Review of Literature
Pre-history man required several thousand years of great efforts to discover the nourishing qualities of plants and to properly collect them. Later, he started cultivating them once agriculture was created. Alongside the acknowledgement of the nutritional value of the plants, experience permitted him to identify the plants, and to discover other qualities related to improving the health for the sick. Subsequently, it was possible for him to identify the advantages of using some plants for the treatment of a given disease and also the hazardous, and even the mortal effects, or the psychoactive ones.

2.1. History of plants in medicine

The earliest known medical document is a 4000-year-old Sumerian clay tablet that recorded plant remedies for various illnesses. The ancient Egyptian medical document, Ebers papyrus from 3500 year ago lists hundreds of remedies. The Chinese medical document, Pen-T Sao Keng Mu contains thousands of herbal cures attributed to China's legendary emperor, Shen-nung, who lived 4500 years ago. In India, herbal medicine dates back several thousand years to the Rig-Veda. Western medicine can be traced back to the Greek physician Hippocrates, who believed that disease had natural causes and used various herbal remedies in his treatments. Early Roman writings also influenced the development of western medicine, especially the works of Dioscorides, who compiled information on more than 600 species of plants with medicinal value in De Materia Medica. Many of the herbal remedies used by the Greeks and Romans were effective treatments that have become incorporated into modern medicine (e.g., willow bark tea, the precursor to aspirin). Then on, Dioscorides' work remained the standard medical reference in most of Europe for the next 1500 years. The beginning of the Renaissance saw a revival of herbalism, the identification of medicinally useful plants. This coupled with the invention of the
printing press in 1450 ushered in the Age of Herbals. Many of the herbals were richly illustrated. All of them focused on the medicinal uses of plants, but also included much misinformation and superstition. The Doctrine of Signatures, for example, held that the medicinal use of plants could be ascertained by recognizing features of the plant that corresponded to human anatomy. For example, the red juice of bloodwort (Achillea millefolium) suggests that it should be used for blood disorders; the lobed appearance of liverworts suggests that it should be used to treat liver complaints; the "humanoid" form of mandrake root (Mandragora officinarum) suggests that it should be used to promote male virility and ensure conception. Many of the remedies employed by the herbalists provided effective treatments. Studies of foxglove (Digitalis purpurea) for the treatment of dropsy (congestive heart failure) set the standard for pharmaceutical chemistry. In the 19th century, scientists began purifying the active extracts from medicinal plants (e.g., the isolation of morphine from the Papaver somnifera). Advances in the field of pharmacology led to the formulation of the first purely synthetic drugs based on natural products in the middle of the 19th century. In 1839, for example, salicylic acid was identified as the active ingredient in a number of plants known for their pain-relieving qualities; salicylic acid was synthesized in 1853, eventually leading to the development of aspirin. It is estimated that 50% of prescriptions written in the U.S. contain plant-derived ingredients; an even greater percentage are based on semisynthetic or wholly synthetic ingredients originally isolated from plants. While Western medicine strayed away from herbalism, 75% to 90% of the rural population of the rest of the world still relies on herbal medicine as their only health care. In many village marketplaces, medicinal herbs are sold alongside vegetables and other wares. The People's Republic of China is the leading country for incorporating traditional herbal medicine into a modern health care system; the result is a blend of herbal medicine, acupuncture, and Western
medicine. Plantations exist in China for the cultivation of medicinal plants. Thus thousands of species are available for the Chinese herbalists. Their prescriptions are filled with measured amounts of specific herbs rather than with pills or ointments. In India, traditional systems have remained quite separate from Western medicine. In addition to Ayurvedic medicine, Unani medicine is another widely practiced herbal tradition in India. The famous Indian herbal therapy ‘Ayurveda’ is based mainly on herbal system.

2.2. Conservation of medicinal plants.

Traditional systems of medicine and modern pharmaceutical industries are hugely dependent on medicinal plants at their natural stands. This has led to over-exploitation of medicinal plants. Many species of medicinal plants have become rare, threatened or endangered due to over-exploitation, habitat loss and non-judicious use. In addition to this, the medicinal plants are highly affected by climate change, such as increase in carbon dioxide concentration which favours C3 plants over C4 plants, increase in diseases and pest, high rain fall and high salt content in soil etc. There are two methods of conservation of medicinal plants: (i) ex situ conservation, and (ii) in situ conservation, but these techniques are natural and time-consuming. Therefore, biotechnological techniques can be applied for the conservation of critical genotypes of medicinal plants. Biotechnological approaches are imperative for rapid multiplication and genetic improvement of medicinal plants.

2.3. Plant tissue culture

Each plant cell is the repository of all the information necessary for its subsequent growth into a multicellular, highly organized, complex but co-ordinated system. This ability of a plant cell to develop into a complete plant is called totipotency. The tiny totipotent cell conceals the potential for differentiation. The
concept of totipotency is an inherent part of the cell theory of Schleiden (1838) and Schawn (1839), is the basis for plant tissue culture. Later, Haberlandt during 1902 developed the concept of *in vitro* culture by culturing the cells of carrot stamen hairs, thus he has been regarded as the father of tissue culture. Haberlandt’s attempt to grow vegetative cells in an artificial medium did not succeed due to lack of proper techniques and unfortunate choice of highly specialized materials, but it opened up new vistas in morphogenesis. The formulation of nutrient medium played a vital role in the micropropagation. In subsequent years, different culture media for plant tissue culture were formulated by the work of Gautheret (1940), Hildebrandt (1946), Nitsch (1951), Reinert and White (1956), Murashige and Skoog (1962), Gamborg *et al.* (1968) etc. Among these various media formulations, Murashige and Skoog (1962) medium is the most used medium for culturing large number of medicinal plants. Then the discovery of auxins and cytokinins played a very important role in the plant tissue culture. Skoog and Miller (1957) revealed the hormonal control of shoot and root regeneration from tobacco callus and established the basis for manipulating organ initiation and provided the principle on which micropropagation depends.

2.3.1. Micropropagation

Propagation of plants using tissue culture technology is called micropropagation. Micropropagation techniques have been employed early especially for the propagation of economically important ornamental and foliage plants, while micropropagation of medicinal plants is also getting increased interest in the present era. The problems associated with conventional seed propagation and vegetative methods can be overcome to a great extent by micropropagation. During the last few years there has been considerable emphasis in propagating medicinal plants through *in vitro* culture techniques (Prakash *et al.*, 1999; Komalavalli and Rao, 2000; Tang
2000; Prabhakar et al., 2001; Tyagi and Prakash 2001; Choi et al., 2002). Presently 20% of the medicinal plants are propagated though this technique (Rajendra and D’Souza, 1999). Hence, the micropropagation has become a useful tool to produce elite genotypes with higher quantity of an active ingredient in the plant parts.

2.3.1.1. Explant

Tissue cultures are started from pieces of whole plants called explants (George, 1993). Choice of explant plays an important role in determining the efficiency of propagation (Abbasi et al., 2007). The part of the plant (stock plant) from which explants are obtained depends on the kind of the culture to be initiated, the purpose of the culture and the plant species to be used. The influence of plant material or explants on growth and development depends on several factors including genotype, the age of explants, size of the explants and the method of inoculation. Dicotyledons regenerate better than monocotyledons, and gymnosperms have very limited regenerative capacity except when juvenile. In woody plants juvenile tissues, stump sprouts, sprouts from pruned trees, zygotic embryos or seedling parts (epicotyls, hypocotyls and cotyledon) are the best explants if regeneration of plants is to be achieved. Very small structures such as cells, clumps of cells and meristems are much more difficult to induce growth than larger structure such as leaf, stem or tuber explants (Rout et al., 2000). Basically, explants used in the micropropagation of medicinal plants are leaf, shoot tips (Hussein et al., 2005; Martin et al., 2006) and buds (Vincent et al., 1992), apical meristems (Sahoo et al., 1997). Saba et al. (1999) reported use of nodal segments in *Ammi majus* L., nodal explants of *Bacopa monnieri* were propagated in vitro using liquid cultures (Tiwari et al., 2000). Shoot tip, nodal and internodal segments were used for regeneration of *Phyllanthus amarus* (Ghanti et al, 2004). The morphogenetic potential of node, internode and leaf explants of
Bacopa monniera (L.) Wettst. was investigated to develop reliable protocols for shoot regeneration and somatic embryogenesis (Tiwari et al., 1998). Regeneration from callus cultures of Centella asiatica obtained from stem and leaf explants from greenhouse grown mother plant (Patra and Rai, 1998). Regeneration through cotyledonary node was reported in Commiphora wightii (Tarun et al., 2010). Dhar and Joshi (2005) found leaf explants better for callus mediated shoot regeneration when they tested the influence of various explants like root, hypocotyl, cotyledon and leaf on the plant regeneration through callus in Saussurea obvallata. Both organogenesis and somatic embryogenesis was successfully induced in Semecarpus anacardium L. using cotyledonary explants (Panda and Hazra, 2012).

2.3.1.2. Growth regulators

Plant hormones are relatively small molecules that are effective at low tissue concentrations. At present, five groups of plant growth regulators, auxin, cytokinin, gibberellin, abscisic acid and ethylene are regarded as plant hormones. Cytokinins produce two immediate effects on undifferentiated cells: the stimulation of DNA synthesis and increased cell division. Although low tissue concentrations of cytokinins have noticeable effects, higher concentrations are found in actively dividing tissues such as those of plant embryos and developing fruits. Auxins stimulate cell expansion, particularly cell elongation they also promote xylem and phloem differentiation, adventitious root development, parthenocarpy and inhibit bud formation (Takahashi, 1986). The gibberellins stimulate growth of organs but generally do not favour organ initiation (Rout et al., 2000). Plant hormones do not function in isolation within the plant body instead they function in relation to each other. Hormone balance is apparently more important than the absolute concentration of any one hormone. Both cell division and cell expansion occur in actively dividing tissue,
therefore cytokinin and auxin balance plays a role in the overall growth of plant tissue. Since hormone balance is presumably important to the overall effect on growth and morphological changes, then the hormone differentials in each of the experimental media should produce somewhat different effects on the growth and development of excised explants. Many reports on micropropagation indicate that cytokinins are prime important in promoting direct shoot initiation or multiplication of various explants. Auxins such as IAA, IBA and NAA rarely stimulate shoot induction. NAA in *Linum usitassimum* (Bretagne et al., 1996), IBA and 2,4-D in *Sesbania aculeata* (Bansal and Pandey, 1993) has induced the shoots. The effectiveness of NAA in rooting of in vitro regenerated shoots is well documented in plants like *Cucumis sativus* (Ahmad and Anis, 2005). Among the cytokinins, BAP is most widely used for *in vitro* shoot regeneration. (Harris et al., 1989; Juliani (Jr) et al., 1999; Fracaro and Echeverrigaray 2001; Salvi et al., 2001; Tiwari et al., 2001; Dias et al., 2002). The superiority of BAP over other cytokinins in a tissue culture system has been well demonstrated in a number of previous studies using variety of explants like *Adhatoda vasica* (Abhyankar and Reddy, 2007); *Gynura procumbens* (Keng et al., 2009), *Vitex negundo* (Ahmad and Anis, 2010). The naturally occurring ribosides and nucleotides in the BAP are relatively more stable in comparison to other cytokinins (Letham and Palni, 1983). A comparison of relative effectiveness of different cytokinins for multiple shoot formation in *Withania somnifera* revealed the order of effectiveness as BAP>KN> 2-iP (Fatima and Anis, 2012). Reduction in shoot number at higher than the optimal level has been reported in various medicinal plants including *Ruta graveolens* (Faisal et al., 2005); *Eclipta alba* (Hussain and Anis, 2006). Combinations of cytokinins were also reported to be effective for the induction or multiplication of shoots (Komalavalli and Rao 1997, 2000). Barna and Whaklu (1988) indicated that the production of multiple shoots was higher in *Plantago ovata*.
on a medium having 4-6 μM kinetin along with 0.5 μM NAA. A threefold increase in shoot growth was achieved in *Gentiana kurroo* with 1.1 μM NAA and 8.9 μM BAP (Sharma et al., 1993). Mehra and Cheema (1980) reported multiple shoots (75 – 80 per culture) or immature lamina discs of female *Populus ciliata* on MS medium with optimal amount of BAP in dark. TDZ has been used to micropropagate a wide array of woody species because of its great ability to stimulate shoot proliferation and regeneration (Hurtteman and Preece, 1993; Lu, 1993). TDZ induced the organogenesis in tamarind (Mehta et al., 2004), *Camellia sinensis* (Mondal et al., 1996). In *Acacia sinuata* GA₃ induced shoot elongation (Vengadesan et al., 2000).

2.3.1.3. Organogenesis

Organogenesis during *in vitro* culture may be direct or through the intervention of callus. Direct organogenesis results in the formation of shoot buds, which may further elongate to form the multiple shoots that phenotypically and genotypically resemble the mother plant. Direct shoot organogenesis from primary tissue is more desirable than indirect organogenesis (Larkin and Scowcroft 1981; George et al. 1993; Jasraj et al., 1999; Shirin et al., 2000; Sivakumar and Krishnamurthy 2000) and it has been reported in many medicinal plants from various explants especially from shoot tips or nodal explants. Explants such as leaf, root etc. have also been used for direct shoot regeneration of many medicinal plants (Harris et al., 1989; Wawrosch et al., 1999; Pereira et al., 2000; Salvi et al., 2001; Das and Rout 2002). Chen et al., (2001) reported adventitious shoot formation from the stem internode explants of *Adenophora triphylla*-an important medicinal plant. Kantia and Kothari (2002) reported adventitious shoot bud formation directly on the surface of the leaf explants in *Dianthus chinesis*. Koroch et al. (2002) reported multiple shoot regeneration and Agrobacterium mediated transformation of *Echinacea purpurea* leaf
explants. Martin et al., (2003) reported direct shoot regeneration from lamina explants of two commercial cut flower cultivars of Anthurium andraeanum. Uddin et al. (2005) established a protocol for rapid multiplication of shoots from cotyledonary node of Peltophorum sps. Indirect organogenesis results in somaclonal variations, hence the method is less desirable for large scale clonal propagation (Thorpe et al., 1991). However, the variations may be useful for crop improvement (George 1993; Suryanarayan and Pai, 1998). Indirect organogenesis and subsequent micropropagation have been reported in many medicinal plants, Mentha arvensis (Shasany et al., 1998), Tulipa gesneriana (Famelaer et al., 1996), Psoralea corylifolia (Saxena et al., 1997), Typhonium trilobatum (Das et al., 1997), Saussurea obvallata (Dhar and Joshi, 2005), Semecarpus anacardium (Panda et al., 2011)

2.3.1.4. Somatic embryogenesis

Somatic embryogenesis is the development of embryos from somatic cells without going through a sexual cycle. The process is developmentally similar to zygotic embryogenesis, except that it occurs in the artificial environment of a culture vessel. The process was first described in Daucus carota by Reneirt (1958) and Steward et al. (1958). Sandal wood (Santalum album) was the first angiospermic tree species to show somatic embryogenesis. Cloning of trees through somatic embryogenesis is an important technology for commercial mass propagation of improved genotypes (Gupta et al., 1993; Sutton and Polonenko, 1999). In addition to providing uniform stocks of selected genotypes for direct use in planting (synthetic seed technology), these cultures are valuable tools for testing: (a) the resistance of clonal material to pathogens and environmental stress; (b) virus elimination; (c) metabolite production; and (d) in vitro mycorrhizal initiation (Vicient and Martinez, 1998). Whereas zygotic embryos develop under a highly restrictive environment of the maternal tissue, somatic embryo development can be affected dramatically by
changes in the physical and chemical environment of the medium (Goldberg et al., 1989; Carman, 1990). Increased osmolarity can affect the efficiency of induction as well as maturation of somatic embryos. Addition of various sugars and polyethylene glycol (PEG) has been shown to promote somatic embryo maturation in both angiosperms and gymnosperms (Brown et al., 1989; Attree et al., 1991; Tautorus et al., 1991). Plant regeneration via somatic embryogenesis has been reported in *Aesculus hippocastanum* (Gastaldo et al., 1996), *Gymnema sylvestre* (Kumar et al., 2002), *Punica granatum* (Nataraja and Neelambika, 1996). Plant regeneration via somatic embryogenesis is the *in vitro* process used to reduce multiplication time and potentially offers an efficient system for mass propagation (Redenbaugh et al., 1987).

Somatic embryogenesis may be either direct or indirect. Direct somatic embryogenesis has been reported in many medicinal plants like *Calliandra tweedi* (Kumar et al., 2002), *Camellia japonica* (Pedroso and Pais, 1995), *Trachyspermum ammi* (Seghal and Abbas, 1994), Ginseng (Choi et al., 1998, 1999) etc. In direct somatic embryogenesis pre-embryogenic determined cells develop into somatic embryos directly (Konar and Nataraj, 1965) and greater genetic and cytological fidelity is associated with this process (Binsfield et al., 1999; Choi et al., 1999; Iantcheva et al., 1999). Direct somatic embryogenesis and plant regeneration was achieved in *Garcinia indica* by Thengane et al. (2006) from immature seeds on woody plant medium supplemented with BAP alone or in combination with NAA. Plant regeneration via somatic embryogenesis is also reported in *Piper colubrinum* (Yusuf et al., 2001), Japanese larch (Kim et al., 1999), *Lyceum barbarum* (Hu et al., 2008), *Musa acuminata* (Wang et al., 2007) and *Guizotia abyssinica* (Naik and Murthy, 2010).

Indirect embryogenesis requires dedifferentiation and acquisition of embryonic state (Sharp et al., 1982) and it has been reported in many medicinal plants.
like *Panax ginseng* (Tang, 2000), *Cuminum cyminium* (Tawfik and Noga, 2002), *Thevetia peruviana* (Kumar, 1992), *Gymnema sylvestre* (Kumar *et al*., 2002), *Simmondsia chinensis* (Hamama *et al*., 2001) *Eschscholzia californica* (Park and Facchini, 1999), *Eleutherococcus senticosus* (Choi *et al*., 2002), *Cymbopogon pendulus* (Bhattacharya *et al*., 2010). MS is the most widely used medium for somatic embryogenesis (Tsay and Huang, 1998; Choi *et al*., 1998). Growth regulators in the media influence the embryonic response of cultured cells. Among the different auxins, 2,4-D has been widely used for somatic embryogenic calli (Patil 1998; Wakhulum and Sharma, 1998; Kitamia *et al*., 2000; Kim *et al*., 2000; Choi *et al*., 2002). However, other auxins also have been reported to be effective for somatic embryogenesis of many medicinal plants (Hiraoka *et al*., 2001; Monteiro *et al*., 2002). Synergic effect of auxin-cytokinin interaction has also been reported for the induction of somatic embryogenesis in many medicinal plants (Hamama *et al*., 2001; Gallo-Meagher and Green, 2002; Kumar *et al*., 2002; Tawfik and Noga, 2002). In most of the reports indirect somatic embryogenesis involves induction of callus from suitable explants in a medium rich with or without cytokinin and differentiation of somatic embryos upon transfer of these calli into a medium containing relatively low auxin or hormone free medium. Maturation and germination of somatic embryos occur on transfer of embryos to the medium without growth regulators (Rout *et al*., 1995, 2001; Kumar, 1992) or with low levels of growth regulators (Sehgal and Abbas, 1994; Sinha *et al*., 2000; Kumar *et al*., 2002).

2.3.1.5. Synthetic seeds

Synthetic seeds are artificially encapsulated somatic embryos, shoot buds, cell aggregates or any other tissue that can be used for sowing as a seed and that possess the ability to convert into a plant under *in vitro* or *ex vitro* conditions, which retain the potential also after storage (Ara *et al*., 2000). Artificial seeds were first developed and

2.3.1.6. Liquid cultures

The micropropagation technique has certain demerits which have limited the use and exploitation of this technique at industrial level. The major limitation is the higher cost of plant production. Hence, the most challenging aspect at present is to reduce the production cost, thereby improving the production efficiency (Anderson and Meagher, 1977; Sluis and Walker, 1985; Donnan, 1986; Levin and Vasil, 1989; Aitken-Christie, 1991). Therefore, to overcome this limitation, a number of cost reduction strategies have now been developed. The use of shake cultures utilizing liquid medium alone (Weathers and Giles, 1988) or in combination with solid culture medium (Debergh and Mane, 1981; Aitken-Christie and Jones, 1987) have been developed and used by various workers (Earle and Langhans, 1975; Takayama and Misawa, 1981; Takayama, 1991; Paque *et al.*, 1992; Chu *et al.*, 1993). Liquid cultures are basically used for plant cells, somatic embryos and organ cultures, both in agitated flask and bioreactors (Smart and Fowler, 1984; Attree *et al.*, 1994; Tatorus and Dunstan, 1995), the liquid medium has been relatively less used for the purpose of micropropagation, however certain merits of this technique are helpful in proving it now as one of the important methods for cost reduction during micropropagation. In liquid medium, the close contact of the tissue with the medium may stimulate and facilitate the uptake of nutrients and phytohromones, leading to better shoot and root
growth (Ziv, 1989; Smith and Spomer, 1994; Sandal et al., 2001). The disappearance or lesser expression of activity of apical dominance due to continuous shaking condition of the tissues in the medium is another important feature of liquid cultures, which generally leads to the induction and proliferation of numerous axillary buds. This leads to the development of bud clusters which are amenable to the control of medium components, to mechanical separation and to automated inoculation as an efficient delivery system to the final stage for plant growth (Levin et al., 1997; Ziv et al., 1998). The formation of condensed organized structures in which the shoots are reduced to buds/meristematic tissue in liquid media has been reported for several plant species. These clusters are made up of densely packed meristematic cells, actively dividing and forming new meristemoids on outer surface (Ziv et al., 1998, 1990; Young et al., 2000). Promotion of larger number of axillary bud development favours the production of large number of plants which are more or less true-to-type (Takayama and Misawa, 1981; Harris and Mason, 1983; Douglas, 1984; Pierik, 1987; Chu et al., 1993) Furthermore, within the shake culture conditions, the growth and multiplication rate of the shoots is enhanced by forced aeration, since continuous shaking of the medium provides sufficient oxygen supply to the tissue, which ultimately leads to their faster growth. In addition to these advantages, the preparation of liquid medium and handling of shake cultures is easier as compared to the semi-solid one. On the other hand, vitrification in the tissues is the common disadvantage in plant tissues during their in vitro propagation using liquid medium (Kevers et al., 1984; Gasper et al., 1987; Debergh et al., 1992). The tissue culture environment has large negative osmotic potential, high sucrose and ammonium content, high relative humidity and unusual plant growth regulators compared to field conditions. While some plants can adapt to these in vitro conditions, some show abnormal characteristics, such as translucent appearance, chlorophyll deficiency, and high water content. These symptoms were originally called ‘vitrification’ (Franck et al., 2004),
but now this physiological disorder is named as ‘hyperhydricity’ (Ziv, 1995). Leaves of hyperhydric shoots are curled, elongated, wrinkled and brittle (Saheer et al., 2004). Hyperhydricity is often described related to water relations between the culture environment and developing shoot. Hyperhydricity frequently affects herbaceous as well as woody shoots during their in vitro vegetative propagation (Kevers et al., 2004). The culture medium is the cause of vitrification of tissues. There are many evidences, which suggest that the reduction of agar concentration or its absence in the culture medium causes vitrification during the growth of the tissue (Hakkaart and Versluijs, 1983; John, 1986; Densco, 1987; Kevers et al., 1987; Bottcher et al., 1988).

A number of studies have been undertaken suggesting that not all the plant species show vitrification when cultured in liquid medium. In some culture systems where the problem of asphyxiation exists, it can be avoided by taking advent-age of the surface tension and floating explants, so that developing tissue do not get submerged in the liquid medium. (Debergh et al., 1981; Leshem, 1983; Skidmore et al., 1988; Dillen and Buysens, 1989)

2.4. Accumulation of secondary metabolites in cell and organ cultures

Certain chemical substances present in the plants are not directly concerned with their primary metabolic process are usually termed as secondary metabolites or secondary products. The disease curing property of the medicinal plants is found to be due to the presence of the secondary metabolites. Since the middle of the century there had been several reports representing the presence or accumulation of secondary metabolites in plant cell cultures or micropropagated plants. Discoveries of tissue cultures capable of producing specific medicinal compounds at a rate similar or superior to that of intact plants have accelerated in the last few years. New physiologically active substances of medicinal interest have been found by bioassay. It has been demonstrated that the biosynthetic activity of cultured cells and tissues can
be enhanced by regulating environmental factors, as well as by artificial selection or the induction of variant clones. Some of the medicinal compounds localized in morphologically specialized tissues or organs of native plants have been produced in culture systems not only by inducing specific organized cultures, but also by undifferentiated cell cultures. In 1989, Christen et al. reported for the first time the production of taxol by Taxus cell cultures. A biotechnological process has been developed for the production of capsaicin from Capsicum frutescens cells (Lindsey et al., 1983). The possible use of plant cell and tissue cultures for the specific biotransformations of natural compounds has been demonstrated (Cheetham, 1995; Scragg, 1997; Krings and Berger, 1998; Ravishankar and Rao, 2000). The production of secondary metabolites is generally higher in differentiated tissues (organs) and attempts have been made to cultivate shoot cultures and root cultures for the production of medicinally important compounds. These organ cultures are relatively more stable. Due to these advances, research in the area of tissue culture technology for the production of plant metabolites has bloomed beyond expectations.

2.5. Molecular techniques for testing genetic fidelity of regenerants

Molecular techniques are at present powerful and valuable tools used in analysis of genetic fidelity of in vitro propagated plants. It has been reported that in vitro cultured plantlets might exhibit somaclonal variations (D’Amato, 1978; Skirvin, 1978; Larkin and Scowcroft, 1981; Earle and Demarly, 1982; Rani et al., 1995; Hashmi et al., 1997). This variation is often heritable (Larkin et al., 1984; Breiman et al., 1987) and is therefore unwanted in clonal propagation. Many workers have tried to assess these variations in various plant species through morphological, biochemical and molecular analyses (Patel and Berlyn 1982; Renfroe and Berlyn 1984; Mo et al., 1989; Berlyn et al., 1987; Sabir et al., 1992). Genetic molecular markers are considered to be reliable in monitoring variability in the DNA sequences
of plants. Several authors have applied the Random amplified length polymorphism (RAPD) technique to investigate genetic variability and found it very efficient and reliable (Brown et al., 1993; Munthali et al., 1996)

2.6. *Nothapodytes nimmoniana* Graham

*Nothapodytes nimmoniana* is a small, spreading, sub-canopy tree distributed in many parts of the Western Ghats, South India, some parts of the Himalayan foot hills, Sri Lanka, Myanmar and Thailand (Suhas et al., 2007). This tree is rich source of camptothecin, isoquinoline alkaloid, which is being used for treating colorectal and ovarian cancer (Lilenbaum et al., 1995; Romanelli et al., 1998; Vladu et al., 2000). It is believed that camptothecin is the third most important alkaloid sought after by the pharmaceutical companies around the world. Perhaps this has led to the large scale exploitation of the species from its wild habitat in the recent years, despite the ban on green felling (Verma and Indira, 1998). This has led to decrease in the population of this species in the Western Ghats, India, and, in fact due to the extremely high pressure the species has been declared as endangered (Suhas et al., 2007). An estimated 20% of the population of this species is believed to have declined over the last decade. The tree growth is very slow, and propagation is usually achieved by seeds. The seeds are recalcitrant due to high sensitivity to dessication and freezing, and have a short shelf life.

Camptothecin, a monoterpane indole alkaloid is a promising plant based metabolite known for its antitumour activity (Wall et al., 1966; Cragg et al., 1999; Yan et al., 2003; Nalawade et al., 2003), Irinotecan and topotecan, two water soluble derivatives of camptothecin, have been approved by the Food and drug Administration of the United states of America for treating colorectal and ovarian cancers. Camptothecin has been shown to be an effective drug in the new direction of
AIDS chemotherapy (Priel et al., 1991) and in treatment of lung and breast cancer (Takeuchi et al., 1991), uterine cervical cancer (Potomesil, 1994). Bodley et al., (1998) reported the molecular and cytotoxic effects of camptothecin on Plasmodium falciparum. Comparative studies carried out by Roja (2006) on the camptothecin content from the indigenous plants namely Notapodytes nimmoniana, Ophiorhiza mungos and O. rugosa indicated highest yields of camptothecin and 9-methoxy camptothecin in N. nimmoniana. The other two plants Ophiorhiza mungos and O. rugosa contained low levels of alkaloids. Wu et al. (2008) reported camptothecinoids, including two new ones, 9-methoxy-18,19-dehydrocamptothecin and hydroxymappicine-20-O-β-glycopyranoside, have been identified and quantified by HPLC, in different parts of the N. nimmoniana, including mature and immature seeds, leaves, stems, and roots. The structures of two new camptothecinoids were elucidated by spectroscopic analyses and cytotoxicity of camptothecinoids toward six cancer cell lines was investigated. Fulzele and Satdive (2005) analysed distribution of camptothecin in mature and immature seeds and found the accumulation of highest concentration of camptothecin and 9-methoxycamptothecin in the cotyledons of immature seeds. Amna et al. (2006) detected the camptothecin by gradient reverse phase HPLC method with diode array and quantified by MS/MS detection from the extract of the fungus Entrophospora infrequens isolated from the inner bark of N. nimmoniana. Wu et al. (1995) isolated two new alkaloids, nothapodytines A and B, from stems of this species. Rehman et al. (2008) isolated endophyte (Neurospora crassa) and presence of camptothecin was confirmed by chromatographic and spectroscopic methods in comparison with authentic camptothecin. Li et al. (2005) analysed camptothecin derivatives and trigonelline using H-NMR method in N. nimmoniana root, stem and leaves. Pan et al. (2004) studied the effects of eight microelements (I, BO$_3$$^-$, MoO$_4$$^{2-}$, Co$_2$$^{3+}$, Cu$^{2+}$, Mn$^{2+}$, Fe$^{3+}$, Zn$^{2+}$) on the biosynthesis of
camptothecin and the growth of suspension cultures of *Camptotheca acuminata*.

Lorence and Nessler (2004) described the distribution of camptothecin and its metabolites, the present understanding of its mechanism of action, the biosynthetic route leading to camptothecin formation in plants with emphasis on the enzymes/gens involved in the pathway. Srinivas and Das (2003) isolated 9-methoxy-20-O-acetylcamptothecin from a natural source. Pan *et al.* (2004) studied the effects improvement in growth and camptothecin yield by altering nitrogen source supply in cell suspension cultures of *Camptotheca acuminata*.

2.6.1. *In vitro* studies of *Nothapodytes nimmoniana* Graham.

Thengane *et al.* (2001) reported the effect of thidiazuron on adventitious shoot regeneration from seedling explants like leaf, hypocotyls and cotyledon. Multiple shoot regeneration from the hypocotyl explants was reported by Rai (2002). Fulzele and Satdive (2003) reported somatic embryogenesis, plant regeneration and evaluation of camptothecin from the zygotic embryos. Further, there are only few reports on the production of camptothecin from the *in vitro* cultures, Ciddi and Shuler (2000) established callus cultures from excised embryos and determined the concentration of camptothecin and 9-methoxycamptothecin by TLC, UV, HPLC and electron spray mass spectral analysis. Thengane *et al.* (2003) analysed the concentration of camptothecin and 9-methoxycamptothecin by HPLC in relation to the media composition in callus cultures derived from cotyledons. Sundarvelan *et al.* (2004) reported the production of camptothecines from callus cultures derived from the leaves. However calluses and cell suspensions were found to produce small amounts of camptothecin and 9-methoxy camptothecin, but again the level of alkaloid production was 100 to 1000 fold lower than that from the soil grown plants. Singh *et al.* (2010) carried out studies on analysis of camptothecin by RP-HPLC from different
parts of both in vivo and in vitro plants of *N. nimmoniana* and found out maximum camptothecin content in the seeds followed by dried leaf samples. Seed coat recorded least camptothecin content. Hsiao *et al.* (2008) developed a rapid capillary electrophoresis procedure for determining the camptothecins from *N. nimmoniana*. Chandrika *et al.* (2010) used DNA based, inter simple sequence repeats (ISSR) to monitor genetic stability in micropropagated plantlets of *N. nimmoniana*. The banding pattern was uniform and comparable to mother plant from which the culture had been established.

2.7. *Andrographis paniculata* Nees

*Andrographis paniculata* Nees, is a small, erect branched and herbaceous to semi woody annual plant, known as “King of bitter”, is widely distributed in tropical Asia; in plains of Uttar Pradesh, West Bengal, Karnataka, Assam, Kerala and many parts of South India. *Andrographis* is seed propagated medicinal plant. *Andrographis paniculata* is an ideal plant for prophylactic and therapeutic hepatoprotective herbal preparations. This plant possesses astringent and alexipharmic properties and is used against intermittent fevers, dysentery, diabetes, influenza, bronchitis, swellings, piles and gonorrhoea. Leaves and roots have febrifuge, tonic, alterative and cholagogue action (Kurian and Sankar, 2007). Inflammation plays an important role in the pathogenesis of several neurodegenerative diseases including Parkinson’s disease. Andrographolide has been reported to possess an anti-inflammatory effect in vitro by modulating macrophage and neurotrophin activity (Sheeja *et al.*, 2006). Madav *et al.* (1995) in their studies on immuno modulatory effect of andrographolide confirmed its anti allergic activity. Herbal preparations containing *Andrographis paniculata* lowered levels of serum total cholesterol, phospholipids and triglycerides as reported by Kar *et al.* (2000). Zhang *et al.* (2000) observed the ethanolic extracts of aerial parts of *Andrographis paniculata* suppressed elevated levels of glucose in normal and
diabetic rats. Extracts of *Andrographis paniculata* exhibited significant alteration in behavior pattern and reduction in spontaneous motility (Mandal *et al.*, 2001). Trivedi and Rawal (2001) in their studies confirmed the antioxidant action andrographolide, wherein activities of antioxidant enzymes like catalase, superoxide dismutase and glutathione peroxidase decreased. Upadhyay (2001) observed the drugs containing *Andrographis paniculata* have cell membrane stabilizing property that prevents toxic effects of bile salts in various hepatic disorders. The extract is reported to cure thread worm infestation in children (Bondya *et al.*, 2002). Antimicrobial activity of the aqueous extracts of *Andrographis paniculata* was reported by Singha *et al.* (2003). Ethanolic extracts of *Andrographis paniculata* showed considerable inhibitory activity against *Micrococcus luteus*, *Micrococcus roseus* and *Staphylococcus aureus* (Ram *et al.*, 2004). Xu *et al.* (2006) reported the anti malarial activity of *Andrographis paniculata*. Roy *et al.* (2009) reported that chloroform extract of *Andrographis paniculata* showed anti microbial activity against pathogenic clinical strains of bacteria. Andrographolide has been reported for its anti cancer (Sheeja and Kuttan, 2007), anti HIV (Nanduri *et al.*, 2003) and cardio protective (Yoopan *et al.*, 2007) properties. Previous investigations on the chemical composition of *Andrographis paniculata* showed that it is rich source of diterpenoids and 2-oxygenated flavonoids including andrographolide, neoandrographolide, 14-deoxy-11, 12-didehydroandrographolide, 14-deoxyandrographolide, 19-beta-D-glucoside, homoandrographolide, andrographan, andrographosterin and stigmasterol (Pramanick *et al.*, 2006; Jarukamjom and Nemato, 2008). The primary bioactive component of *Andrographis paniculata* is andrographolide (Siripong *et al.*, 1992), which is a colourless diterpene lactone insoluble in water. Andrographolide content varied among plant parts and different accessions. Farooqi *et al.* (1999) reported a maximum of 2.5% andrographolide in leaves and minimum of 2% in stem. Rajani *et al.* (2000) reported a simple method for isolation of andrographolide from the leaves of
Andrographis paniculata. Saxena et al. (2000) developed a rapid and simple HPTLC method for simultaneous quantitative estimation of andrographolide, neoandrographolide and 14-deoxy-11, 12-didehydroandrographolide from leaves of Andrographis paniculata. A reverse phase HPLC method was developed by Li and Fitzloff (2002) for determination of andrographolide in commercial Andrographis products. Pan et al. (2003) studied the effect of pressure and temperature on extracting and crystallizing andrographolide by using super critical carbon dioxide. Besides the diterpene lactones, Polyphenols, caffeic, chlorogenic acids, tannins and saponins were also isolated from Andrographis paniculata. α-sitosterol was reported to occur in Andrographis paniculata roots by Govindachari et al. (1969).

2.7.1. In vitro studies of Andrographis paniculata

Martin (2004) reported in vitro regeneration of Andrographis paniculata through somatic embryogenesis. Purkayastha et al. (2008) reported a method for high frequency direct shoot regeneration from nodal explants of Andrographis paniculata. Praveen et al. (2009) successfully induced adventitious roots from the leaf explants of Andrographis paniculata for the production of andrographolide. Gangopadhyay et al. (2002) reported multiplication and rooting through use of coir in liquid culture media in ten plant species including Andrographis paniculata. Higher number of roots with profuse root hairs were recorded in all the plant materials in liquid medium with coir compared to conventional agar gelled media.

2.8. Spilanthes oleracea L.

Spilanthes oleracea is an important medicinal herb, native to South America, occurring in tropical and sub tropical parts of the world. Fresh leaves are eaten for their medicinal properties or used by the cosmetic industry for their spilol contents. The flowers and leaves have a pungent taste accompanied by tingling and numbness, and have been used as a spice for appetizers and as folk medicine for stammering,
toothache and stomatitis. (Tiwari et al. 2011). The plant shows antioxidant, anti-inflammatory, antipyretic and hepatoprotective properties (Prachayasittikul et al., 2009). The antimicrobial activity of this species is due to the presence of a highly valuable biologically active compound spilanthol and scopoletin.

Scopoletin is one of the major compounds of S. oleracea. Scopoletin is 6-methoxy-7-hydroxycoumarin. It possesses interesting activities, in particular, vasorelaxant, antimicrobial, anti-inflammatory, antipyretic, antiplatelet aggregation and antidiabetic properties. In addition, it exerts neuroprotective and hypotensive activities in addition to applications in cardiovascular disease, antitumor, antiproliferation and antithyroid treatment. Several studies have shown that scopoletin has hepatoprotective activity (Kang et al., 1998), antioxidant activity (Shaw et al., 2003), spasmylytic action (Oliveira et al., 2001) and the inhibitory effect of nitric oxide synthesis (Kang et al., 1999). Scopoletin was also shown to inhibit the cell proliferation by inducing cell cycle arrest and increase apoptosis in PC 3 cells i.e. human androgen-independent prostate adenocarcinoma cell (Liu et al., 2001). Furthermore, scopoletin isolated from Micromelum integerrium exhibited antitumoral activity on p-388 lymphocytic leukaemia (Cassady et al., 1979). Immunomodulatory effects were reported for a traditional Hawaiian herb, Morinda citrifolia, rich in scopoletin, which was found to enhance the host immune system involving macrophages and lymphocytes (Hirazumi et al., 1994). This important compound has been isolated from in vitro plantlets of S. acmella by Singh and Chaturvedi, (2011). There are no reports on the isolation of this compound from S. oleracea. Prachayasittikul et al. (2009) reported various bioactive metabolites from S. acmella. Greger et al. (1985) reported new amides from the flower heads and roots of S. oleracea. Maosso (2008) reported the multiple shoot regeneration from nodal explants in Spilanthes oleracea.
2.8.1. *In vitro* studies of *Spilanthes*.