.000±0.000^{a}
IAA 0.1+Kn 2.0	1.000±0.365^{ab}	5.833±2.056^{b}	0.000±0.000^{a}	2.433±0.809^{c}	0.000±0.000^{a}
IAA 0.5+Kn 0.1	1.166±0.307*^{ab}	2.666±0.333^{a}	0.000±0.000^{a}	1.016±0.140+*^{ab}	0.000±0.000^{a}
IAA 0.5+Kn 0.5	1.000±0.258^{ab}	1.666±0.333^{a}	0.000±0.000^{a}	0.683±0.090+*^{ab}	0.000±0.000^{a}
IAA 0.5+Kn 1.0	1.166±0.307*^{ab}	1.500±0.341^{a}	0.000±0.000^{a}	1.016±0.153+*^{ab}	0.000±0.000^{a}
IAA 0.5+Kn 2.0	1.166±0.307*^{ab}	2.333±0.494^{a}	0.000±0.000^{a}	2.716±0.572^{d}	0.000±0.000^{a}
IAA 1.0+Kn 0.1	1.333±0.333*^{b}	1.666±0.421^{a}	0.166±0.166^{a}	1.800±0.252^{ab}	0.133±0.133^{a}
IAA 1.0+Kn 0.5	0.666±0.210^{ab}	1.833±0.401^{a}	0.000±0.000^{a}	1.616±0.479^{ab}	0.000±0.000^{a}
IAA 1.0+Kn 1.0	1.333±0.333*^{b}	2.333±0.760^{a}	0.000±0.000^{a}	2.983±0.591^{d}	0.000±0.000^{a}
IAA 1.0+Kn 2.0	1.166±0.307*^{ab}	2.833±0.945^{a}	0.666±0.494^{a}	2.083±0.879^{b}	0.516±0.203^{ab}

Root induction and their lengths:

Almost all the concentration combinations in this series also did not induce any root. A few which responded also were very insignificant. Mainly the shoots first induced callus from which the roots differentiated (Fig 49 and 50). Maximum number observed was 0.66 roots per explant on the medium with IAA 1.0 + Kn 2.0 (Fig. 49) and the minimum of 0.16 was in IAA 1.0 + Kn 0.1 mg/l but with only 33.33% and 16.66% success rates respectively. Similarly, the longest roots were found to be of 0.96 cm in IAA 0.1 + Kn 0.1 and the shortest one of 0.13 cm in IAA 1.0 + Kn 0.1 mg/l (Table 4.9).

4.A.2.9. Responses of MS basal medium to different explants:

MS basal medium (without any hormone addition) showed no effects with cladodes, internodes and roots, however a slight secondary callus growth from the primary callus was observed (Fig. 51). In case of shoot tips, mainly the shoot growth (shoot elongation) was observed whereas the nodes frequently induced multiple shoots up to an avarage number of 2.0±0.25 (Fig. 52 and 53). In all cases, no roots, buds or somatic embryoids were observed (Table 4.10).

4.A.3. Agar concentration manipulation:

Since, neither leaves nor roots responded in the initial trials, only the shoots (shoot tips, nodes, and internodes) were innoculated on the agar manipulated media. All the explants were innoculated in the media containing different concentrations of NAA. Since, internodes responded less both in callus as well as root induction and the shoot tips generally showed shoot elongation only, all the data for this experiment were recorded from the node explants. All the explants gave callus in the beginning and later differentiated to roots only. The maximum callus inductions were recorded from 0.6% and 1.0% agar concentration with 100% success rate when the explants were cultured on the MS + NAA 2.0 mg/l whereas all the explants cultured on the same medium with a low NAA concentration (0.1 mg/l) induced minimum callus with 1.33 points (Fig. 57). An average of 11.33 roots were recorded from the MS + NAA 0.1 mg/l + 1.0% agar whereas the minimum number was found on MS + NAA 0.5 mg/l+ 0.6% agar (Fig. 58). Similarly, the root length was at its heighest when the explants were cultured on MS + NAA 0.1mg/l + 0.8% agar with 3.3 cm length. Here, the shortest roots of 1.0 cm were found on the same medium with NAA 0.5 mg/l (Fig. 59). The details are given in the graphs and picture plates below (Fig. 57-65).

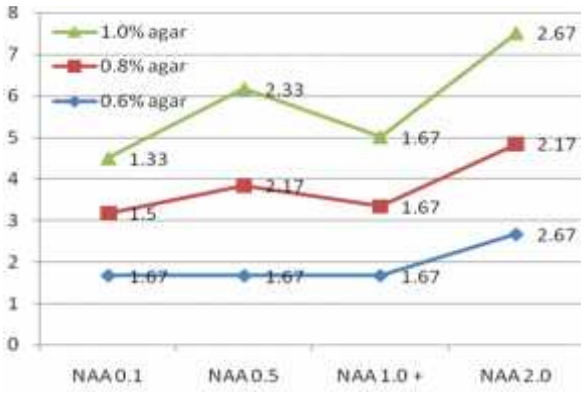


Fig. 57. Effect of agar and NAA concentrations on callus induction

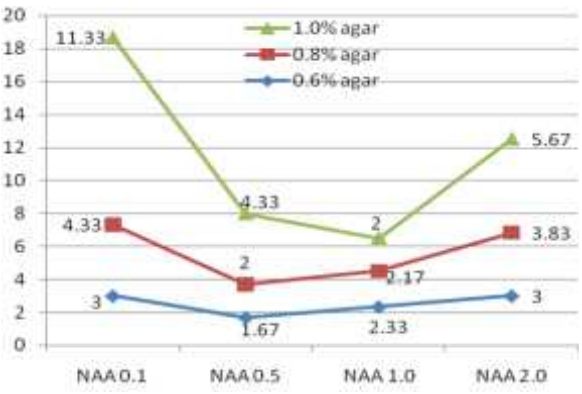


Fig. 58. Effect of agar and NAA concentrations on root induction

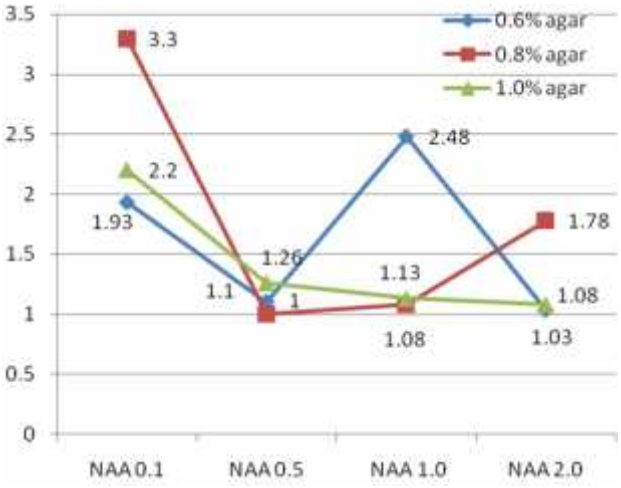


Fig. 59. Effect of agar and NAA concentration on root length

4.A.4 Somatic embryogenesis and Callus analysis:

In MS + NAA 2.0 mg/l, most of the concentrations of BAP and Kn and many other auxin (IAA, IBA or NAA)-cytokinin (BAP or Kn) combinations induced somatic embryoids from the callus as well as shoots with an intermediate callus. In combinations of auxin and cytokinin also, a higher concentrations of both favored the induction of somatic embryoids. As treated individual, BAP was found to induce somatic embryoids more than in presence of Kinetin in the medium (Table 4.10). In the same way the observed maximum number of mature somatic embryoids from the callus were found in the callus from BAP 2.0 mg/l, NAA 0.1 + BAP 1.0 and NAA 0.1 + BAP 2.0 mg/l (Fig. 66 and 67). The plantlets germinated from the somatic embryoids in general were found

to be relatively much thicker and with less cladodes than the normal shoots (Fig. 68 and 69). Similarly, the length of internodes was also longer than the normal ones.

The calli obtained from the shoot explants cultured on all the concentrations were cytologically examined. The friable, tough and greenish yellow calli contained the somatic embryoids at different stages of their development. The watery, brown and black calli were discarded. The friable, tough and greenish calli of 10-12 weeks were studied under the different magnifications of a compound microscope. In some cases, (NAA 0.1 + BAP 2.0 mg/l) the somatic embryoids grew so much that they were easily visible (Fig. 67). The embryogenic calli when spread appeared densely nucleated cells (Fig. 70). Similarly, actively dividing embryogenic cells at various stages of somatic embryoids like 2 celled (Fig. 71 and 72), 4 celled and multicellular proembryoids (8 or more cells) of different shapes were observed. The embryogenic cells generally were found to divide transversely to give a linear four celled pro embryoids (Fig. 73 and 74). It has also been observed that the apical cell then divides vertically and then transversely to form an octant (Fig.75, 76, 77 and 78). Like a typical monocot pro embryo, a large haustorium was also observed (Fig. 79). In the same way, the globular embryoids (Fig. 80 and 81) were also observed. The torpedo as well as cotyledonary stage embryoids were also observed (Fig. 82 - 83 and 84 respectively). The photo micrographs of somatic embryogenesis (Fig. 70-85).

4.A.5. Buds, caulogenesis and vitrification of shoots:

To study the buds, embryogenesis and vitrification, all the possible explants Viz. nodes, internodes, calli and roots were used (Table 4.10).

Buds:

The buds generally started at the nodes (Fig. 87 and 88) in just after 1-2 weeks of culture but in some cases they started even under the callus mass grown under the rapidly growing/multiplying shoots as on IBA 0.5 + BAP 0.5 mg/l (Fig. 86). It has also been observed that in the media containing high concentrations of either cytokinins alone or in combinations with auxins induce shoot buds at the nodes. The shape and size of the buds also varied from media to media. The buds grown on the medium containing NAA 0.1 +

BAP 2.0 mg/l were the biggest among all (Fig. 89). The germination behaviors of these buds were also different. The MS + IBA 0.5 + BAP 0.5 mg/l grown buds produced 1-2 normal shoots, but the buds induced from NAA 0.1 + BAP 2.0 mg/l were slightly thicker. The rest of the media grown buds induced single normal shoots. Under our observation the maximum number of buds/ node was observed to be 11.66 when the explants were treated with NAA 0.1+BAP 2.0 mg/l followed by IBA 1.0 + BAP 2.0 mg/l with 10.33. In general, higher concentrations of cytokinin induced shoot buds at all levels. The detaile data are given in the table 4.11. BAP has been found to be more effective than Kn in bud induction in general. In combinations of auxins and cytokinins, NAA and IBA induced buds with both of the cytokinins tested whereas IAA with Kn showed less response.

Those shoot buds as induced on the medium containing IBA 1.0 + BAP 2.0 mg/l slowly mature on the mother plant and finally get easily detached. These buds after detachment from the mother plants behaved as the normal seeds. They germinated producing single to multiple shoots (mainly1-2 shoots) with or without roots. These buds used as explants showed very good response in the initial trials and possibally can be used as one of the good explants for rapid multiplication of *A. racemosus* (Fig. 86-90).

Caulogenesis:

Similarly, caulogenesis from the callii under all the treatments were also recorded. In most of the cases, the callii when sub cultured in different media induced either shoots or roots or both (Fig. 91-93). From the data available it can be concluded that the cytokinins at lower concentrations induce multiple shoots however kinetin have been found to induce more roots than shoots. The shoot induction decreased as the concentration of cytokinins increased. Similarly, auxins when in low concentration induce multiple shoots but in higher concentrations induce roots. In combinations of auxin and cytokinin NAA band IBA have been found to be good for caulogenesis with BAP than Kn. Here, auxin and cytokinin together in most of the cases shoots were induced. In some cases where auxin concentration was much higher, both shoots and roots were induced from the same explant.The detailed data and information have been given in the table 4.10 and the figures 91-93.

Table: 4.10. Buds, Somatic embryoids, Caulogenesis and Vitrification observation.

Hormone Concentrations	Explant	Mean no. of buds± Std.Error	Vitrification	Caulogenesis	S. E.
Control	Nodes/callus	0.000±0.000	N		
NAA 0.1	Nodes	0.666±0.333[a]	N	(2S)	
NAA 0.5			N	(3R)	
NAA 1.0			N	(2R)	
NAA 2.0	Nodes	1.000±0.577[a]	N	(2R)	Y
BAP 0.1	Nodes	2.333±0.881[a]	N	(3S)	Y
BAP 5.0	Nodes	2.000±0.577[a]	N	(3S)	Y
BAP 1.0	Callus/Nodes	3.666±1.452[ab]	Y	(3S)	Y
BAP 1.5	Callus/nodes	3.333±1.855[a]	Y	(1S)	Y
BAP 2.0	Callus/nodes/internodes	1.333±0.333[a]	Y	(1S)	Y
BAP 3.0	Callus/nodes/internodes	1.000±0.000[a]	Y		Y
NAA 0.1+BAP 0.1			N	(3S1R)	
NAA 0.1+BAP 0.5	Callus/nodes	2.333±0.881[a]	N	(2S)	Y
NAA 0.1+BAP 1.0	Callus	3.000±2.081[a]	N	(2S)	Y
NAA 0.1+BAP 2.0	Callus	11.666±6.009*[c]	Y	(3S)	Y
NAA 0.5+BAP 0.1	Callus/nodes	0.333±0.333[a]	N		
NAA 0.5+BAP 0.5	Callus/nodes	2.000±0.577[a]	N	(2S1R)	Y
NAA 0.5+BAP 1.0	Callus/nodes	0.666±0.333[a]	Y	(1S1R)	
NAA 0.5+BAP 2.0	Callus	4.000±1.154[ab]	N	(2S1R)	Y
NAA 1.0+BAP 0.1			N	(2S2R)	Y
NAA 1.0+BAP 1.0	Callus/nodes	9.000±6.658*[b]	Y	(1S)	
NAA 1.0+BAP 2.0			Y	(2S)	Y
Kn 0.5			N	(3R)	Y
Kn 1.0	Callus	3.666±0.881[ab]	Y	(2S1R)	Y
Kn 2.0	Callus	1.666±0.333[a]	Y	(2R)	Y
N 0.1+Kn 0.5	Callus	0.666±0.666[a]	N	(1S)	Y
N 0.1+Kn 1.0			N	(2S)	Y
N 0.1+Kn 2.0		4.000±4.000[ab]	N		Y
N 0.5+Kn 0.1		0.666±0.666[a]	N	(1R1S)	Y
N 0.5+Kn 0.5		1.333±0.333[a]	N	(2R1S)	Y
N 0.5+Kn 1.0			N	(2R1S)	Y
N 0.5+Kn 2.0	Nodes	3.333±0.881[a]	N	(2S)	Y
N 1.0+Kn 0.1	Nodes/callus	2.666±0.881[a]	N	(2S1R)	
N 1.0+Kn 0.5	Nodes/callus	2.333±0.333[a]	N	(2S1R)	Y
N 1.0+Kn 1.0			N	(1S)	Y
N 1.0+Kn 2.0	Callus	1.666±1.666[a]	Y	(1S)	Y
N0.5+2,4-D 0.5	NA	NA	NA	NA	Y
IAA 0.1+BAP 0.5	Nodes	2.000±1.000[a]	Y		Y
IAA 0.1+BAP 1.0	Nodes	4.000±2.309[ab]	Y		Y

IAA 0.1+BAP 2.0	Nodes	3.000±0.577[a]	Y		Y
IAA 0.5+BAP 0.1	Nodes	1.000±1.000[a]	N		
IAA 0.5+BAP 0.5	Nodes	1.333±1.333[a]	N		Y
IAA 0.5+BAP 1.0	Nodes	3.000±1.527[a]	Y		
IAA 0.5+BAP 2.0	Nodes	1.000±0.577[a]	Y		
IAA 1.0+BAP 0.1	Nodes	0.333±0.333[a]	N		
IAA 1.0+BAP 0.5	Nodes	1.666±1.666[a]	N		Y
IAA 1.0+BAP 1.0	Nodes	1.333±0.666[a]	Y		Y
IAA 1.0+BAP 2.0	Nodes	3.000±1.732[a]	Y		Y
IAA 0.1+Kn 0.1	Callus/nodes	1.00±1.000[a]	N	(1R)	
IAA 0.1+Kn 0.5	Nodes	2.333±0.577[a]	N		
IAA 0.1+Kn 1.0	Nodes	3.666±1.527[ab]	N		
IAA 0.1+Kn 2.0	Nodes	4.00±1.000[ab]	Y		
IAA 0.5+Kn 2.0	Nodes	1.000±0.000[a]	N		
IAA 1.0+Kn 0.1			N	(1R)	
IAA 1.0+Kn 2.0			N	(1R)	
IBA 0.1+BAP 0.1	Callus/nodes	0.333±0.333[a]	N		Y
IBA 0.1+BAP 0.5	Callus/nodes	6.333±4.841[abc]	N	(3S)	
IBA 0.1+BAP 1.0	Nodes/internodes	3.666±2.185[ab]	Y		
IBA 0.1+BAP 2.0	Nodes	4.000±0.577[ab]	Y	(3S)	Y
IBA 0.5+BAP 0.1	Callus/nodes	1.333±0.333[a]	N	(3S)	
IBA 0.5+BAP 0.5	Callus/nodes	1.333±0.333[a]	Y	(3S)	Y
IBA 0.5+BAP 1.0	Callus/nodes	2.333±0.881[a]	Y	(1R2S)	Y
IBA 0.5+BAP 2.0	Callus/nodes	5.000±0.577*[abc]	Y	(2S)	Y
IBA 1.0+BAP 0.1	Callus/nodes	2.666±0.666[a]	N	(2S)	Y
IBA 1.0+BAP 0.5	Callus/nodes	3.333±1.333[a]	Y	(1S)	Y
IBA 1.0+BAP 1.0	Callus/nodes	1.333±0.333[a]	Y	(1R3S)	Y
IBA 1.0+BAP 2.0	Callus/nodes	10.333±3.527*[b]	Y	(2S)	Y
IBA 0.1+Kn 0.1	Callus/nodes	3.000±0.577[a]	N	(1S)	
IBA 0.1+Kn 0.5	Callus/nodes	1.000±0.577[a]	N	(1S)	
IBA 0.1+Kn 1.0	Callus/nodes	2.000±1.154[a]	N	(3S)	
IBA 0.1+Kn 2.0	Callus/nodes	3.666±2.185[ab]	Y		
IBA 0.5+Kn 0.1	Callus/nodes	2.000±2.000[a]	N		
IBA 0.5+Kn 1.0			Y		Y
IBA 0.5+Kn 2.0	Callus/nodes	2.000±1.154[a]	N		
IBA 1.0+Kn 0.1	Callus/nodes	2.666±0.881[a]	N	(3S)	Y
IBA 1.0+Kn 0.5	Callus/nodes	1.666±0.333[a]	N	(1R2S)	Y
IBA 1.0+Kn 1.0	Callus/nodes	5.666±1.201*[abc]	Y	(2S)	Y
IBA 1.0+Kn 2.0	Callus/nodes	3.000±0.577[a]	N	(2R1S)	Y

Y = Yes, N = No, Blank = Data not available, S = Shoot, R = Root, 1 = 1-2, 2 = 3-5, 3 = more than 5, S. E. = Somatic Embryoids.

Vitrification:

Shoots formed from the media containing BAP either singly or in combinations with any of the auxins greatly reduce the number of cladodes as well as the shoots get widely vitrified (especially when BAP is above 1.0 mg/l). Similar treatments of kinetin induced normal shoots although in some cases vitrifications were observed. Hence in case of shoot multiplication, Kn is superior to BAP. The abnormal vitrified shoots from the media containing high BAP concentrations were also used as the explants and they also produced normal multiple shoots (very high upto 10 shoots/ explant) on various media containing either low concentrations of cytokinin or auxins or both in combinations. Hence, the vitrified shoots that are normally discarded in tissue culture have also been used to multiply the plant fastly.

4.A.6. Rooting and acclimatization:

NAA was effective both in *in vitro* as well as *in vivo* conditions whereas other auxins like IAA and IBA did not induce roots at any concentrations. The best rooting was observed at NAA 0.1 mg/l *in vitro* (Fig. 12 and 15). *In vivo* rooting was very difficult and a very few one out of 10 rooted with pulse treatment of NAA 100 mg/l. A 100% coco-peat acclimatization was found to be better than any other substrate (Fig. 54 and 55). The *in vitro* rooted micro shoots survived, grew and branched in the plastic shade house for about 3 months and they gradually died in the winter (Fig. 56). The final survival percentage of both *in vitro* and *in vivo* rooted shoots in nature was 60 and 20 respectively.

***Asparagus racemosus* Willd.**

Fig. 4A and 4A$_1$: Habitat and seeds of *Asparagus racemosus* Willd.

Fig. A- Habitat of *Asparagus racemosus* plant growing in the garden of CDB, TU.

Fig. A$_1$. Germinating seeds of *A. racemosus* in the petridish on a moist filter paper for germination experiments.

Figure 4.A.1: Seed germination of *Asparagus racemosus* on MS medium:

Fig. 1- Emergence of root (with no secondary root) and no shoot after 8 weeks of culture.

Fig. 2- Emergence of shoot without roots after 8 weeks of culture.

Fig. 3- Emergence of one shoot with rudimentary root after 8 weeks of culture.

Fig. 4- Growth of root and shoot after 8 weeks of culture.

Fig. 5- Multiple shoot (5) with single root system after 8 weeks of inoculation.

Fig. 6- Multiple shoot (4) with single root system after 8 weeks of inoculation.

Fig. 7- Multiple shoots (4) emerging from the single point after 8 weeks of culture.

Fig. 8 - Multiple shoot formation from the cut bases of shoots after 7 weeks of culture.

Fig. 9- Multiple shoot and root induction from the cut bases of shoots after 3 weeks of culture.

Asparagus racemosus Willd.

Fig. A. Habitat of *A. racemosus*

Fig. A_1. Seeds on germination trial.

Fig. 1.

Fig. 2.

Fig. .3

Fig. 4.

Fig. 5.

.Fig. 6

Fig. 7.

Fig. 8.

Fig. 9.

Figure 4.A.2: Various effects of Auxins in *A. racemosus*.

Fig. 10 – Callus from the node on MS + NAA 0.5 mg/l after 10 weeks of culture.

Fig. 11- Callus from the node on MS + NAA 2.0 mg/l after 10 weeks of culture.

Fig. 12- Multiple shoot and root from node on MS + NAA 0.1 mg/l after 9 weeks.

Fig. 13 – Multiple shoots from the node on MS+IBA 0.5 mg/l after 12 weeks.

Fig. 14- Normal multiple shoots from vitrified shoot on MS+IAA 0.5 mg/l in 10 weeks.

Fig. 15- Root differentiation from a single shoot on MS + NAA 0.1 mg/l after 8 weeks.

Fig. 4.A.3: Various effects of Cytokinins on *A. racemosus*.

Fig. 16- Callus formation from the shoot on MS + Kn 2.0 mg/l after 12 weeks of culture.

Fig. 17- Multiple shoots from the vitried mass of tissue on BAP 0.1 mg/l after 10 weeks.

Fig. 18- Multiple shoots from vitrified tisses on MS + BAP 1.0 mg/l after 12 weeks.

Fig. 19- Abnormal (vitrification) shoot formation on MS + BAP 2.0 mg/l after 8 weeks.

Fig. 20- Root formation from the marginal cells of the shoot induced callus on MS + Kn 0.5 mg/l after 12 weeks of culture.

Fig. 4.A.4: Various effects of NAA and BAP in combination.

Fig. 21- Hard callus induction followed by formation of somatic embryoids on MS + NAA 0.1 + BAP 2.0 mg/l after 12 weeks of culture.

Fig. 22- Multiple shoots from vitrified shoot after subculture on NAA 0.1+BAP 2.0 mg/l.

Fig. 23- Long and healthy roots and shoots with callus at the base from a node on MS + NAA 1.0 + BAP 0.1 mg/l after 12 weeks of culture.

Fig. 24- Vitrified shoots from callus on MS + NAA 1.0 + BAP 2.0 mg/l after 12 weeks.

Fig. 25- Roots from callus on MS+NAA 1.0+BAP 0.1 mg/l in 10 weeks of sub culture.

Fig. 4.A.5: Various effects of NAA and Kinetin in combination:

Fig. 26- Friable callus from node on MS+NAA 0.5+Kn 0.5 mg/l after 12 weeks.

Fig. 27- Multiple shoots from node on MS + NAA 0.1 + Kn 2.0 after 12 weeks.

Fig. 28- Multiple shoots from a node on MS + NAA 0.1 + Kn 1.0 mg/l after 10 weeks.

Fig. 29- Healthy branched shoots from node on MS+NAA 1.0+Kn 0.5 mg/l in 20 weeks.

Fig. 30- Multiple shoots from node on MS + NAA 1.0 + Kn 2.0 mg/l after 10 weeks.

Fig. 31- Multiple root formation from callus on MS + NAA 0.5+K 0.5 mg/l in 12 weeks.


Fig. 10


Fig. 11


Fig. 12


Fig. 13


Fig. 14


Fig. 15


Fig. 16


Fig. 17


Fig. 18


Fig 19


Fig. 20

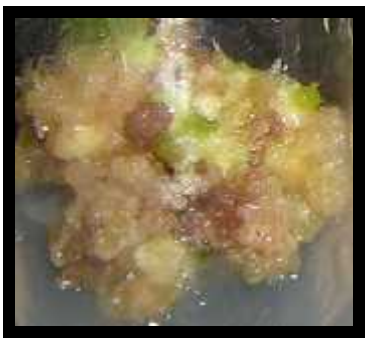


Fig. 21


Fig. 22


Fig. 23


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Fig. 29


Fig. 30


Fig. 31

Fig. 4.A.6: Various effects of IBA and BAP in combination.

Fig. 32- Primary and secondary callus from node in 12 wks. On MS+IBA 1.0+BAP 2.0.

Fig. 33- Multiple shoot induction from node on MS+IBA 1.0+BAP 1.0 mg/l in 8 weeks.

Fig. 34- Healthy multiple shoots from node on MS+IBA 0.1+BAP 0.1 mg/l in 12 weeks.

Fig. 35- Multiple shoots with heavy branching from a node erpenti on MS + IBA 0.5 + BAP 0.5 mg/l after 14 weeks of culture.

Fig. 36- Root induction from a node on MS + IBA 1.0 + BAP 1.0 mg/l after 10 weeks.

Fig. 4.A.7: Various effects of IBA and Kn in combinations.

Fig. 37- Green callus from a shoot on MS+IBA 1.0+Kn 2.0 mg/l after 10 week.

Fig. 38- Long, branched and healthy multiple shoots induced from nodes on MS + IBA 0.5 + Kn 2.0 mg/l after 14 weeks of culture.

Fig. 39- Vitrified shoots induced from node on MS+IBA 0.1+Kn 2.0 mg/l in 12 weeks.

Fig. 40- Root induction from the shoot with small amount of callus on the explants surface on MS + IBA 1.0 + Kn 2.0 mg/l after 10 weeks of culture.

Fig. 4.A.8: Various effects of IAA and BAP in combinations.

Fig. 41- Callus from the surface of a shoot on IAA 1.0+BAP1.0 mg/l in 8 weeks.

Fig. 42- Long healthy multiple shoot formation on IAA 0.1+BAP 0.1 mg/l in 12 weeks.

Fig. 43- Vitrified shoots induced from the normal node explant on IAA 0.5 + BAP 1.0 mg/l after 15 weeks of culture.

Fig. 44- Normal as well as abnormal (vitrified) multiple shoots formed from the node explant on IAA 0.1 + BAP 0.1 mg/l after 12 weeks of culture.

Fig. 45- Weak multiple roots from the shoot on IAA 1.0 + BAP1.0 mg/l after 12 weeks.

Fig. 4.A.9: Various effects of IAA and Kn in combinations.

Fig. 46- Friable callus formed from the node on IAA 1.0 + Kn1.0 mg/l after 13 weeks.

Fig. 47- Long multiple shoots from the node on IAA 0.1 + Kn 2.0 mg/l after 12 weeks.

Fig. 48- Normal healthy shoots formed from node on IAA 0.5+Kn 2.0 mg/l in 12 weeks.

Fig. 49- Multiple roots from a node induced callus on IAA 1.0+Kn 2.0 mg/l in 10 weeks.

Fig. 50- Root formation from shoot induced callus on IAA 0.1+Kn 0.1 mg/l in 12 weeks.


Fig. 32

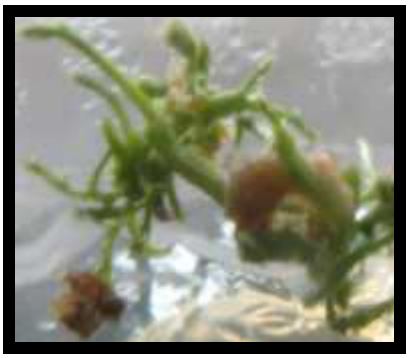
Fig. 33


Fig. 34


Fig. 35


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Fig. 37


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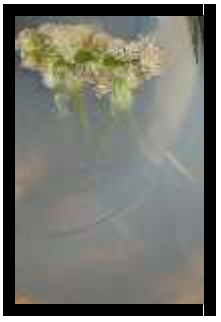
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Fig. 49


Fig. 50

Fig. 4.A.10-: Various effects of MS hormone free medium on different explants.

Fig. 51- Slight growth of secondary callus from the sub culture of shoot induced calli explants on hormone free MS medium after 12 weeks of culture.

Fig. 52- Normal shoot induction and branching from the node explant after 10 weeks of culture on hormone free MS medium.

Fig. 53- Normal multiple shoot formation from a node on MS medium after 15 weeks.

Fig. 54- Acclematization of *in vitro* rooted shoots of .*A. recemosus* on sand soil mixture 50% each

55. Acclematization of *A. recemosus* shoot on 100% coco-peat after *in vivo* rooting using NAA 100 mg/l pulse treatment.

Fig. 56- A well eatablished *A. recemosus* plant growing on the garden soil after acclematization for 13 weeks inside the shade house.

Fig. 4.A.11: Callus and root induction by NAA and agar concentrations.

Fig. 60- Root formation from the shoot induced callus on MS + NAA 1.0 mg/l with 0.6% agar after 15 weeks of culture.

Fig. 61- Heavy friable callus formation from the shoot explant on MS + NAA with 2.0 mg/l with 0.6% agar after 12 weeks of culture.

Fig. 62- Heavy rooting from the node on MS + NAA 0.1 mg/l with 1.0% agar in 15 weeks.

Fig. 63- Friable callus induction after 10 weeks of culture of a node and the root formation from the same callus after 14 weeks of culture on NAA 0.5 mg/l with 1.0% agar.

Fig. 64- Heavy friable callus from a node on NAA 1.0 mg/l with 1.0% agar after 12 weeks.

Fig. 65- Friable callus mass induced from a shoot on NAA 2.0 mg/l with 1.0% agar after 10 weeks and root formation from the same callus on the same medium after 12 weeks.

Fig. 4.A.12: Somatic embryoid induction and germination.

Fig. 66- Induction of somatic embryoids from the friable callus induced from the node on MS + NAA 0.1 + BAP 1.0 mg/l after 12 weeks of culture.

Fig- 67- Formation of somatic embryoids from the shoot induced tough callus on MS + NAA 0.1 + BAP 2.0 mg/l after 13 weeks of culture.

Fig. 68- Germination of embryoids from the shoot induced callus after 8 weeks of callus on MS + IBA 1.0 + BAP 1.0 mg/l.

and 69- A germinated embryoid with distinct shoot and root after 8 weeks on NAA 0.1 mg/l.


Fig. 51


Fig. 52


Fig. 53


Fig. 54


Fig 55


Fig. 56


Fig. 60


Fig. 61


Fig. 62


Fig. 63


Fig. 64


Fig. 65


Fig. 66


Fig. 67


Fig. 68


Fig.69

Fig. 4.A.13. Photomicrographs:

Fig. 70- Embryogenic cells of callus spread (12 weeks) from MS + BAP 1.0 mg/l (10×4).

Fig. 71 and 72- Actively dividing embryogenic cells of the callus (10×40).

Fig. 73 and 74- Longitudinal cell divisions forming 4 celled pro embryoids (10×40).

Fig. 75-78- Transverse and vertical divisions to give octant pro-embryoids (10×40).

Fig. 79- A pro embryoid showing a big houstorium cell (10×40).

Fig. 80-81- Globular embryoids (10×40).

Fig. 82-83- Torpedo stages of somatic embryoids (10×40).

Fig. 84- A cotyledonary stage somatic embryoid and

Fig. 85- A mature somatic embryo exposed (10×4).

Fig. 4.A.14- Bud induction from the nodes.

Fig. 86- Different stages of buds induced from the node induced shoots on the MS medium containing IBA 0.5 + BAP 0.5 mg/l after 11 weeks of culture.

Fig. 87- Multiple and different shapes of buds at the nodes from MS + BAP 1.0 mg/l after 8 weeks of culture.

Fig. 88- A cone shaped bud from the MS + BAP 3.0 mg/l after 8 weeks of culture.

Fig. 89- Multiple buds (large number) of buds of different lengths induced from a node on MS + NAA 0.1 + BAP 2.0 mg/l after 12 weeks of culture.

Fig. 90- Emergence of shoot from the bud (inside the bud).

Fig. 4.A.15- Caulogenesis (Shoot and root formation from the callus).

Fig. 91- Multiple shoots regeneration from the callus after 12 weeks of culture on MS + NAA 0.1 mg/l.

Fig. 92- Massive root formation from the callus on the MS + NAA 2.0 mg/l.

Fig. 93- Emergence of shoot (shoot meristem clearly visible) from callus (organogenesis) cultured on MS + NAA 0.1 + BAP 2.0 mg/l after 12 weeks of culture.


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Fig. 73


Fig. 74


Fig. 75.


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Fig. 78


Fig. 79


Fig. 80

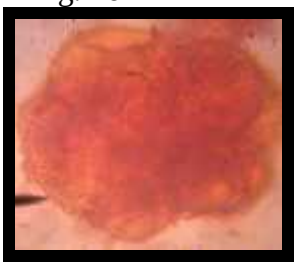
Fig. 81


Fig. 82


Fig. 83

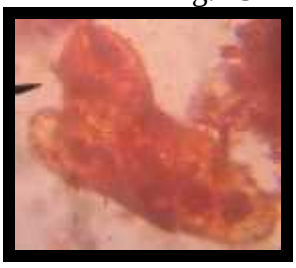
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Fig. 86


Fig. 87


Fig. 88

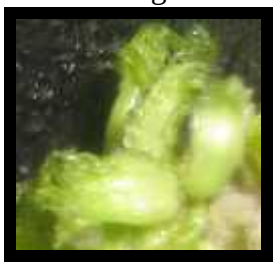
Fig. 89

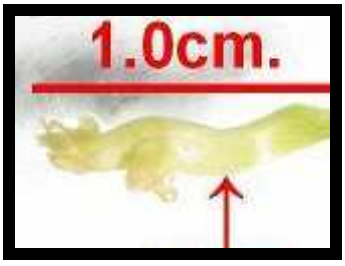

Fig. 90


Fig. 91


Fig. 92

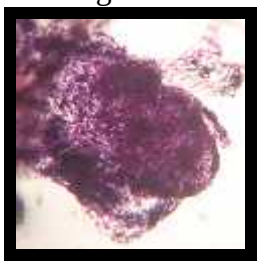
Fig. 93

4.B. *Rauvolfia serpentina* (L.) Benth. ex. Kurz.

4.B.1 Seed germination experiment*:*

All together 11.1% seeds germinated after 8 weeks of culture. The seeds were also tried to germinate on petri-dishes and soil but none of the seeds showed any sign of germination even after 16 weeks. The *in vitro* germinated plants were the source of all explants. Those were multiplied in the MS basal medium mainly through nodes and shoot tips. Later, the different explants (leaves, roots, nodes, internodes and shoot tips) from the MS basal medium multiplied shoots were used to test the effects of different hormones either singly or in combinations.

4.B.2. Effects of different PGRs either singly or in combinations on nodes.

4.B.2.1. Effects of auxins on nodal explants:

Callus induction:

From the data recorded NAA and 2,4-D were found to be highly effective in inducing the callus from both shoot and leaf explants. All the concentrations of 2,4-D above 0.5 mg/l induced the callus but the maximum induction was observed on MS + 2,4-D 2.0 mg/l (data not shown).All the concentrations of NAA above 0.5 mg/l and 2,4-D above 1.0 mg/l induced callus significantly at 1% level of significance. Among these, 2,4-D 2.0 mg/l induced maximum callus with 100% frequency (Fig. 101 and 102). However, 2,4- 0.1 mg/l did not produce any callus. IAA and IBA did not show any good results although they produced small amounts of callus at all concentrations with very low frequencies (Table 4.11).

Shoot multiplication and their lengths:

None of the treatments of any of the auxins were found to be significant in shoot multiplication. The maximum average number of shoots 1.5/explant was induced was by NAA 0.1 with 100% occurance (Fig. 103) whereas at high concentrations of 2,4-D (2 and 3 mg/l), no induction was observed. Similar was case in shoot length. NAA 0.1 mg/l yielded an average shoot length of 4.58 cm whereas 2,4-D 0.5 mg/l and above were

observed to retard the growth of the shoots. From the table below, shoot length and shoot number show similarity in selection of media (Table 4.11).

Table 4.11: Effects of auxins on nodal explants:

	callus induction	Shoot number	Root Number	Shoot length	Root length
PGR	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error
Control	0.166±0.166^{a}	1.166±0.166^{bc}	0.500±0.341^{ab}	2.716±0.591^{bc}	1.683±0.769^{b}
NAA 0.1	1.000±0.258^{bc}	1.500±0.223^{c}	7.833±1.424*^{c}	4.583±0.546^{d}	2.683±0.459^{b}
0.5	2.000±0.258*^{c}	1.000±0.000^{bc}	12.500±3.947*^{d}	1.500±0.367^{ab}	4.766±0.808*^{d}
1.0	2.000±0.258*^{c}	1.166±0.166^{bc}	10.166±2.982*^{cd}	3.616±0.661^{cd}	3.266±0.877^{c}
2.0	2.666±0.210*^{d}	0.666±0.210^{ab}	7.833±1.137*^{c}	2.466±0.779^{bc}	2.966±0.731^{bc}
IAA 0.1	0.333±0.210^{ab}	0.666±0.210^{ab}	2.333±0.557^{ab}	3.266±1.069^{c}	3.383±0.767*^{c}
0.5	0.333±0.210^{ab}	0.666±0.210^{ab}	1.333±0.333^{ab}	2.566±0.832^{bc}	2.300±0.375^{b}
1.0	0.500±0.223^{ab}	0.833±0.166^{b}	2.500±0.562^{ab}	2.333±0.533^{bc}	4.016±0.396*^{cd}
2.0	0.833±0.166^{b}	0.666±0.210^{ab}	2.333±0.494^{ab}	2.316±0.783^{bc}	2.316±0.558^{b}
IBA 0.1	0.500±0.223^{ab}	0.666±0.210^{ab}	0.000±0.000^{a}	1.383±0.440^{ab}	0.000±0.000+*^{a}
0.5	0.833±0.307^{b}	1.166±0.401^{bc}	2.166±0.980^{ab}	1.333±0.366^{ab}	1.366±0.535^{ab}
1.0	0.333±0.210^{ab}	0.666±0.210^{ab}	3.666±1.382^{b}	1.700±0.587^{b}	2.883±0.661^{bc}
2.0	0.166±0.166^{a}	0.666±0.210^{ab}	3.666±1.382^{b}	1.216±0.387^{ab}	1.933±0.354^{b}
2,4-D 0.1	0.000±0.000^{a}	0.833±0.166^{b}	0.000±0.000^{a}	1.750±0.416^{b}	0.000±0.000+*^{a}
0.5	0.500±0.341^{ab}	0.500±0.223^{ab}	0.833±0.654^{ab}	0.666±0.300*^{ab}	3.050±0.897^{bc}
1.0	1.833±0.401*^{c}	1.166±0.477^{bc}	0.000±0.000^{a}	1.433±0.546^{ab}	0.000±0.000+*^{a}
2.0	2.833±0.166*^{d}	0.000±0.000^{a}	0.000±0.000^{a}	0.000±0.000+*^{a}	0.000±0.000+*^{a}

Root multiplication and their lengths:

Except NAA all the auxins were not found to be significantly inducing the roots. All the concentrations of NAA were found to be highly significant in inducing the number of roots per explants, NAA 0.5 being the best with 12.5 roots/ explants and 100% response. IBA 0.1 mg/l and almost all concentrations of 2,4-D except 0.5 mg/l failed to produce any root in any of the replications. Similarly, NAA 0.5 mg/l induced the

significantly longest roots with 4.76 cm length (Fig. 104) although IAA 0.1 and 1.0 mg/l (Fig. 106) were also significant (Table 4.11).

4.B.2.2. Effects of cytokinins on nodal explants:

Callus induction:

Among all the concentrations of BAP and Kinetin only Kn 1.0 and 2.0 mg/l were found to be significantly inducing the callus with 66.66% and 83.33% success respectively (Fig. 107). The minimum callus induction was observed on MS and the medium containing Kn 0.1 mg/l (Table 4.12).

Shoot multiplication and their lengths:

The maximum shoot number of 5.16 with 100% frequency was observed with BAP 1.0 mg/l in the medium which was also significant at 1% level (Fig. 108). The average number of shoots per explant was found to be higher than that of control in all the concentrations of cytokinin containing media however, the least number among cytokinin concentrations was 1.66 shoots/explants on the MS medium containing Kn 0.1 mg/l. Shoot lengths were not found to be significantly long at any concentrations of both the cytokinins. However, the longest shoots were recorded when Kn 2.0 mg/l was added in the MS medium (Fig. 109). The minimum number of shoots were found under the controlled condition whereas the shortest length of shoots were recorded on medium with BAP 1.0 mg/l (Table 4.12).

Root multiplication and their lengths:

The maximum number of roots were observed to be 3.0 per explants with BAP 0.1 and Kn 1.0 mg/l (Fig. 111) with 100% and 66.66% occurance respectively. However most of the concentrations did not induce any root. In case of root length Kn 1.0 mg/l gave the longest roots of 3.56 cm and the shortest ones of 1.61 cm were found in BAP 0.1 mg/l containing media. From the table it can be said that root induction and their growth is supported by the similar hormone (Table 4.12).

Table 4.12:Effects of cytokinins on nodal explants:

	Callus induction	Shoot number	Root Number	Shoot length	Root length
PGR	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error
Control	0.166±0.166[a]	1.166±0.166[a]	0.500±0.341[a]	2.716±0.591[ab]	1.683±±0.769[b]
BAP 0.1	1.000±0.365[ab]	2.333±0.714[ab]	3.000±0.632[b]	2.050±0.437[ab]	1.616±±0.522[b]
0.5	0.666±0.333[ab]	3.000±1.064*[ab]	0.000±0.000[a]	2.066±0.465[ab]	0.000±0.000+*[a]
1.0	0.833±0.307[ab]	5.166±0.792*[c]	0.000±0.000[a]	1.200±0.315[ab]	0.000±0.000+*[a]
2.0	0.833±0.307[ab]	4.500±0.991*[bc]	0.000±0.000[a]	2.383±0.900[ab]	0.000±0.000+*[a]
Kn 0.1	0.333±0.210[ab]	1.666±0.210[ab]	0.000±0.000[a]	1.666±0.345[ab]	0.000±0.000+*[a]
0.5	0.666±0.210[ab]	3.333±0.714*[b]	0.000±0.000[a]	2.483±0.477[ab]	0.000±0.000+*[a]
1.0	1.166±0.307*[b]	2.166±0.477[ab]	3.000±0.966[b]	2.966±0.410[b]	3.566±0.583*[c]
2.0	1.333±0.210*[b]	3.166±0.401*[ab]	2.500±0.619[b]	2.783±0.497[b]	2.683±0.396[c]

4.B.2.3. Effects of NAA and BAP in combinations on nodal explants:

Callus induction:

All the concentration combinations of BAP with NAA higher than 0.5 mg/l were found to be significantly inducing the callus mainly from the shoots and leaves. The highest callus induction with 100% success was found at NAA 1.0 + BAP 0.5 mg/l. Besides control, the least callus production was recorded from the MS medium containing NAA 0.1 + BAP 0.5 mg/l in 50% of the cultures (Table 4.13 and Fig. 112).

Shoot multiplication and their lengths:

For shoot induction a lower concentration (0.1 or 0.5 mg/l) of NAA along with moderate levels (0.5 or 1.0 mg/l) of BAP were found to be significant in inducing the multiple shoots from the shoot explants. The maximum average number of shoots induced by all the cutures was 7.16 shoots per explant when the MS medium was supplemented with NAA 0.1 + BAP 1.0 mg/l (Fig. 113). Only 33.33% of the cultures from MS medium supplemented with NAA 1.0 + BAP 0.5 mg/l were able to produce 0.5 shoots per explant which were also the shortest ones of 0.56 cm in average Similarly the longest shoots of 4.36 cm were recorded in the medium containing NAA 0.5 + BAP 1.0 mg/l (Table 4.14 and Fig. 113).

Root multiplication and their lengths:

The highest number of roots per explant counted from the medium containing NAA 0.5 + BAP 0.5 mg/l was 5.5 and the success percentage was 50 (Fig. 115). The least number of 0.33 roots per explant from the least (16.66) percentage of cultures as well as the shortest roots of 0.68 cm were found one step above the maximum number of roots i.e NAA 0.5 + BAP 0.1 mg/l. Similarly, the maximum length of 2.7 cm was observed in the tubes containing NAA 0.1 + BAP 2.0 (Table 4.13).

Table 4.13: Effects of NAA and BAP in combinations on nodal explants:

	callus induction	Shoot number	Root Number	Shoot length	Root length
PGR	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error
Control	0.166±0.166^{a}	1.166±0.166^{a}	0.500±0.341^{a}	2.716±0.591^{b}	1.683±0.769^{ab}
NAA 0.1+BAP 0.1	0.666±0.210^{a}	1.333±0.210^{ab}	0.500±0.500^{a}	1.416±0.363^{ab}	2.100±0.959^{ab}
NAA 0.1+BAP 0.5	0.500±0.223^{a}	4.166±±1.137*^{c}	1.000±0.683^{a}	1.883±0.562^{ab}	2.133±0.266^{ab}
NAA 0.1+BAP 1.0	1.500±0.428*^{b}	7.166±1.351*^{d}	0.833±0.833^{a}	1.266±0.285^{ab}	1.900±0.446^{ab}
NAA 0.1+BAP 2.0	0.833±0.307^{ab}	1.500±0.500^{ab}	1.833±1.327^{a}	2.666±0.460^{b}	2.700±0.441^{ab}
NAA 0.5+BAP 0.1	1.666±0.333*^{c}	0.666±0.210^{a}	0.333±0.333^{a}	1.366±0.518^{ab}	0.683±0.496^{a}
NAA 0.5+BAP 0.5	1.500±0.223*^{b}	3.666±1.282*^{c}	5.500±3.334^{a}	4.300±1.095^{c}	2.166±0.351^{ab}
NAA 0.5+BAP 1.0	1.500±0.223*^{b}	3.333±1.145*^{b}	1.333±0.614^{a}	4.366±1.078^{d}	2.233±0.428^{ab}
NAA 0.5+BAP 2.0	1.833±0.542*^{cd}	1.500±0.846^{ab}	3.666±2.231^{a}	2.516±0.364^{b}	3.116±0.258^{b}
NAA 1.0+BAP 0.1	2.166±0.166*^{cd}	1.333±0.210^{ab}	4.166±2.023^{a}	2.566±0.799^{b}	1.500±0.193^{ab}
NAA 1.0+BAP 0.5	2.666±0.210*^{e}	0.500±0.341^{a}	4.666±1.926^{a}	0.566±0.348+*^{a}	1.950±0.463^{ab}
NAA 1.0+BAP 1.0	2.500±0.223*^{d}	1.333±0.494^{ab}	4.833±2.613^{a}	1.683±0.409^{ab}	1.483±0.219^{ab}
NAA 1.0+BAP 2.0	2.166±0.166*^{cd}	1.000±0.000^{a}	3.833±2.688^{a}	1.600±0.263^{ab}	2.033±0.439^{ab}

4.B.2.4. Effects of NAA and Kn in combinations on nodal explants:

Callus induction:

The callus induction was found to be highly significant when NAA concentration was high i.e. 0.5 and 1.0 mg/l. The maximum callus formation from 100% of the cultures was recorded from the medium containing NAA 1.0 + Kn 2.0 mg/l (Fig. 116). Besides MS basal medium, the least callus induction was observed from only 33.33% of the

cultures when cultured on the MS medium containing NAA 0.1 + Kn 0.1 mg/l however, NAA 0.1 + Kn 0.5 mg/l did not produce any callus (Table 4.14).

Shoot multiplication and their lengths:

Among the series only NAA 0.1 + Kn 2.0 mg/l induced significant number of shoots per explant i.e. 4.16 as well as the longest shoots of the average length of 3.95 cm from 100% of the cultures (Fig. 117 and 118). Similarly, the least number of shoots per explants but with 100% frequency was 1.0 with the medium containing NAA 1.0 + Kn 0.1 mg/l and the shortest ones were recorded from the medium containing NAA 1.0 + Kn 0.1 mg/l. Here, none of the media were significantly elongating the shoots (Table 4.14).

Table 4.14 : Effects of NAA and Kn in combinations on nodal explants:

	callus induction	Shoot number	Root Number	Shoot length	Root length
PGR	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error
Control	0.166±0.166^{a}	1.166±0.166^{a}	0.500±0.341^{a}	2.716±0.591^{b}	1.683±0.769^{bc}
NAA 0.1+Kn 0.1	0.333±0.210^{a}	1.833±0.833^{a}	0.000±0.000^{a}	2.700±0.146^{ab}	0.000±0.000+*^{a}
NAA 0.1+Kn 0.5	0.000±0.000^{a}	1.666±0.210^{a}	0.000±0.000^{a}	1.883±0.195^{ab}	0.000±0.000+*^{a}
NAA 0.1+Kn 1.0	1.000±0.365^{b}	2.000±0.365^{ab}	0.000±0.000^{a}	1.983±0.496^{ab}	0.000±0.000+*^{a}
NAA 0.1+Kn 2.0	1.500±0.223*^{bc}	4.166±0.749*^{c}	0.166±0.166^{a}	3.950±0.647^{b}	0.333±0.333^{ab}
NAA 0.5+Kn 0.1	1.666±0.210*^{c}	1.166±0.166^{a}	0.500±0.341^{a}	2.800±0.543^{ab}	0.666±0.311^{ab}
NAA 0.5+Kn 0.5	1.000±0.365^{b}	1.333±0.210^{a}	0.333±0.333^{a}	2.716±0.725^{ab}	0.883±0.565^{ab}
NAA 0.5+Kn 1.0	1.500±0.223*^{bc}	1.833±0.477^{a}	2.166±1.376^{a}	2.400±0.357^{ab}	2.216±0.461^{c}
NAA 0.5+Kn 2.0	1.500±0.223*^{bc}	1.666±0.333^{a}	0.500±0.500^{a}	3.000±0.411^{ab}	1.183±0.540^{b}
NAA 1.0+Kn 0.1	1.666±0.210*^{c}	1.000±0.000^{a}	0.333±0.333^{a}	1.333±0.088^{a}	0.300±0.196^{ab}
NAA 1.0+Kn 0.5	1.500±0.223*^{bc}	1.333±0.210^{a}	1.166±0.980^{a}	1.366±0.363^{a}	1.500±0.134^{bc}
NAA 1.0+Kn 1.0	1.666±0.210*^{c}	1.666±0.421^{a}	3.666±1.960^{a}	1.900±0.961^{ab}	2.083±0.186^{c}
NAA 1.0+Kn 2.0	2.166±0.166*^{d}	3.166±0.542^{b}	4.000±2.840^{a}	3.900±1.070^{b}	2.083±0.554^{c}

Root multiplication and their lengths:

In case of root induction (both root number and root length), no media tested in this series showed any positive sign of significance. However, NAA 1.0 + Kn 2.0 mg/l in

the medium showed 33.33% response but a maximum number of roots i.e. 4 roots per explant (Fig. 119). The longest roots of 2.21 cm were measured from the medium supplemented with NAA 0.5 + Kn 1.0 mg/l. Similarly, the least numer and shortest roots were found in the media with NAA 0.1 + Kn 2.0 mg/l (16.66%) and NAA 1.0 +Kn 0.1 respectively (Table 4.14).

4.B.2.5. Effects of IAA and BAP in combinations on nodal explants:

Callus induction:

Almost all the concentrations in this series were found to be insignificant in inducing the callus except IAA 1.0 + BAP 2.0 mg/l from all the cultures (Fig. 121). Most of the concentration combinations either yielded the callus in a very low amount or not at all. The least induction of callus from only 16.66% cultures was recorded from the MS medium containing IAA 0.1 + BAP 1.0 (Table 4.15).

Shoot multiplication and their lengths:

Although shoot induction was observed from all the media of this series statistically significant, shoot multiplications were observed when BAP concentration was raised to 1.0 – 2.0 mg/l. The maximum average number of shoots was recorded from the medium with IAA 1.0 + BAP 2.0 mg/l with 100% success (Fig. 122) whereas the minimum number of 1.66 shoots per explant also with 100% success rate was observed from the MS medium containing IAA 1.0 + BAP 0.1 mg/l. Similarly, the medium that induced least number of shoots (IAA 1.0 + BAP 0.1 mg/l) supported the elongation of shoots to 4.31 cm (Fig. 123) but the medium that significantly induced the shoot number (IAA 0.1+BAP 2.0 mg/l) reduced shoot length to its minimum i.e 1.06 cm (Table 4.15).

Root multiplication and their lengths:

All the concentration combinations of this series failed to induceany root (Table 4.15).

Table 4.15: Effects of IAA and BAP in combinations on nodal explants:

	callus induction	Shoot number	Root Number	Shoot length	Root length
PGR	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error
Control	0.166±0.166^{ab}	1.166±0.166^{a}	0.500±0.341^{b}	2.716±0.591^{b}	1.683±0.769^{b}
IAA 0.1+BAP 0.1	0.000±0.000^{a}	2.166±0.477^{ab}	0.000±0.000^{a}	2.783±0.766^{bc}	0.000±0.000+*^{a}
IAA 0.1+BAP 0.5	0.000±0.000^{a}	2.500±1.310^{ab}	0.000±0.000^{a}	2.516±0.413^{b}	0.000±0.000+*^{a}
IAA 0.1+BAP 1.0	0.166±0.166^{ab}	4.333±1.626*^{ab}	0.000±0.000^{a}	1.650±0.487^{ab}	0.000±0.000+*^{a}
IAA 0.1+BAP 2.0	0.333±0.210^{ab}	4.833±1.447*^{b}	0.000±0.000^{a}	1.066±0.221^{a}	0.000±0.000+*^{a}
IAA 0.5+BAP 0.1	0.000±0.000^{a}	2.166±0.600^{ab}	0.000±0.000^{a}	2.966±0.504^{c}	0.000±0.000+*^{a}
IAA 0.5+BAP 0.5	0.333±0.210^{ab}	2.500±0.885^{ab}	0.000±0.000^{a}	3.216±0.571^{cd}	0.000±0.000+*^{a}
IAA 0.5+BAP 1.0	0.666±0.210^{b}	2.500±0.763^{ab}	0.000±0.000^{a}	1.933±0.343^{ab}	0.000±0.000+*^{a}
IAA 0.5+BAP 2.0	0.666±0.210^{b}	4.166±0.792^{ab}	0.000±0.000^{a}	1.416±0.241^{ab}	0.000±0.000+*^{a}
IAA 1.0+BAP 0.1	0.333±0.210^{ab}	1.666±0.210^{ab}	0.000±0.000^{a}	4.316±0.282^{d}	0.000±0.000+*^{a}
IAA 1.0+BAP 0.5	0.666±0.210^{b}	2.666±0.333^{ab}	0.000±0.000^{a}	2.900±0.446^{c}	0.000±0.000+*^{a}
IAA 1.0+BAP 1.0	0.666±0.210^{b}	4.500±0.718*^{b}	0.000±0.000^{a}	1.566±0.277^{ab}	0.000±0.000+*^{a}
IAA 1.0+BAP 2.0	1.166±0.166*^{c}	6.000±1.460*^{c}	0.000±0.000^{a}	2.350±0.326^{ab}	0.000±0.000+*^{a}

4.B.2.6. Effects of IAA and Kn in combinations on nodal explants:

Callus induction:

From the data recorded it is clear that higher concentration of IAA supported the callus induction to a significant level. IAA 1.0 mg/l along with higher concentrations of Kn (0.5 – 2.0 mg/l) induced the callus significantly but IAA 1.0 + Kn 2.0 mg/l incorporated MS medium produced maximum callus with 100% occurance (Fig. 124). Other than the hormone free MS medium, IAA 0.1 + Kn 0.1 mg/l produced the least amount of callus from only 33.33% of the cultures (Table 4.16).

Shoot multiplication and their lengths:

Generally it has been observed that, as the concentration of Kn increases the number of shoots also increase; however, the maximum number of shoots recorded was 5.50 shoot per explant from the medium containing IAA 0.5 + Kn 2.0 with 100% success rate (Fig. 125). Other than the MS hormone free basal medium, the medium containing

IAA 1.0 + Kn 0.1 mg/l induced minimum number of 1.5 shoots per explant from all the cultures. Similarly, the maximum shoot elongation was observed on the medium containing 0.5 + 0.5 mg/l with an average length of 4.33 cm (Fig. 126) but the shortest shoots of 2.06 cm were found when the medium was supplemented with IAA 1.0 + Kn 1.0 mg/l (Table 4.16).

Table 4.16: Effects of IAA and Kn in combinations on nodal explants:

	callus induction	Shoot number	Root Number	Shoot length	Root length
PGR	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error
Control	0.166±0.166^{a}	1.166±0.166^{a}	0.500±0.341^{a}	2.716±0.591^{b}	1.683±0.769^{b}
IAA 0.1+Kn 0.1	0.333±0.210^{ab}	2.000±0.365^{ab}	0.000±0.000^{a}	4.150±0.447^{ab}	0.000±0.000+*^{ab}
IAA 0.1+Kn 0.5	0.500±0.223^{ab}	2.333±0.614^{ab}	0.000±0.000^{a}	2.516±0.452^{ab}	0.000±0.000+*^{ab}
IAA 0.1+Kn 1.0	0.666±0.210^{ab}	3.666±0.954^{ab}	0.000±0.000^{a}	3.116±0.737^{ab}	0.000±0.000+*^{ab}
IAA 0.1+Kn 2.0	0.666±0.210^{ab}	5.333±1.563*^{b}	0.000±0.000^{a}	2.650±0.749^{ab}	0.000±0.000+*^{ab}
IAA 0.5+Kn 0.1	0.666±0.210^{ab}	2.833±0.654^{ab}	0.000±0.000^{a}	3.050±0.466^{ab}	0.000±0.000+*^{ab}
IAA 0.5+Kn 0.5	0.666±0.210^{ab}	2.500±0.619^{ab}	0.000±0.000^{a}	4.333±0.565^{b}	0.000±0.000+*^{ab}
IAA 0.5+Kn 1.0	1.166±0.166*^{bc}	2.833±1.013^{ab}	0.000±0.000^{a}	2.666±0.628^{ab}	0.000±0.000+*^{ab}
IAA 0.5+Kn 2.0	0.500±0.223^{ab}	5.500±1.688*^{b}	0.000±0.000^{a}	2.466±0.645^{ab}	0.000±0.000+*^{ab}
IAA 1.0+Kn 0.1	0.666±0.210^{ab}	1.500±0.341^{ab}	3.500±2.247*^{b}	3.916±0.531^{ab}	1.866±0.416^{b}
IAA 1.0+Kn 0.5	0.833±0.166*^{ab}	3.166±1.108^{ab}	0.000±0.000^{a}	2.483±0.434^{ab}	0.000±0.000+*^{ab}
IAA 1.0+Kn 1.0	1.000±0.258*^{b}	3.833±1.351^{ab}	0.000±0.000^{a}	2.066±0.438^{a}	0.000±0.000+*^{ab}
IAA 1.0+Kn 2.0	1.333±0.210*^{c}	4.833±1.579*^{ab}	0.000±0.000^{a}	2.266±0.540^{ab}	0.000±0.000+*^{ab}

Root multiplication and their lengths:

Almost all the media in this series failed to induce any root. Only 33.33% of the cultures of the MS medium containing IAA 1.0 + Kn 0.1 gave 3.50 shoots per explant with an average length of 1.86 cm (Fig. 127 and Table 4.16).

4.B.2.7. Effects of IBA and BAP in combinations on nodal explants:

Callus induction:

The data revealed that the IBA at 0.5 and 1.0 mg/l concentrations were highly significant in inducing the callus mainly from the shoot explants although some other combinations were also found to induce callus. It has also been observed that all the concentrations of IBA along with higher BAP levels might be suitable for callus induction. The 100% of the cultures produced maximum callus from the media containing IBA 0.5 + BAP 1.0, IBA 0.5 + BAP 2.0 (Fig. 128) and IBA 1.0 + BAP 2.0 mg/l. Besides MS basal medium (control), the lowest callus formation was observed from the medium containing IBA 0.1 + BAP 0.1 mg/l (Table 4.17).

Shoot multiplication and their lengths:

The minimum shoot number of 1.16 per explants was recorded from the MS basal medium whereas among the IBA and BAP combination, MS + IBA 0.1 + BAP 0.1 produced 2.00 shoots per explants in average. A 100% of the cultures from the medium containing IBA 0.1 + BAP 2.0 mg/l yielded the maximum number of 7.83 shoots per explant (Fig. 129). In case of shoot elongation none of the treatments showed any significant result statistically. The maximum length of shoot was 4.38 cm and the minimum was 1.01 cm on the media IBA 0.1 + BAP 1.0 mg/l (Fig. 130) and IBA 1.0 + BAP 1.0 mg/l respectively (Table 4.17).

Root multiplication and their lengths:

The shoots cultured on MS + IBA 0.1 + BAP 1.0 with 16.66% frequency (Fig. 132) and IBA 1.0 + BAP 0.1 mg/l with 33.33% showed 0.5 roots on average which was equal to that of control. The maximum root number of 1.16/shoot were observed from 66.66% of the cultures containing MS + IBA 1.0 + BAP 1.0mg/l. Most of the media here, were either incapable or less capable than that of control in inducing the root from the shoots. Most of the media in this series were statistically found to be retarding the root elongation. The maximum length of 1.08 cm and a minimum of 0.266 cm long roots were recorded from the media containing IBA 0.1 + BAP 1.0 and IBA 0.5 + BAP 1.0 mg/l respectively (Table 4.17).

Table 4.17: Effects of IBA and BAP in combinations on nodal explants:

	callus induction	Shoot number	Root Number	Shoot length	Root length
PGR	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error
Control	0.166±0.166^{a}	1.166±0.166^{a}	0.500±0.341^{ab}	2.716±0.591^{b}	1.683±0.769^{c}
IBA 0.1+BAP 0.1	0.333±0.210^{ab}	2.000±±0.365^{ab}	0.000±0.000^{a}	2.100±0.378^{ab}	0.000±0.000+*^{a}
IBA 0.1+BAP 0.5	0.833±0.166*^{bc}	3.833±0.477^{b}	0.000±0.000^{a}	3.200±0.473^{c}	0.000±0.000+*^{a}
IBA 0.1+BAP 1.0	0.500±0.223^{ab}	3.333±1.563^{b}	0.500±0.500^{ab}	4.383±0.793^{d}	1.083±0.499^{b}
IBA 0.1+BAP 2.0	0.833±0.166*^{bc}	7.833±1.013*^{d}	0.000±0.000^{a}	3.416±0.448^{c}	0.000±0.000+*^{a}
IBA 0.5+BAP 0.1	1.000±0.000*^{c}	2.666±0.333^{ab}	0.166±0.166^{a}	3.450±0.536^{c}	0.433±0.433+*^{a}
IBA 0.5+BAP 0.5	1.000±0000*^{c}	4.166±0.477^{bc}	1.833±0.542^{a}	3.200±0.379^{c}	1.316±0.185+*^{a}
IBA 0.5+BAP 1.0	1.166±0.166*^{d}	2.333±0.614^{ab}	0.500±0.500^{a}	1.466±0.240^{ab}	0.266±0.120+*^{a}
IBA 0.5+BAP 2.0	1.166±0.166*^{d}	6.166±0.600*^{cd}	1.000±0.258^{a}	3.366±0.676^{c}	0.566±0.080+*^{a}
IBA 1.0+BAP 0.1	1.000±0.000*^{c}	4.333±0.421*^{c}	0.500±0.341^{ab}	2.333±0.405^{ab}	0.616±0.322+*^{ab}
IBA 1.0+BAP 0.5	1.000±0.000*^{c}	3.00±0.516^{ab}	0.166±0.166^{a}	1.983±0.329^{ab}	0.283±0.283+*^{a}
IBA 1.0+BAP 1.0	0.666±0.210^{b}	2.833±0.477^{ab}	1.166±0.477^{a}	1.016±0.203^{a}	1.033±0.042+*^{a}
IBA 1.0+BAP 2.0	1.166±0.166*^{d}	6.833±1.194*^{d}	0.000±0.000^{a}	2.633±0.606^{b}	0.000±0.000+*^{a}

4.B.2.8. Effects of IBA and Kn in combinations on nodal explants:

Callus induction:

The media containing IBA irrespective of its concentration when added with high Kn concentration induced the callus significantly. The maximum callus induction with 100% success rate was recorded from the media containing IBA 1.0 + Kn 1.0 (Fig. 133) and IBA 1.0 + Kn 2.0 mg/l. Among the IBA + Kn series besides control, the lowest amount of callus formation was observed only from the 33.33% of the cultures on the medium containing IBA 0.1 + Kn 0.1 mg/l (Table 4.18).

Shoot multiplication and their lengths:

The maximum numbers of shoots 2.83 per explant and the longest shoots of 3.11 cm have been recorded from the medium with IBA 1.0 + Kn 2.0 with 100% frequency (Fig. 134); whereas, the lowest number of 0.83 per explant from the 66.66% of the cultures and the shortest shoots of 1.15 cm were noted from the media with IBA 0.5 + Kn

0.5 mg/l and IBA 0.1 +Kn 0.1 mg/l respectively. Here, no concentration combination was found to be statistically significant in either increasing the shoot number or shoot length (Table 4.18).

Table 4.18: Effects of IBA and Kn in combinations on nodal explants:

	callus induction	Shoot number	Root Number	Shoot length	Root length
PGR	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error	Mean±Std.Error
Control	0.166±0.166[a]	1.166±0.166[a]	0.500±0.341[a]	2.716±0.591[ab]	1.683±0.769[b]
IBA 0.1+Kn 0.1	0.333±0.210[ab]	1.333±0.333[a]	0.666±0.666	1.150±0.379[a]	1.133±0.375[b]
IBA 0.1+Kn 0.5	0.666±0.210[ab]	1.500±0.223[a]	0.000±0.000	2.750±0.509[ab]	0.000±0.000+*[a]
IBA 0.1+Kn 1.0	0.833±0.166*[b]	1.500±0.341[a]	1.333±1.333	1.816±0.362[ab]	1.250±0.296[b]
IBA 0.1+Kn 2.0	0.833±0.166*[b]	1.666±0.494[ab]	0.000±0.000	2.233±0.758[ab]	0.000±0.000+*[a]
IBA 0.5+Kn 0.1	0.500±0.223[ab]	1.333±0.210[a]	0.000±0.000	1.583±0.324[ab]	0.000±0.000+*[a]
IBA 0.5+Kn 0.5	0.666±0.210[ab]	0.833±0.307[a]	0.000±0.000	2.283±0.295[ab]	0.000±0.000+*[a]
IBA 0.5+Kn 1.0	0.666±0.210[ab]	1.500±0.341[a]	0.000±0.000	2.200±0.334[ab]	0.000±0.000+*[a]
IBA 0.5+Kn 2.0	0.833±0.166*[b]	2.000±0.258[ab]	0.000±0.000	2.366±0.394[ab]	0.000±0.000+*[a]
IBA 1.0+Kn 0.1	0.833±0.166*[b]	1.500±0.341[a]	0.000±0.000	2.266±0.353[ab]	0.000±0.000+*[a]
IBA 1.0+Kn 0.5	0.833±0.166*[b]	1.833±0.307[ab]	0.000±0.000	2.650±0.510[ab]	0.000±0.000+*[a]
IBA 1.0+Kn 1.0	1.000±0.000*[b]	1.333±0.210[a]	0.000±0.000	1.583±0.415[ab]	0.000±0.000+*[a]
IBA 1.0+Kn 2.0	1.000±0.000*[b]	2.833±0.654[b]	0.000±0.000	3.116±0.438[b]	0.000±0.000+*[a]

Root multiplication and their lengths:

Only a few concentration combinations of this series were able to induce very little roots. Only IBA at 0.1 mg/l level showed some sign of root induction with Kn either at 0.1 or 1.0 mg/l. The maximum number of 1.33 from only 16.66% of the cultures and a minimum number of 0.50 roots per explant were recorded from IBA 0.1 + Kn 1.0 mg/l (Fig. 135) and hormone free MS medium. Similarly, the maximum length of 1.68 cm and minimum length of 1.13 cm were measured from MS basal medium and IBA 0.1 + Kn 0.1 (Fig. 136) containing medium respectively (Table 4.18).

4.B.2.9. Effects of hormone free MS medium on shoot explants:

The nodal explants responded to the hormone free MS medium in almost all rerspects. From the study it has been observed that the shoots were able to produce small amount of callus with very little frequency. In case of shoots, this medium produced 1.16 shoots in average from all the replications. This medium also was capable of increasing the length of shoots remarkably upto an average of 2.71 cm which was more than many of the hormone supplemented media.The roots produced from this medium were of 0.5/ explants from the 33.33 % of the explants. Although with very low frequency, the roots formed were of 1.68 cm which is also of good length than that produced from many other hormone supplemented media (Fig. 138-141).

4.B.3. Somatic embryogenesis and caulogenesis:

In case of Somatic embryogenesis and caulogenesis, only NAA series, 2,4-D series, BAP series and NAA + BAP combination series were studied. Here also, the all the treatments were observed regularly for every possible change on the explants. We could not find either buds (2 buds in one explant on media IBA 0.1 + BAP 1.0 mg/l and BAP 3.0 mg/l were recorded but they failed to develop further) or a single sign of vitrification. But caulogenesis and somatic embryogenesis from different calli were recorded.

Somatic embryogenesis:

The callus obtained from the shoot explants cultured on all the concentrations of either BAP or NAA or 2,4-D singly, or NAA + BAP were analysed under compound microscope for cytological study. The calli of 10-12 weeks were studied under microscope. Under different magnifications of a compound microscope only a few of the friable and greenish yellow calli reveled different stages of somatic embryoids. The calli from the MS medium with the following PGR/s were found to be embryogenic viz. NAA 0.5 + BAP 0.1, NAA 0.5 + BAP 0.5, NAA 0.5 + BAP 1.0, NAA 1.0 + BAP 0.5, NAA 1.0 + BAP 1.0 and 2,4-D 3.0 mg/l. The photo micrographs of the different stages of somatic embryogenesis are given below (Fig. 147-154).

Caulogenesis:

Caulogenesis in *R serpentina* has also been studied from the MS medium with NAA series, 2,4-D series, BAP series and NAA + BAP combination series were studied. Among them, some induced roots whereas some other induced shoots. Roots and shoots at the same time from the same explants were were also observed in *R. serpentina.* In this case a clear and regular influence of auxin cytokinin relation is established. Auxin alone induce roots whereas cytokinin alone induced shoots. In combination of auxin and cytokinin also the same rule was followed i.e. higher auxin vs lower cytokinin induced roots, lower auxin vs higher cytokinin induced shoots and an average of both induced both roots and shoots.The media that induced roots were MS with NAA 0.5, NAA 1.0, NAA 2.0, NAA 0.5 + BAP 0.5, NAA 0.5 + BAP 1.0, NAA 0.5 + BAP 2.0, NAA 1.0 + BAP 0.1, NAA 1.0 + BAP 0.5, NAA 1.0 + BAP 1.0 And NAA 1.0 + BAP 2.0 mg/l. Similarly, the MS medium with BAP 0.5, BAP 1.0, BAP 2.0, NAA 0.1 + BAP 0.5, NAA 0.1 + BAP 1.0, NAA 0.1 + BAP 2.0, NAA 0.5 + BAP 0.5, NAA 0.5 + BAP 1.0, NAA 0.5 + BAP 2.0 mg/l induced shoots (Fig. 142-146).

4.B.4. *In Vivo* Rooting and Acclimatization:

The shoots rooted *in vitro* were acclimatized in the coco peat survived well with 90% success. The acclimatization on sand soil mixture (1:1) as a substrate for the *in vitro* rooted plants did not show a good result. The *in vitro* grown shoots were pulse treated with 100 mg/l IAA, IBA and NAA and a total of about 68% rooted after 2-3 weeks in pure sand and they were planted in the coco-peat for 2 months for hardening. Among all the rooted shoots more than 80% stabilized in the coco peat whereas on the sand soil mixture 50/50 after 2 months gave 60% survival (Fig. 155-160, Table 4.19).

Table 4.19: *In vivo* rooting and acclimatization:

Bed	IAA	IBA	NAA
Sand rooting (Pulse Treatment)	66.6%	53.3%	86.6%
Coco peat stabilizing	80.6%		
Sand soil mixture in shade condition	60.0%		

B. *Ruvolfia serpentina* (L.) Benth. ex. Kurz.

Habit and Habitat of *Rauvolfia serpentina*.

Fig. B- Natural habitat of *Rauvolfia serpentina* growing in the forest of Nawalparasi.

Fig. B_1- A flowering and fruiting branch on the natural habitat.

Fig. 4.B.1: Various effects of auxins at different concentrations.

Fig. 101- Heavy friable callus formation from the leaf on MS + 2,4-D 2.0 mg/l after 12 weeks of culture.

Fig. 102- Heavy primary as well as secondary callus formation from the shoots on MS + 2,4-D 2.0 mg/l after 12 weeks of culture.

Fig. 103- Healthy multiple shoot as well as roots induced from a node on MS + NAA 0.1 mg/l after 12 weeks of culture.

Fig. 104- Heavy root induction from an *in vitro* multiplied shoot on MS + NAA 0.5 mg/l after 11 weeks of culture.

Fig. 105- Roots induced from an *in vitro* multiplied shoot on MS + IBA 1.0 mg/l after 11 weeks of culture.

Fig. 106- Root induction from an *in vitro* multiplied shoot on MS + IAA 1.0 mg/l after 11 weeks of culture.

Fig. 4.B.2: Various effects of Cytokinins at different concentrations.

Fig. 107- Heavy and hard callus formation at the shoot base on MS + Kn 2.0 mg/l after 12 weeks of culture.

Fig. 108- Multiple shoot induction from the node on MS + BAP 1.0 mg/l after 10 weeks of culture.

Fig. 109- Long and healthy multiple shoots induced from pieces of calli on MS + BAP 2.0 mg/l after 15 weeks of culture.

Fig. 110- Healthy multiple shoots induced from noded on MS + BAP 1.5 mg/l after 18 weeks of culture.

Fig. 111- Fine and long roots induced from the *in vitro* multiplied shoot on MS + Kn 1.0 mg/l after 15 weeks of culture.

Rauvolfia serpentina (L.) Benth. ex Kurz.

Fig. B. Habit

Fig. B_1. A flowering and fruiting branch

Fig. 101

Fig. 102

Fig. 103

Fig. 104

Fig. 105

Fig. 106

Fig. 107

Fig. 108

Fig. 109

Fig. 110

Fig. 111

Fig. 4.B.3: Various effects of NAA and BAP in combinations.

Fig. 112- Callus formation from the node explants after 8 weeks and root formation from the same callus after 14 weeks of culture on MS + NAA 1.0 + BAP 0.5 mg/l.

Fig. 113- Multiple shoots induced from the node on MS + NAA 0.1 + BAP 1.0 mg/l after 12 weeks of culture.

Fig. 114- Long and healthy shoots with callus at the base induced from a node on MS + NAA 0.5 + BAP 1.0 mg/l after 12 weeks of culture.

Fig. 115- Root induction from an *in vitro* multiplied shoot base on MS + NAA 0.5 + BAP 0.5 mg/l after 12 weeks of culture.

Fig. 4.B.4: Various effects of NAA and Kinetin in combinations.

Fig. 116- Callus formation from the shoot on MS + NAA 1.0 + Kn 2.0 mg/l after 12 weeks.

Fig. 117- Multiple shoots induced from a node on MS + NAA 0.1 + Kn 2.0 mg/l after 12 weeks.

Fig. 118- Healthy multiple shoots induced from a node on MS + NAA 0.1 + Kn 2.0 mg/l after 12 weeks of culture.

Fig. 119- Roots induced from the shoot base along with callus on MS + NAA 1.0 + Kn 2.0 mg/l after 12 weeks of culture.

Fig. 120- Long roots induced from the shoots on MS + NAA 0.5 + Kn 1.0 mg/l after long term culture of 16 weeks.

Fig. 4.B.5: Various effects of IAA and BAP in combinations.

Fig. 121- Callus formation from a shoot on MS + IAA 1.0 + BAP 2.0 mg/l after 10 weeks.

Fig. 122- Multiple shoots induced from a node on MS+IAA 1.0+BAP 2.0 mg/l after 10 weeks.

Fig. 123- Long healthy shoots induced from nodes on MS+IAA 1.0+BAP 0.1 mg/l in 12 weeks.

Fig. 4.B.6: Various effects of IAA and Kinetin in combinations.

Fig. 124- Callus induction from a shoot on MS + IAA 1.0 + Kn 2.0 mg/l after 11 weeks.

Fig. 125- Multiple shoot induced from a node on MS + IAA 0.5 + Kn 2.0 mg/l after 10 weeks.

Fig. 126- Long healthy shoots induced from nodes on MS+IAA 0.5+Kn 0.5 mg/l after 12 weeks.

Fig. 127- Roots from the *in vitro* grown shoots on MS + IAA 1.0 + Kn 0.1 mg/l after 12 weeks.


Fig. 112


Fig. 113


Fig. 114


Fig. 115


Fig. 116


Fig. 117


Fig. 118


Fig. 119


Fig. 120


Fig. 121


Fig. 122


Fig. 123


Fig. 124


Fig. 125


Fig. 126


Fig. 127

Fig. 4.B.7: Various effects of IBA and BAP in combinations:

Fig. 128- Callus formation from the shoot on MS + IBA 0.5 + BAP 2.0 mg/l after 12 weeks.

Fig. 129- Multiple shoot induction from the nodes on MS + IBA 0.1 + BAP 2.0 mg/l after 12 weeks of culture.

Fig. 130- Long multiple shoots formed from node on MS+IBA 0.1+BAP 1.0 mg/l in 12 weeks.

Fig. 131- Multiple long and short shoots induced from a node on MS + IBA 1.0 + BAP 2.0 mg/l after 12 weeks of culture.

Fig. 132- Root induction from the *in vitro* multiplied shoot on MS + IBA 0.1 + BAP 1.0 mg/l after 14 weeks of culture.

Fig. 4.B.8: Various effects of IBA and Kinetin combinations:

Fig. 133- Callus formation from the shoot on MS + IBA 1.0 + Kn 1.0 mg/l after 10 weeks.

Fig. 134- Multiple shoots induced (tips wilting) from a node on MS + IBA 1.0 + Kn 2.0 mg/l after 12 weeks of culture.

Fig. 135- Root induction from a shoot on MS + IBA 0.1 + Kn 1.0 mg/l after 12 weeks.

Fig. 136- Fine long roots induced from a shoot on MS+IBA 0.1+Kn 0.1 mg/l after 12 weeks.

Fig. 137- Multiple shoots with a single long root induced from a nodes on MS + IBA 1.0 + Kn 0.1 mg/l after 12 weeks of culture.

Fig. 4.B.9: Various effects of hormone free MS medium:

Fig. 138- Multiple shoot along with slight amount of callus formation from a node 12 weeks.

Fig. 139- Initiation of multiple shoot formation from a node after 6 weeks of culture.

Fig. 140- Formation of root from the *in vitro* multiplied shoot after 7 weeks of culture.

Fig. 141- Formation of roots from the multiple shoots after 12 weeks of culture.

Fig. 4.B.10: Caulogenesis.

Fig, 142- Massive root induction from shoot induced callus on MS+NAA 0.5 mg/l in 20 weeks.

Fig. 143- Hairy roots coming directly from the leaves on medium containing MS + NAA 1.0 + BAP 0.1 mg/l after 12 weeks of culture.

Fig. 144- Heavy induction of roots from the shoot induced callus 10 weeks after after subculture on the same MS + NAA 1.0 + BAP 1.0 mg/l.

Fig. 145- Profuse rooting with hairs from shoot induced callus on MS+NAA 1.0+BAP 1.0 mg/l.

Fig. 146- Root and shoot formation from callus on MS+NAA 1.0+BAP 0.1 mg/l after 15 weeks.


Fig. 128


Fig. 129


Fig. 130


Fig. 131


Fig. 132


Fig. 133


Fig. 134


Fig. 135


Fig. 136


Fig. 137


Fig. 138


Fig. 139


Fig. 140


Fig. 141


Fig. 142


Fig. 143


Fig. 144


Fig. 145


Fig. 146

Fig. 4.B.11: Photomicrographs:

Fig. 147- Embryogenic callus from NAA 0.5 + BAP 1.0 mg/l.

Fig. 148- Actively dividing embryogenic cell (10×40).

Fig. 149- A Globular stage of somatic embryoid (10×40).

Fig. 150 and 151- Elongation of pro-embryois (10×40).

Fig. 152-. Embryoids at different stages (Globular, heart and torpedo from NAA 1.0+ BAP 0.5 mg/l at 10×10).

Fig. 153- T. S. of a mature embryo (10×10).

Fig. 154- A typical mature germinating somatic embryo with two distinct cotyledons (10×10).

Fig. 4.B.12: Rooting and acclimatization.

Fig. 155- Numerous thick roots formed from the *in vitro* multiplied shoot on MS + NAA 1.0 mg/l after 10 weeks of culture.

Fig. 156- Complete plant obtained from the shoot on NAA 0.5 + BAP 0.5 mg/l after 12 weeks.

Fig. 157- Sand rooting of shoots with 2 or more nodes after pulse treatment with different auxins (IAA, IBA and NAA).

Fig. 158- Sand rooted shoot with NAA 100 mg/l pulse treatment after 10 weeks.

Fig. 159- Acclimatization in the glass house on different substrates (soil: sand, coco-peat etc).

Fig. 160- A well acclimatized plant after 12 weeks in the shade house on coco-peat.

Photo Plates. Photomicrographs from the callus study of *Rauvolfia serpentina*


Fig. 147

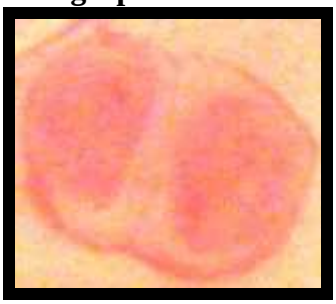
Fig. 148

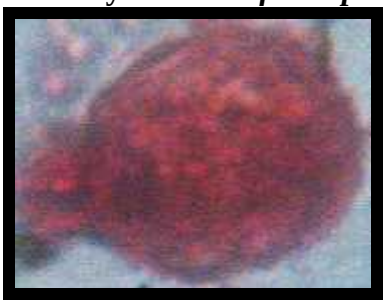
Fig. 149


Fig. 150

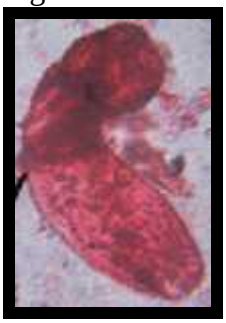
Fig. 151


Fig. 152


Fig.153


Fig. 154


Fig. 155


Fig. 156

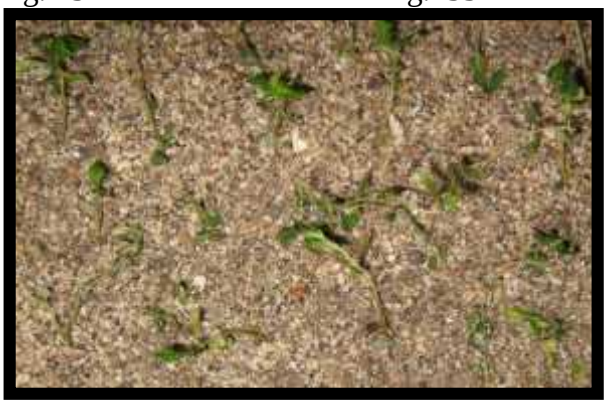
Fig. 157


Fig. 158


Fig. 159


Fig. 160

CHAPTER – 5

DISCUSSION

5.1. *Asparagus racemosus:*

In the present investigation, the roots did not respond to any of the hormone media. Similarly, internodes and cladodes gave slight amount of callus in some of the media which were not found to be significant, hence these were discarded. In the same way, almost all the shoot tips elongated to give out a single shoot in most of the media. Nodes responded the best in all respects (i.e. callus and multiple shoot induction). Hence, nodes were selected for sub cultures and data recording in all the media.

5.1.1 Seed germination:

From the study so far, culture and sub culture of seeds or different explants in hormone free MS medium is the monetarily cheapest and best time saving method of *in vitro* multiplication of *Asparagus racemosus* has been identified. In the present study the seeds germinated on hormone free MS medium. The germination started after two weeks and continued until eight weeks with a maximum percentage of 62.5 *in vitro* but *in vivo* the germination percentage was quite low i.e. only 30% (Pant and Joshi, 2009a). Gupta *et al.,* (2002) observed 17 to 60 % germination in *A. racemosus* but they achieved up to 86% after treatment of seeds with 20% H_2SO_4. The seeds presoaked in water overnight performed much better than those not pre soaked ones under both the conditions. The time for the germination process to complete was also relatively faster in *in vitro* condition where light and temperature were maintained. In similar experiments by Cermano *et al.,* (2002) and Bittencourt *et al.,* (2005) found better percentage in germination and vigor of the seedlings of *A. officinalis* at 20-30^{0}C (12 hour cycle) and water and temperature stress priming respectively.

In present study multiple shoot formation *in vitro* from the seeds has been observed in the MS medium during germination process. The multiple shoots obtained

were morphologically similar to natural plant. Ye *et al.,* (2002) found similar results and they recognised MS medium to be better than B5 for multiple shoot induction from the seeds of *Lens culinaris*. The number of shoots per seed ranged from 0-5 with a single root system in general. The maximum number observed was 5 and all the shoots appeared to emerge from the same point. Shimomura *et al.,* (1998) observed 2-4 shoots in 67% of the seeds of *Reineckea carnia* and he concluded that the seeds were of polyembryonic nature. In a few cases either root or shoot only have also been observed which might be of abnormal nature since we found such cases very rarely. On the MS medium shoot bases either detached from the seeds or intact with the seed showed both root and shoot initiation in just four days of culture. Similar results have been observed by Hisajima, (1982) in almond embryos. The number of shoots gradually decreased from 5 in every successive detachment of the shoots and sub-culture in the same fresh medium. Multiple shoot buds have been observed at the bases of shoots which later give rise to new shoots. The maximum of 13 shoots have been achieved from a single seed. Most of the workers like Yadav *et al.,* (2007) in *Datura metel*, Palanivel and Jayabalan, (2002) in *Arachis hypogaea,* Polisetty *et al.,* (1997) in *Cicer arietinum*, Hisajima, (1982) in almond have achieved similar multiple shoot induction in various plants using different plant growth regulators (PGRs) mostly BAP, BA or Kin. But in the present study PGRs were not used to induce multiple shoots from the seeds. Similar results have been achieved by Te-chato *et al.,* (2005) in Mangosteen seeds using different gelling agents and not PGRs. All these observations show that for the better performance of seeds of *A. racemosus* pre soaking and *in vitro* germination is much more efficient than without soaking and *in vivo* process from where a large number of shoots can be multiplied in a short period of time.

5.1.2 Callus Induction

Present investigation showed that the auxins play a significant role in callus induction from the shoot explants of *A. racemosus.* In *A. racemosus* 2,4-D containing medium acted differently in the induction of callus unlike many other investigators like Kohmura *et al.,* (1990); Li and Wolyn, (1995); Li and Wolyn, (1996); Li and Wolyn (1997); Kunitake *et al.,* (1997) etc. in *A. officinalis.* In most of the previous literatures

the researchers have used mainly 2,4-D in induction of callus of this genus. Although auxins are considered as the most important factors to induce callus, the data of present study show that the shoot explants of *A. racemosus* can initiate and grow callus in a significant amount even without an external supply of auxin if the cytokinins are incorporated in the media. In general it has been seen that up to a certain concentration the amount and quality of callus increases above which they decrease. This might be because of the indogenous hormone the explants already have. Generally, when the auxins are used signally, the callus induction increases with the increase in auxin concentration up to a certain limit. In tissue culture experiments, change in auxin concentrations may change the type of growth, like stimulation of root formation may switch to callus induction etc. Hence, each tissue culture system is unique, and the effect of different concentrations of auxins must be tested for each case individually (Machakova *et al.,* 2008). In the present study NAA was found to be the best (Pant and Joshi, 2009b) although other auxins also induced a good amount of callus. Probably due to its high instability, IAA is usually less effective than synthetic auxins like 2,4-D or NAA (Machakova *et al.,* 2008). All active auxins are weak organic acids and the relative degree of activity of individual auxins in different growth processes is very variable. It differs not only from plant to plant, but also from organ to organ, tissue to tissue, cell to cell and, moreover, also with the age and physiological state of the plant (tissue) (Davies, 2004). Although other concentrations also gave a significant amount of callus, the highest positive effect was recorded when the shoots were inoculated on MS medium supplemented with either 0.5 or 2.0 mg/l of NAA with 100% frequency. Kumar and Vijay, (2008) reported maximum callogenesis of *A. recemosus* on MS supplemented with NAA and they also observed very little and infrequent callus occurrence even with 3.0 mg/l IAA from the nodes of *A. racemosus*. Similar results were obtained by Levi and Sink, (1991) and Delbreil and Jullien, (1994) using 10 mg/l NAA + MS from Cladodes, Crown and lateral buds; Delbreil *et al.,* (1994) from lateral shoots of *A officinalis* using 1.0 mg/l NAA however, Liu *et al.,* (1997) got best callus induction and growth with MS + NAA + Kn from the hypocotyle of *Glysine max*. In the present investigation, the lowest effect was recorded when the shoots were subjected to IBA 0.1 mg/l. Besides NAA, other auxins IAA and IBA showed good results at 1.0 mg/l. whereas, 2,4-D

showed significant effects at 0.5 and 1.0 mg/l. For the induction of callus from the shoot explants IAA and IBA at lower concentrations were ineffective. However, their higher concentrations were found to be significant for callus initiation. Similar to our result, Tang and Newton, (2007) observed callus induction by NAA and 2,4-D but they did not see any induction with IAA and IBA in slash pine (*Pinus elliottii*). Similarly, Prasad *et al.,* (2007) induced callus with 0.25-1.0 mg/l 2,4-D alone in *Chlorophytum borivilianum*. Most of the literatures available reveal that most of the researchers prefer 2,4-D to initiate the callus in most of the plants like: Kar and sen, (1985) observed callus induction with 2,4-D 1.0 + Kn 1.0 mg/l in *A. racemosus*; Saito *et al.,* (1991) induced calli from the crown or lateral buds of *A. officinalis* on LS medium + 5 µM 2,4-D; Mehta and Subramanian, (2005) induced callus from the media containing various concentrations of 2,4-D + Kn in *A. adscendens* node cultures but the calli they observed were similar to our observation with 2,4-D i.e pale yellow creamy type of callus. Pontaroli and Camadro, (2005) used 1.0 mg/l Kn + 2,4-D 1.5,5.0 and 10.0 mg/l + 0.9% agar to induce callus from the shoot explants of *A. officinalis*. It can be assumed that almost all the auxins induce callus from the shoot explants of this species at a certain level (especially at higher concentrations) but in a very few cases the previous investigators have used IAA or IBA to induce callus. Another reason behind not selecting these two auxins might be because of the quality of the callus they induce. It has been observed in our experiment that the calli induced by these auxins are generally non embryogenic brown to black coloured.

Similarly among cytokinins, Kn was found to be better than BAP which above 1.0 mg/l in the MS medium induced the maximum callus. However, BAP at 0.1 -0.5 mg/l in the MS medium induced fairly high amount of callus. These data indicate that BAP has its optimum point at lower concentration whereas higher Kn concentrations induce more callus. All the concentrations of cytokinins tested except Kn 0.1 mg/l induced significant amount of callus. In most of the literatures investigators have been found to prefer Kn than BAP either singly or in combination with 2,4-D to induce callus from the shoot explants of the plants similar to *A. racemosus.* This supports the works of previous researchers and their selection of Kn to induce callus. Hence, it can be

concluded that cytokinins are capable of inducing callus even if there is no external supply of auxin.

In the present study, among NAA, IAA and IBA in combination with either BAP or Kn, NAA was found to be the best auxin in case of callus induction (Pant and Joshi, 2009b). NAA at higher concentration was suitable for callus induction either with BAP or Kn. All the concentration combination of NAA + BAP induced significant amount of callus except NAA 0.5+ BAP 0.1 mg/l. Among NAA + Kn, all the treatments above NAA 0.5 mg/l induced the callus significantly. Here, NAA 0.5 + Kn. 0.5, NAA 0.5+ Kn. 1.0, NAA 0.5 +Kn 2.0 and NAA 1.0 + Kn. 2.0 mg/l showed similar effects. In a similar experiment, Ghosh and Sen 1991 induced callus from the sub apical region of spears of *A. cooperi* using NAA and Kn in MS medium. Ghosh and Sen, (1998) reported the induction of callus was affected by light intensity and combinations of 2,4-D and Kn, 2,4-D and BAP and NAA and Kn in *A. verticillatus*. Odake *et al.,* (1993) observed good embryogenic callus from the internodal segments of *A. officinalis* using MS + NAA 3.0 + K 1.0 mg/l. From the present research data NAA 1.0 + BAP 2.0 mg/l was found to be the best among all the media tested in inducing the callus from the nodal explants. In a similar experiment Saensouk and Suddee, (2004) observed high callus induction from the leaves as well as from the nodes while using NAA 0.5 + BA 2.0 mg/l which is very similar to our result. Hence, from the result it can be understood that the auxin concentration seems to be playing the major role in callus induction. In general, high auxin + high cytokinin favor the callus induction.

In case of IBA + BAP, BAP concentration when increased from 0.1-2.0 mg/l the callus induction also increases. Here, IBA 0.5 + BAP 2.0, IBA 1.0 + BAP 0.5 and IBA 1.0 +BAP 2.0 mg/l gave better results. In this series, cytokinin concentration played the major part in callus induction. Here also a high auxin + high cytokinin increase the callus induction because most of the treatments at IBA 1.0 mg/l induced callus at a highly significant level of 1%.but the Kn concentration generally did not play a significant role in it. Here also, IBA in combination with Kn were found to be less effective than IBA +

BAP although a few concentration combinations like IBA 1.0 +Kn. 0.5 and IBA 1.0 + Kn. 2.0 mg/l were significant.

None of the concentration combinations of IAA and BAP were found to be inducing the callus significantly in the present work. A high IAA + high BAP level (1.0 + 1.0 mg/l) induced slightly more callus than other treatments. Statistically, all the treatments in this series were similar in case of callus induction. Hence, IAA along with BAP can not be considered as good combination for callus induction. IAA when combined with Kn, a lower IAA concentration with higher Kn concentration (IAA 0.1 + Kn 1.0) induced significant amount of callus but at lower concentrations of both the induction level was also low. Higher IAA concentrations above 0.5 mg/l when used with all the concentrations of Kn, most of the treatments induced significant amount of callus. In IAA + Kn series, IAA 0.1 + Kn. 1.0, IAA 1.0 + Kn. 0.1 and IAA 1.0 + Kn. 1.0 mg/l induced significant amount of callus. Hence, in this experiment both the PGRs (IAA and Kn) seem to be equally active in callus induction.

From the overall discussion it can be assumed that almost all the auxins induce callus from the shoot explants of this species at a certain level (especially at higher concentrations). Among all the auxins tested NAA proved to be the best either singly or in combinations and among cytokinins, both responded somewhat equally but in different ways. Lots of previous literatures support the use of NAA + BAP or Kn but in a very few cases the previous investigators have used IAA or IBA to induce callus. Here, from our experiment as well, the use of NAA + BAP or Kn for the induction and growth of callus is verified. NAA is followed by IBA and IAA respectively in combinations with cytokinins but when used singly, IAA and 2,4-D were behind NAA. With NAA and IBA, BAP showed slightly better performance than Kn whereas with IAA, Kn showed slightly better than BAP.

5.1.3. Shoot Multiplication and Shoot length

In the present work the best medium for shoot multiplication has been recognized as MS + IBA 1.0 + BAP 1.0 mg/l with a mean shoot number of 10.83 per explant. In a

similar experiment by Hurgoiu and Blidar, (2008) the same medium as ours induced a lot of plantlets from the calli of *A. officinalis* but after 12 weeks of culture, they observed plantlets vitrification.

Among the auxins NAA 0.1 mg/l have shown the highest number of multiple shoots (i.e. 7.83 shoots/explant). In IBA series also 0.1 mg/l came up with a highest number of 2.5 shoots/explant whereas among IAA concentrations 0.5 mg/l gave 4.8 shoots per explant. 2,4-D showed no response in shoot multiplication at any concentration levels. In general all the auxins except 2,4-D showed shoot multiplication at lower concentrations between 0.1 and 0.5 mg/l above which the effect gradually decreased. In general the shoot number and length seems to be inversely proportional to the concentration of auxin supplied.Here it can also be concluded that the auxins alone either are not suitable to use for shoot multiplication or can be used at very low concentrations. Within the cytokinin group, BAP 0.1 mg/l showed the maximum number of shoots (7.50) per explant which was the only statistically significant concentration among all the cytokinin concentrations at 1% level [Wala and Jasrai, (2003)]. In contrast Kn 0.1 gave the lowest number of shoots i.e. 1.33. Here BAP proved to be better in shoot multiplication than Kn. Kumar and Vijay, (2008); Guo *et al.*, (2004); Quraishi *et al.*, (2004); Liao *et al.,* (2004); Pandey *et al.*, (2004) and Hassanein and Azooz, (2003) have also reported the superiority of BAP` over Kn in case of multiple shoot induction in different plant species. The maximum number of shoots induced by Kn was 4.66 when the explants were cultured on the MS + Kn 1.0 mg/l. Here also BAP at lower concentration favored the shoot proliferation. As the concentration increased the number of shoots decreased. In contrast, Kn induced the shoots at relatively higher concentration than BAP and the trend was somewhat opposite to BAP. In a similar experiment Bopana and Saxena, (2008) induced a maximum of 5.89 shoots per node with Kn 6.97 µM. Bopana and Saxena, (2008) also found that in combination of BA 3.33 + Kn 3.48 µM they observed an average of 9.49 shoots per node. They also observed a maximum of 2.31 cm long shoots on the MS + Kn 2.32 µM.

In combinations of auxin and cytokinin, IBA 1.0 + BAP 1.0 mg/l induced the maximum number of 10.83 shoots/explants. Mehta and Subramanian, (2005) recorded the maximum of 8.45 shoots per node on MS supplemented with 0.27 µM NAA and 0.46 µM Kn after four weeks of incubation and an increase in the concentration of NAA and Kn did not show any improvement. When IAA and IBA were used in place of NAA with Kn, all explants died. On the other hand in present work IBA and IAA also induced multiple shoots when combined with either BAP or Kn. Although many of the IAA and IBA concentrations along with cytokinins induced very little number of shoots per explant, some cases of IBA + Cytokinin were found to be inducing multiple shoots at a highly significant level. In combination of NAA and BAP none of the concentration combinations were found to be statistically significant in inducing the multiple shoots although NAA 0.1 + BAP 2.0 mg/l was able to proliferate an average of 5.33 shoots per expant. Among NAA + Kn combinations, NAA 0.1 + Kn 1.0 and NAA 0.1 + Kn 2.0 mg/l produced 8.16 and 8.83 shoots/explants respectively. Among all concentration combinations only these two combinations were significant. From this we came to a conclusion that NAA at lower concentration along with high cytokinin greatly favor the shoot multiplication. This finding was similar to work of Kumar and Vijay, (2008) with their experiment on the same plant species. This result is also similar to the findings of Kambaska and Santilata, (2009); Azad *et al*., (2005); Sharma *et al.*, (2004); Guo *et al*., (2004) etc. on various plant species. However, Stajner *et al.*, 2002 found the best shoot initiation (11.9 per explant) on MS + BA 0.44 + K 0.93 + NAA 1.07 + ancymidol 3.90 µM from the crown of *A. maritimus*. Similarly, in IBA with BAP, only three combinations IBA 0.1 + BAP 0.1, IBA 0.5 + BAP 0.5 and IBA 1.0 + BAP 1.0 mg/l were highly significant and induced averages of 8.83, 8.66 and 10.83 shoots/explant. From this result it can be assumed that equal concentrations of IBA and BAP favor the shoot induction. Among all the IBA and Kn combinations, only two were found to be significant viz. IBA 0.1 + Kn 1.0 and IBA 0.5 + Kn 2.0 mg/l with average shoots of 6.66 and 8.33 respectively. Here in the present experiment, lower IBA with higher Kn were successful. In combination of IAA and BAP, the maximum number of shoots produced was 5.16/expalnt with IAA 0.1 + BAP 0.1 whereas in IAA and Kn series 5.83 shoots/explant were induced by IAA 0.1 + Kn 2.0 mg/l. None of the concentration

combinations of IAA both with BAP or Kn were significant. In a similar experiment Kar and Sen, (1985) observed that the shoots induced from callus when subcultured on the MS medium containing IAA 0.1 or 0.5 + BAP 1.0 mg/l, an average of 12.0 new shoots per shoot were produced after 6 weeks in *A. racemosus*. In this experiment, among all the combinations, NAA 1.0 + BAP 0.5 could not induce any shoot. In overall observation, all the auxins except 2,4-D showed a gradual increase in shoot number up to a certain concentration and a gradual fall there after. In case of cytokinins, the response data showed reverse trend i.e. low BAP and high Kn concentrations induced more shoots. In combinations IBA showed the best results followed by NAA. Here both the cytokinins have mixed results but Kn proved to be slightly better than BAP. Higher BAP generally in almost all treatments gave short and thick shoots vitrified shoots. Similar observations were also observed by Bopana and Saxena, (2008) in this plant species. Higher concentrations of BA could possibly be responsible for the hyperhydricity seen at this stage (Paek and Hahn, 2000); Armstrong and Johnson, 2001) Theses shoots had very little cladodes and they turned yellow after 10 weeks of culture. Multiple shoot induction *in vitro* is dependent on the proper selection of hormones (auxins and cytokinins) and their proportions in the media.

Among all the auxins used in the present investigation, IBA 0.5 mg/l was the only concentration where shoot elongation occurred significantly (5.96 cm) in the present research work. Higher auxin concentration accumulation might have affected in the proper growth of the shoots because high auxin increases ethylene production and the ethylene accumulated in the culture vessels may then inhibit the growth and development of many *in vitro* grown plants. Conversely, ethylene may affect auxin transport and metabolism (Machakova *et al.,* 2008). Similarly, among cytokinins, BAP 1.0 mg/l was the only significant concentration to elongate shoots with an average length of 5.48 cm. In combinations of NAA and BAP, MS + NAA 1.0 + BAP 0.1 only elongated the shoots significantly with an average length of 5.81 cm. Among NAA + Kn combinations, no treatment was found to be significantly facilitating the shoot elongation. Kumar and Vijay, (2008) while working with the same plant species got maximum elongation on MS + BA 3.0 + Kn 3.0 + NAA 0.1 mg/l. In case of shoot length IBA 0.5 + BAP 0.5 mg/l

gave the longest shoots of 6.36 cm but higher BAP concentration with a relatively lower IBA actually retarded the shoot elongation in our experiments. This has been observed when the explants were cultured on the media containing IBA 0.1 + BAP 2.0 and IBA 0.5 + BAP 1.0 mg/l. This indicates that the PGRs sometimes have a very limited range where they show a positive effect otherwise either they do not have any effect or actually respond negatively. IBA and Kn combination series also elongated shoots significantly only at IBA 0.5 + BAP 2.0 mg/l with 5.60 cm. Most of the concentration combinations of IAA along with BAP were negatively significant in case of shoot elongation i.e. they retarded the growth of shoots but with Kn the result was slightly better when Kn or both were at higher levels. Although in some cases vitrifications were observed, higher Kn concentration generally gave shoot elongation whereas BAP mostly showed vitrification.

5.1.4. *In Vitro* Root Induction and Root length

All the shoots produced from the above experiments when cultured in MS medium containing different concentrations of NAA (0.1-2.0 mg/l) gave normal healthy roots [Kambaska and Santilata, (2009)]. The best rooting was observed at MS + NAA 0.1 mg/l with an average of 4.33 roots per shoot which was the highest number among all the treatments of our experiments. As the concentration of NAA was increased, the callus at the bases increased and all the parameters of roots gradually decreased. Similar to our experiment Kumar and Vijay, (2008) reported good rooting of the shoots only on NAA 1.0 and 5.0 mg/l containing MS medium. Similarly, Wala and Jasrai, (2003) observed good rooting of shoots of *Curculigo* on ½ MS + NAA 0.53µM. We did not observe good rooting with any concentrations of IAA and IBA. Kumar and Vijay, (2008) also did not observe any roots with any concentrations of IAA or IBA in *A. racemosus*. However, in similar experiments by Benmoussa *et al.*, (1997) in *A. densiflorus* and Kar and Sen, (1985) in *A. racemosus* induced roots on medium containing IBA whereas Ghosh and Sen, (1998) in *A. verticillatus* reported good rooting on MS medium containing IAA. Almost all the concentrations of NAA incorporated in the MS medium could produce multiple roots significantly. From our results, it is clear that NAA possibly is the only auxin that singly can induce roots from the shoots of this plant and its low concentration is sufficient for the efficient induction. Among the cytoinins, Kn is better

in root induction either from the callus or from the shoots when used above 0.5 mg/l. Although most of the combinations have failed to induce roots, NAA, IBA and IAA above 0.5 mg/l with low concentrations of both the cytokinins (preferably) have shown a good number of roots. This shows that mainly the concentration of auxin plays vital role in root induction rather than cytokinin. The root elongation also followed the same trend as the root induction. The maximum number of 4.33 roots/explant were recorded from NAA 0.1 mg/l followed by NAA 1.0 + BAP 0.1 and NAA 0.5 + Kn 0.5 mg/l. with 4.0 roots/explant. Similarly, the root elongation followed the root induction pattern. The longest roots of 3.3 cm were found on the medium containing NAA 0.1 mg/l. and this was followed by NAA 1.0 + BAP 0.1 with 2.9 cm long roots. Since, IAA and IBA neither singly nor in combinations with different cytokinins could induce roots properly. The only auxin suitable for root induction in this species is NAA (Pant and Joshi 2009a).

5.1.5. Agar manipulation

This experiment of manipulating agar concentration was intended to induce storage roots from the shoots using NAA as the root inducer as suggested by Shigeta *et al.*, (1996) and to see the effects of agar concentration on vitrification of the shoots as suggested by the workers like Thorpe *et al.*, (2008) and Singha et al., (1990). From the agar manipulation data obtained in the present study it is obvious that the callus induction trend was similar in all agar concentrations and NAA concentrations. Here the callus growth is controlled only by NAA concentration not the agar concentration. In case of root differentiation except at NAA 1.0 mg/l, the number of roots gradually increased with increase in agar concentration i.e. from 0.6 to 1.0%.. Hence, it can be said that the agar concentration influences the root proliferation i.e. root number increase with an increase in the toughness of the media. The root length did not show any relation with the manipulation but the lower concentration of NAA seemed to be positive in root elongation. Similarly, Leshem, (1983) also observed that a higher agar concentration in the medium increased the percentage of normal shoots developing in Carnation. Although, we did not observe any of these responses the water potential of the medium affects morphogenesis of plants, in the whole experiment shoot proliferation was not observed. Hence the effects on vitrification of shoots were not observed. Debergh *et al.*,

(1981) also found a decrease in shoot proliferation from the shoot cultures of *Cynara scolymus* after increasing the agar concentration in the medium.

5.1.6. Somatic embryogenesis:

From the result of this investigation, it can be said that the BAP is one of the best cytokinins to induce somatic embryoids from this plant. Similarly, all the auxins tested in combinations with cytokinins were found to be capable of inducing the embryoids. NAA when tested singally, higher concentration (2 mg/l) was also effective. Mainly in combination of auxin and cytokinin, higher concentrations of both (above 0.5 mg/l) were capable of inducing the somatic embryoids. Auxin concentrations above 0.5 mg/l were found to be inducing somatic embryoids. In combinations of auxin and cytokinin also, a higher concentrations of both favored the induction of somatic embryoids. From the ovarall observation it is clear that BAP (BAP 2.0) proved to be better than Kn among the cytokinins both conditions either singally or in combination with auxins. In the same way in combination of auxin and cytokinin, NAA and BAP proved to be the best one with large number and size of embryoids (NAA 0.1 + BAP 1.0 and NAA 0.1 + BAP 2.0 mg/l). Since the plantlets germinated from the somatic embryoids were found to be relatively much thicker, with less cladodes than the normal shoots, and the longer internodes, there might be the presence of somatic variability. A very few or no literatures are available on the somatic embryogenesis of this species probably because this species is less known to the western world. The previous works on somatic embryogenesis induction in *A. officinalis* have been studied widely by various workers like Kohmura *et al.*, (1990); Levi and Sink, (1992); Odake *et al*., (1993); Delbreil *et al*., (1994); Li and Wolyn, (1995); Li and Wolyn, (1996) etc. using shoot explant induced calli of different varieties of *A. officinalis* either without any addition of any hormone or incorporating small amout of NAA (0.1 mg/l) + Kn or other cytokinin in the MS medium. Milivojevic *et al.*, (2005) observed the maximum embryogenic capacity from the root explants of *Chenopodium rubrum* on 1 µM 2,4-D and in basal parts of cotyledons in *C. murale* plants on 10 µM 2,4-D. similarly, Feitosa *et al.*, (2007) induced somatic embryoids from the shoot apices of Cassava genotypes on MS + Picloram.

From the present study we conclude that the callii of 10-12 weeks are good enough for the study of somatic embryogenesis. For the induction of somatic embyroids, the quality of the callus is important. Not all the calli are potent in embryoid formation. As observed in the experiment friable, tough and greenish yellow calli were good source of embryoids whereas the watery, brown and black callii were non embryogenic.

5.1.7. Buds, Caulogenesis and Vitrification:

5.1.7.1. Buds:

In case of shoots the best explants for initiation of the buds were nodes when cultured on the MS medium with high cytokinin either alone or with an auxin. This was found to be one of the easiest methods of shoot multiplication. The buds produced shoots after 2 weeks of culture. Similar to the present finding, Kumar and Vijay, (2008) reported the induction of buds from the nodes on MS + BA 4.0 mg/l. As observed in some of the treatments like IBA 0.5 + BAP 0.5 mg/l the buds started even under the callus mass grown around the rapidly growing/multiplying shoots. The shape and size of the buds also varied with the media. In present work buds grown on the medium containing NAA 0.1 + BAP 2.0 mg/l were the biggest among all. The germination behaviors of these buds were also different. The MS + IBA 0.5 + BAP 0.5 mg/l grown buds produced 1-2 normal shoots, but the buds induced from NAA 0.1 + BAP 2.0 mg/l were slightly thicker. The other buds grown either on MS + BAP or MS + NAA + BAP containing media induced single normal shoots. Under our observation the maximum number of buds per node were observed to be 11.66 when the explants were cultured on MS + NAA 0.1+BAP 2.0 mg/l followed by IBA 1.0 + BAP 2.0 mg/l with 10.33. Since the higher concentrations of cytokinin induced shoot buds at all levels, it can be concluded that a cytokinin with its higher concentration highly favors the bud induction and BAP proved to be better cytokinin than Kinetin in this case. In combinations of auxins and cytokinins, Among the auxins, NAA and IBA were better than IAA when they were added with cytokinin. The shoot buds as induced on the medium containing IBA 1.0 + BAP 2.0 mg/l slowly mature on the mother plant and finally gets easily detached. These buds after detachment from the mother plants could behave as normal seeds. They germinated producing single to multiple shoots (mainly1-2 shoots) with or

without roots. These buds used as explants showed very good response in the initial trials and possibally can be used as one of the good explants for rapid multiplication of *A. racemosus*. Reuther, (1977) also observed buds from the superficial cells of callus usin NAA 1.0 ppm and BAP 1.0 ppm in the LS medium in *A. officinalis.*

5.1.7.2. Caulogenesis:

The term literally means the **s**tem organogenesis or the induction of shoot development from callus (Zaid *et al.,* 1999). In most of the cases, the callii when sub cultured in different media induced either shoots or roots or both which is a common phenomenon in most of the plants. From the data obtained from this study it can be concluded that the cytokinins at lower concentrations induce multiple shoots. However, kinetin have been found to induce more roots than shoots. The shoot induction decreasd as the concentration of cytokinins increased. Similarly, auxins when in low concentration induce multiple shoots but in higher concentrations induce roots. In combinations of auxin and cytokinin NAA and IBA have been found to be good for caulogenesis with BAP than Kn (Pant and Joshi, 2009b). Kar and Sen, (1985), observed caulogenesis on MS + IAA 0.5 + BAP 1.0mgl/l in *A. racemosus*. Here, auxin and cytokinin together mainly induce shoots but when the concentration of auxin increase the roots are also differentiated. This is another efficient way of rapid multiplication of plants. In a similar experiment by Hurgoiu and Blidar, (2008), induced a considerable amount of calusogenesis on MS + 1.0 mg/l BA + 1.0 mg/l IBA, and a lot of regenerated plantlets from the calli of *A. officinalis*, but finally, this medium-after 12 weeks of culture initiation, induced plantlets vitrification. Similarly, Tripathi and Reddy, (2002) found that among 4 media tried, MS gave the best result with 2 mg/I BA and 2 mg/l IAA in Indian cotton cultivars.

5.1.7.3. Vitrification:

Vitrification literally means the cultured tissue having leaves and sometimes stems with a glassy, transparent or wet and often swollen appearance. The process of vitrification is a general term for a variety of physiological disorders that lead to shoot tip and leaf necrosis (Zaid *et al.,* 1999). From the present research observation, shoots formed from the media

containing BAP either singly or in combinations with any of the auxins greatly reduce the number of cladodes as well as the shoots get widely vitrified especially when BAP is above 1.0 mg/l (Pant and Joshi, 2009b). Similar treatments of kinetin induced normal shoots although in some cases vitrifications were observed. Hence in case of shoot multiplication, Kn is superior to BAP. Singha *et al.,* (1990) also observed both shoot-tip necrosis and severe vitrification on the shoot tip cultures of *Cydonia oblonga* initiated on MS medium containing 5 µM BA and 0.6% agar. The abnormal vitrified shoots from the media containing high BAP concentrations were also used as the explants and they also produced normal multiple shoots (very high upto 10 shoots per explant) on the various media containing either low concentrations of cytokinin or auxins or both in combinations (0.1 and 0.5 mg/l). Debergh, (1983) confirmed that cytokinins under inductive conditions of low agar concentration or high matrix potential are evocators of vitrification and this condition can be overcome by increasing the agar concentration in the media. However, Pearez-Tornero *et al.*, (2001), observed the best remedy to this problem as the bottom cooling system for 2 wk and the use of 0.8% agar as gelling agent in *Prunus armeniaca*. Hyperhydric shoots of this plant even reverted to normal after keeping them for 3 wk in the bottom cooling system. Hence, the vitrified shoots that are normally discarded in tissue culture have also been used to multiply the plant quickly.

5.1.8. *In Vivo* Rooting and Acclematization

As in *in vitro* rooting NAA have also been found to be significantly effective in differentiating the roots from the shoots *in vivo*. *In vivo* rooting was very difficult and a very few (one out of 10) shoots rooted with pulse treatment of NAA 100 mg/l in four to 6 weeks in sand moistened with water. Other auxins tested (IAA and IBA) also at 100 mg/l pulse treatment could not produce any root from the shoots. However, Pandeya *et al.*, 2010 achieved success on *Clitoria ternatea* microshoots with IBA 250 mg/l for half hour. The acclimatization process was highly successful on the 100% coco-peat for 2 months and then on the sand soil (50/50) with a gradual decrease in humidity in the glass/plastic house. Although the plantlets thus produced survived, grew and branched in the plastic/glass house for about 3 months and they gradually died in the winter. The

final survival percentage of both *in vitro* and *in vivo* rooted shoots was 60 and 20 respectively.

5.2. *Rauvolfia serpentina:*

5.2.1. Seed germination:

Since the germination percentage of the seeds of *Rauvolfia serpentina* was very low and was observed only under *in vitro* condition, it can be said that the seed germination of this species is very low. Hence the species generally propagated by root cuttings (Bhatt *et al.,* 2008). The low germination percentage might because of the presence of cinnamic acid and derivatives in the seeds (Sahu, 1979 and Mitra, 1976). The low germination percentage of the seeds of *R.serpentina* has been supported by various workers like Alamgir and Ahmed, (2005), Sudha and Seeni, (2006) in *R. micrantha* etc. According to Baksha *et al.,* 2007, the germination percentage of seed is very poor and variable (25-50 %) and is often as low as 10 percent. Paul *et al.,* (2008) however increased the percentage germination to 48 after scarification with sand paper.

5.2.2. Callus induction:

From the overall data of the present work, the maximum callus induction was recorded when the leaf or shoot explants were cultures on the MS medium containing 2,4-D 2.0 mg/l (Pant and Joshi, 2008) with 100% frequency. Other higher amount of calli formation recorded were from the shoot explants when cultured on MS + NAA 2.0 mg/l or MS + NAA 1.0 + BAP 0.5 mg/l. MS + NAA 1.0 + K 2.0 induced fairly good amount of callus. Very close to our result Salma *et al.,* (2008) and Ahmad *et al.,* (2002) found a very high frequency of callus induction from the nodal or shoot tip explants of *R. serpentina* when they were cultured on MS + NAA 2.0 + BA 0.5 or NAA 0.2 + BA 0.5 mg/l respectively. Similarly, Anitha and Kumari, (2007, 2006 and 2006a) observed callus induction from the leaf explants of *R. tetraphylla* L. using IAA, IBA, NAA and 2,4,D but they observed a maximum of 95% with 2,4-D 9 µM. In a similar experiment by Mungole *et al.,* (2009), observed that MS incorporated with 2,4-D 0.8 + K 0.8 mg/l, NAA 0.2 + BAP 0.2 mg/l, NAA 0.8 + K 0.8 mg/l induced maximum callus from leaves, nodes and buds respectively in *Ipomoea obscura* L. Similarly, the lowest amount of

callus formation was recorded from the MS medium with IBA 2.0 mg/l and hormone free MS medium. Some of the media did not show any response in callus induction such as 2,4-D 0.1, NAA 0.1 + K 0.5, IAA 0.1 + BAP 0.1, IAA 0.1 + BAP 0.5 and IAA 0.5 + BAP 0.1. In general, among auxins it has been noted that all the auxins except IBA showed the gradual incerease in callus production with a gradual rise of auxin concentration. In case of IBA the peak was observed at 0.5 mg/l and a gradual fall with rise in IBA concentration. Here it can be concluded that upto a certain limit a rise in auxin concentration raises the callus induction capacity of the media.

Among cytokinins, Kn was found to be better callus inducer than BAP at high concentration. Bhatt *et al.,* (2008) observed no callus induction either from leaves or shoots of *R.serpentina* with BAP. In the present work, NAA with both the cytokinins in combinations favored callus induction when it was treated at higher concentrations. Here BAP had very lille or no effect but Kn when increased the amount of callus also increased but the lower concentrations of BAP showed very little and irregular trend of callus induction. However, IAA with Kn showed regular and increase in callus with their concentration rise up to a certain level (generally Kn 1.0 mg/l). Singh *et al.,* (2009) observed maximum callus from the juvenile leaf explants of *R serpentina* on media MS + IAA 2.0 + BAP 2.5 mg/l and MS + IAA 0.5 + BAP 1.0 mg/l. In this work, IBA also showed the similar results with BAP (irregular) whereas with Kn the trend was very regular as in the previous cases. Hence here it can be concluded that Kn is superior to BAP in callus induction in this species where as NAA is the best among all the auxins. Sudha and Seeni, (2006), found that ½ MS + 2,4-D 0.3 or 0.5 mg/l from the root explants of *R. micrantha* Hook. F. but we did not see a significant callus from the root explants.

5.2.3. Shoot induction and shoot growth:

From the present work a maximum average number of 7.83 shoots per explant was recorded from the media containing IBA 0.1 + BAP 2.0 mg/l followed by NAA 0.1 + BAP 1.0 mg/l with an average of 7.16 shoots with 100% success. Bhatta *et al.,* (2008) also reported the best shoot multiplication on the medium containing IBA 0.125 + BAP 1.0 mg/l which was very close to our result. Mishra *et al.,* (2010) and Sarker *et al.,*

(1996) also observed the best shoot multiplication on MS + NAA 1.0 µM + BA 1.0 µM and NAA 0.1 + BA 1.0 mg/l respectively on this species with up to 7.66 shoots per node. Similarly, Ahmad *et al.,* (2002) and Baksha *et al.,* (2007) in *R. serpentina* shoot tip cultures observed a maximum of 5.9 and 8 shoots per explant on MS + NAA 0.1 + BA 2.5 and NAA 0.5 + BAP 4.0 mg/l respectively. Faisal *et al.,* (2005) however reported 18.5 shoots per node of *R. tetraphylla* on MS + 5.0 µM TDZ (thidiazuron). In the same way, Sudha and Seeni, (1996) found 77% of the nodes induced up to 3 shoots in 8 weeks in *R. micrantha* on MS + NAA 0.27 µM + BA 13.28 µM. From the above result it is very clear that the best medium for multiple shoot induction from the different shoot explants is none other than MS supplemented with a low auxin (mainly IBA or NAA) and high cytokinin (mainly BAP).

The lowest number of shoots per explant recorded from the present work was 0.5 when the explants were cultured on the media containing 2,4-D 0.5 mg/l and NAA 1.0 + BAP 0.5 mg/l. Similarly, 2,4-D 2.0 and 3.0 mg/l did not produce any shoot. Among all the auxins, NAA was found to be the best shoot inducer although good number of shoots was induced under control condition. This was followed by IAA, IBA and 2,4-D. The shoot elongation trend in all the media with auxins followed the shoot induction trend. The longest shoots were recorded on the medium containing NAA 0.1 mg/l with an average length of 4.58 cm whereas the shortest shoots were found on the medium containing NAA 1.0 + BAP 0.5 mg/l with an average length of 0.56 cm. Here it can be said that the higher auxin concentrations that induces callus at the bases retard the formation as well as normal growth of the shoots. Hence it can be assumed that the similar media are needed for both proper shoot multiplication and growth of the shoots.

Among the cytokinins tested, BAP showed a regular increase up to 1.0 mg/l and a decline above it whereas in Kinetin this trend was not observed. Bhatt *et al.,* (2008) however observed no multiplication from both nodal as well as apical explants of *R. serpentina* with BAP at 1.0 mg/l. In case of number of shoots also BAP proved to be better than Kinetin. In overall response BAP has shown active effects in all the experiments which might be because of its slow degradation and it can be autoclaved

without losing the property (Kumar and Vijay, 2008). Mishra *et al.,* (2010) and Anitha and Kumari, (2006) got up to 9. 16 and 4.56 shoots when they cultured single shoots/ tips on MS + BA 10.0 µM and MS + BAP 4.44 µM respectively. But in case of shoot elongation, the trend was completely opposite to that of shoot induction. As the BAP levels increase up to 1.0 mg/l, the shoot lengths decrease but Kn showed slightly different trend i.e. length increased up to 1.0 mg/l level and then decrease. This effect might be because of vitrification of shoots with high BAP concentration however no evident were available. Kataria and Sekhawat, (2005) multiplied shoots on MS + 4.0 µM BAP to prevent the shoots of *R. serpentina* from hyperhydration.

Similarly, low concentrations of NAA along with any concentration of BAP showed good results especially with 1.0 and 2.0 mg/l BAP. Similar results were observed with NAA and Kn but here a high NAA concentration also favored to some extent. Salma *et al.,* (2008) also observed similar results with 4.0 shoots/ node of *R. serpentina* on MS + NAA 0.2 + BA 1.5 mg/l. The shoot elongation was observed to follow the shoot number trend when NAA was added with either of the cytokinins i.e. the hormone that favors shoot number also favors shoot elongation. In case of IAA + BAP and IAA + Kn a gradual increase in both of their concentrations showed a gradual increase in shoot numbers but with an increase in case of shoot elongation the negative trend was observed. Kataria and Sekhawat, (2005) found 3-5 shoots per node on the MS medium containing 10 µM BAP and 0.5 µM IAA. As the BAP or Kn concentration increased the length of the shoots gradually decreased. This indicates that a low cytokinin level is better for the elongation of shoots and the shoot induction as well as shoot elongation depend more on cytokinin than auxin. In case of IBA + BAP and IBA + Kn also BAP or Kn played the major role in shoot multiplication i.e. as the cytokinin level increases the number of shoots also increase. But in shoot elongation no regular trend was observed. Singh *et al.,* (2009) used 1.0 mg/l GA_3 in the MS medium to induce shoot elongation, whereas Ahmad *et al.,* (2002) found a maximum of 4.2 cm long shoots from shoot tip cultures of *R. serpentina* on MS + BA 2.5 mg/l.

5.2.4. Root induction and root elongation:

The maximum number of roots per explant (12.50) was found from the present work when the shoot explants were cultured on MS + NAA 0.5 mg/l followed by NAA 1.0 with 10.16 (Pant and Joshi, 2008) with 100% frequency. In a similar experiment Ahmad *et al.,* (2002) also achieved 100% rooting with an average of 7.3 roots per shoot in *R. serpentina* on MS + NAA 0.2 + IBA 0.2 mg/l. Similarly, the minimum average number of roots was recorded to be 0.16 per explants on MS + IBA 1.0 + BAP 0.5, MS + IBA 0.5 + BAP 0.1 and MS + NAA 0.1 + Kn 2.0. The average longest roots were also recorded on MS + NAA 0.5 mg/l with 4.76 cm and the shortest ones of 0.26 cm were found on the medium with IBA 0.5 + BAP 1.0 mg/l. Ahmad *et al.,* (2002) observed a maximum of 2.5 cm long roots in 14-16 days in the same species on MS + NAA 0.2 + IBA 0.2 mg/l. Among all the auxins NAA was found to be the best in rooting when it was incorporated in a lower dose upto 0.5 mg/l. Sudha and Seeni, (1996) also observed roots from the excised shoots of *R. micrantha* on MS medium containing 2.6 µM NAA on the other hand, Baksha *et al.,* (2007) observed roots from the micropropagated shoots on ½ MS + NAA 0.5 mg/l. Salma *et al.,* (2008) found ½ MS + IAA 1.0 + IBA 1.0 mg/l as the best rooting medium for *R. serpentina* shoots. In the present work NAA is followed by IBA and NAA when used singly. Faisal *et al.,* (2005) however found MS + IBA 0.5 µM as the best rooting medium for *R. tetraphylla*. Similarly, Patil and Jayanthi, (1997) found best rooting from the shoots of *R. tetraphylla* and *R. micrantha* on MS + IBA 2.0 mg/l. Singh *et al.,* (2009) however observed 100% rooting frequency on MS + IAA 0.5 + NAA 0.5 + BAP 2.5 mg/l. Almost all the concentrations of the 2,4-D were found to retard the root growth. From the data recorded, we came to a conclusion that root length and root number show similar response in the media. Hence it can be assumed that the similar media are needed for good root induction and proper growth.

The root length also follows the similar trend as those of root number i.e. the same hormone concentration favor both root induction and growth. The cytokinins supported the root induction and root elongation at a very small range. BAP supported at 0.1 mg/l whereas Kn supported at a higher concentration (1.0 mg/l). In combination of NAA with BAP at low NAA and high BAP levels root elongation and root number

gradually increased but in presence of high concentration of NAA on the medium, the number of roots increased while their lengths decreased. In case of cultures on MS + NAA + Kn, there was gradual increase in both number and length of roots when NAA concentration was high (above 0.5 mg/l). In presence of IAA roots were not induced with any of cytokinins. But on the MS + IAA 1.0 + Kn 0.1 mg/l the explants showed a slight sign of root induction. MS + IBA + Kn also showed slight sign of root induction when the IBA level was at its lowest (0.1 mg/l) whereas MS + IBA + BAP showed different effect. IBA concentrations at all levels with BAP between 0.5 and 1.0 mg/l induced more as well as long roots. Both the extreme concentrations of BAP mixed with IBA showed negative root induction trend. The root differentiation as well as elongation favored the similar concentrations as observed in many other treatments. In case of root induction the statistical data analysis shoed that most of the concentration combinations of the series IBA +Kn are retarding the growth of roots. Hence, they are not good rooting medium. Similarly, the root induction in the series IBA + BAP was very irregular and the percentage of induction in all combinations was very low. This might be because of the presence of residual auxin in the explants from the previous cultures. Since all the concentrations of IAA + BAP failed to induce any root from any of the explants, this combination can be regardes as a bad combination for root induction.

5.2.5. Somatic embryogenesis:

In our study, calii of age between 10 to 12 weeks induced from the shoots reveled different stages of somatic embryoids. The calli formed from the leaf explants did not show any sign of embryoids. The calli from the MS medium with these PGR/s were found to be embryogenic: NAA 0.5 + BAP 0.1, NAA 0.5 + BAP 0.5, NAA 0.5 + BAP 1.0, NAA 1.0 + BAP 0.5, NAA 1.0 + BAP 1.0 and 2,4-D 3.0 mg/l. Sudha and Seeni, (2006) observed direct somatic embryogenesis from the *in vitro* grown root segments of *R. micrantha* in ½ MS + NAA 0.5 mg/l and MS + NAA 0.1 + BA 0.2 mg/l after six weeks of culture. They only observed cotyledonary embryos occasionally but did not find any globular and heart shaped ones. Similarly, Jocelyne and Chenieux, (1991) reported that the leaf mesophyll protoplast culture of *R. vomitoria* on Murashige and Tucker medium containing 0.17 M sucrose, 9 g/l agar and 4.5 Zeatin gave the highest

percentage (22%) of embryogenic callus in four weeks of culture. Since there is no literature available on the somatic embryogenesis of this species, our initial work can be a step towards further research.

5.2.6. Caulogenesis:

Although the callii were obtained from the same explant they exhibited different effects. Most of the callii obtained from the leaves were white coloured which generally failed to give any shoot in any media. But Ahmad *et al.,* (2002) reported caulogenesis from the leaf derived calli. Singh *et al.,* (2009), have noted that the callii that are light green and fragile are capable of organogenesis. Caulogenesis in *R. serpentina* has also been studied from the MS medium with NAA series, 2,4-D series, BAP series and NAA + BAP combination series were studied. Similarly, Singh *et al.,* (2009) studied on the various combinations of BAP and IAA and found the highest 75% on MS + IAA 0.4 + BAP 2.5 mg/l. Among all, some induced roots whereas others induced shoots. Roots and shoots at the same time from the same explants were also observed. In this case a clear and regular picture of auxin cytokinin relation is established. Auxin alone induce roots whereas cytokinin alone induced shoots. In combination of auxin and cytokinin also the same rule was followed i.e. higher auxin vs lower cytokinin induced roots, lower auxin vs higher cytokinin induced shoots and an average of both induced both roots and shoots.

The media that induced roots mentioned in the results indicate that NAA above 0.5 mg/l either alone or in combinations with BAP at almost all levels are good for root differentiation. Similarly, MS medium with BAP above 0.5 mg/l either alone or in combinations with NAA induce shoot regeneration from the callus frequently. Ahmad *et al.,* (2002) observed a maximum of 6.4 shoots per culture with 81.25% frequency and 8.7 shoots per culture with 94.44 % frequency from the calli obtained from internode and leaf explants after 12 and 16 weeks respectively on MS + NAA 0.05 + BA 2.0. Sarker *et al.,* (1996) regenerated the shoots from the calli formed at cut bases of the explants on MS containing low concentration of BA (0.5 or 0.1 mg/l) and NAA (0.1 mg/l).

5.2.7. *In Vivo* Rooting and Acclematization:

The shoots rooted *in vitro* in all the media survived with an average of 80% on the coco peat substrate under glass or plastic shades. Similarly Baksha *et al.,* (2007) and Sudha and Seeni, (1996) achieved 80% success for rooting of *R. serpentina* and *R. micrantha* respectively whereas Singh *et al.,* (2009) achieved only 67% in *R. serpentina*. The acclimatization on sand soil mixture as a substrate for the *in vitro* rooted plants did not show a good result. The *in vitro* grown shoots were pulse treated with 100 mg/l IAA, IBA and NAA and a total of about 68% rooted after 2-3 weeks in pure sand and they were planted in the coco-peat for 2 months for hardening. In the similar manner Kataria and Sekhawat, (2005) pulse treated the *in vitro* grown shoots of *R. serpentina* with 50 µM each of IBA and NAA and got 98% rooting on the solrite moistened with ½ strength MS macrosalts and 80% hardening. Similarly, Mishra *et al.,* (2010) treated the shoots with NAA 50 µM for 24 hours achieved 100% rooting in pure sand within 30 days and final survival rate of 90% after 2 months of hardening. Patil and Jayanthi, (1997) however achieved only 60% rooting on *R. tetraphylla* and *R. micrantha* using IBA 100 mg/l. Among all the rooted shoots about 80% stabilized in the coco peat in the present experiment. The rooted shoots hardened on the sand soil mixture 50/50 after 2 months gave 60% survival. The decrease in survival rate from coco peat to sand and soil to pure soil might be because of the fragile nature of the *in vitro* plants and desiccation due to low humidity in an open environment.

CHAPTER- 6

CONCLUSION

6.1. Conclusion

From the overall study, we have come to a conclusion that the detailed studies in the plant resources are necessary. The plants that are well known are in a danger of extinction due to various reasons like habitat destruction, over exploitation and lack of conservation strategies. Helle and Olsen 2007 have reported that the medicinal plants are open acces resource in Nepal. Hence, it is essential to conserve them for the future generation in a proper way without losing their natural properties. For conservation, using plant tissue culture method might be the most suitable technique because it is easy and lots of plants can be produced in a short time and space without losing any property (true to type) and without damaging the mother plant . For the multiplication, the time and money requirement is high in the beginning but as the protocol is established, the cost can be greatly reduced. From the present study we have come to a conclusion that the easiest and fastest methods of multiplication of all the plants are possible. It is suggested to rapidly multiply the disappearing plant species (mainly over harvested medicinal and aromatic plants) *in vitro* and rehabilitate them in the forests to maintain the biodiversity as well for our future generations. From the present investigation, the whole experiment can be summarized in the following points:

Asparagus racemosus

- Multiple shoot induction from the seeds *in vitro* can be a good way of multiplying.
- Calli for various purposes can be induced from the nodes of this species using auxins specially NAA in MS medium.
- The tough friable calli induced from the nodes on high auxin containing media were capable of inducing somatic embryoids.
- Multiple shoots can be induced from the calli using low auxin and high cytokinin in the medium.

- Buds induced on different media are unique.
- Rooting was highly supported by NAA.
- Acclimatization and survival % of this plant was not very encouraging.

Rauvolfia serpentina

- Callus induction from the shoot and leaf explants were supported by higher 2,4-D or NAA (1.0 mg/l and 0.5 mg/l respectively).
- The calli from the leaf were not embryogenic and caulogenic.
- Somatic embryogenesis is the least studied part.
- The multiple shoot induction by low auxin+high cytokinin. The maximum induction was on IBA 0.1+BAP 2.0 mg/l (7.83±1.01).
- NAA at all conc. Induce roots significantly (0.5 mg/l=12.50 after 12 wks).
- Acclematization was successful.

Finally, the government and or the concerned authorities/agencies should come forward to conserve these species not only in the laboratories but also in the natual forests as well as in the farmers' fields. The protocols developed from the present research may be applied in the multiplication of similar medicinal as well as other important plants.

CHAPTER- 7

RECOMMENDATIONS

- A joint effort of scientic, governmental and local communities is essential for formulation and implementation of the conservation plans.
- Establishment of a well equipped laboratory is urgent.
- Young researchers should be encouraged to come in the field of conservation.
- Sustainable use of natural resources within the country is important.
- A detailed studies on somatic embryogenesis of these species is necessary
- Further studies regarding rapid multiplication through bud induction can be a great achievement.
- Detailed bio-chemical study of these species from Nepal is essential because the preliminary investigations have shown some symptoms to reveal new as well as useful compounds.
- The outcomes of the present research may be used to rapidly multiply the endangered medicinal plants.

CHAPTER- 8

SUMMARY

Asparagus racemosus willd. and *Rauvolfia serpentina* (L.) Benth. ex. Kurz. Belonging to the families Lilliaceae and Apocynaceae are among the most important natural resources of Nepal. Both these plants have high medicinal value and are decreasing from the natural forests due to unsustainable collection and destruction of their habitats due to various reasons. *R. serpentina* is listed in the "Appendix II" of the CITES and is under the category "E" of IUCN. Similarly, *A. racemosus* is facing towards its extinction due to its increasing demand in the local as well as international markets. Understanding these facts, the Government of Nepal has also prioratised these species for research in the areas of multiplication and cultivation. Considering these facts, the present research was undertaken to support the national mission of conservation through *ex- situ* way. The method of tissue culture was selected for the present study because this is one of the most promising methods of rapid plant propagation without damaging any plant in short time and in short space.

From the present study, we have come to a conclusion that the different explants of the same plant respond differently to the type of medium of culture. It has also been concluded that the most effective explant for the rapid multiplication these plant species using tissue culture technique is probably the shoot node. Calli induced from different explants especially from the shoot explants are suitable for organogenesis as well as somatic embryogenesis. Induction of buds from the shoot explants in *Asparagus racemosus* is another potential area where more works are necessary. In the overall experiments, NAA was found to be the most effective hormone among the auxins in all respects. Similarly, among the cytokinins tested, BAP was found to act better than Kinetin in most of the cases. Although acclimatization part is very difficult, it was successfully completed.

Asparagus racemosus, being a widely used medicinal plant, it is one of the least studied plants using different aspects of tissue culture. From the seed germination experiments of *Asparagus racemosus* on MS basal medium a single seed produced up to 13 shoots and the pre soaked seeds performed better than the non soaked ones. In the tissue culture experiments, the nodes produced more callus when cultured on the medium containing NAA alone in higher concentrations or when combined with BAP. Hence, NAA can be regarded as the best callus inducer for this species. The tough friable calli induced from the shoot explants were capable of inducing somatic embryoids. Generally, high auxin either singally or in combination with higher concentration of cytokinin produced large number of somatic embryoids from the calli. Organogenesis or caulogenesis was observed to be affected by the concentration of cytokinin in the media. It has been observed that the lower cytokinin concentration induce shoots whereas higher concentrations induce roots. In case of multiple shoot induction either a combination of low NAA (0.1 mg/l) and high Kn (1.0 or 2.0 mg/l) concentration or various combinations of IBA and BAP in the MS medium were promising. Similarly, for the root induction, NAA at low concentration (0.1 mg/l) was found to be the best although its other higher concentrations also induced roots at significant levels.

Agar manipulation experiment for the induction of storage roots produced insignificant result. Besides these, a preliminary study on bud induction from the shoots has shown very interesting results. This can be an alternative method of rapid multiplication of plants. Vitrification of shoots is one of the major problems in tissue culture and generally the vitrified shoots are discarded. Vitrified shoots were marked from the media containing high amount of cytokinin generally BAP. We here, have used the vitrified shoots as explants and produced normal shoots using very low amount of cytokinin or auxin in the sub-culture medium.Sand rooting using NAA 100 mg/l pulse treatment, acclimatizing in a shade house on the coco-peat and transferring the acclimatized plantlets on the gaden soil in an open environment were the final steps.

Another important and threatened medicinal plant *Rauvolfia serpentina* also was taken for the present research. Here, callus induction from the shoot and leaf explants

were supported by the incorporation of 2,4-D and NAA above 1.0 mg/l and 0.5 mg/l singally in the MS medium respectively. Incombination of auxin and cytokinin, NAA 1.0 mg/l along with all concentrations of BAP yielded highly significant amount of callus especially from the shoots. The calli obtained from the leaf were rarely embryogenic as well as caulogenic. The shoot induced friable green calli were able to go for organogenesis as well as somatic embryohenesis. The multiple shoot induction was generally supported by a lower auxin and higher cytokinin concentrations. The maximum induction was observed on the MS medium incorporated with IBA 0.1 + BAP 2.0 mg/l with 7.83±1.01 shoots per explant. For the induction of roots from the shoots, NAA singly at almost all concentrations were found to be significant, however NAA o.5 mg/l induced the maximum average roots upto 12.50 per explants after 12 weeks of culture. IAA and IBA did induce some roots when alone but in combination with cytokinins their performances were very poor. Finally, the shoots rooted both *in vitro* and *in vivo* after pulse treatment with 100 mg/l auxin survived in the open environment after acclimatization in the shade house.

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