Chapter - 1

Introduction

No plant on earth is without any application. From the time immortal, plants have been used as a source of drugs for the benefit of mankind.

The knowledge of medicines comes to modern pharma industries from the ancient systems of medicine from various parts of world. India, in particular has large number of diverse medicinally important plants mentioned in the indigenous system of medicine. ‘The Ayurveda’, which means the science of life; enlists and explain the role of medicinal plants in the treatment of diseases for thousands of years. The Chinese traditional system of medicine has also been curing people suffering from various ailments and feeding drug industry for the development of new drugs. As a result of these important leads from great traditions from the corners of world, out of 252 drugs considered as basic and essential by WTO, 118 are exclusively from plant origin (Rates, 2001).

However, in the recent years, discoveries are being made to find out alternative therapeutic use of natural product or newer sources are being searched in light of fact that the existing sources are not sufficient and synthetic drugs have proved inefficient in terms of side effects and price.

Biotechnology, known as; the application of scientific and engineering principles to the processing of materials by biological agents (Gavrilescu and Chisti, 2005) offer opportunity to exploit plant parts to get desired products by growing them in vitro.

Bioactive compounds from plants not only help plants for adaptation to their environment but provide important source of pharmaceuticals (Ramachandra Rao and Ravishankar, 2002) and defence against pathogens and predators (Bennet and Wallsgrove, 1994).

The industrial production of bioactive plant metabolites using plant tissue culture techniques can be exploited as alternative to traditional agricultural practices (Ramachandra Rao and Ravishankar, 2002).

Large scale cultures of plant cells are considered as potential source of secondary products. Tissue cultures on large are convenient and reliable in terms of
secondary metabolites production as cell cultures are free from fluctuations resulting from supply of raw plant material (Collin, 2001).

Pharmaceutical industries are facing increasing difficulties in securing an ample supply of medicinal plants because of significant decrease in plant resources (Kavi Kishor, 1999).

The totipotent nature of plant cell makes them an alternative source to whole plant culture for production of secondary metabolites (Ravishankar et al., 1999; Dornenburg and Knorr, 1997). Plant tissue culture which is being used for propagation of medicinal plants proves to be alternative method for commercial propagation (Geroge and Sherington, 1984).

The growth and morphogenesis response of plant cell or tissue in vitro varies from one plant species to other depending upon their nutritional requirement. In addition, tissue from different parts of same plant may also have different requirements for growth (Murashige and Skoog, 1962).

There are more than 50 different devised media formulations used for in vitro culture of various plant species (Gamborg et al., 1976; Huand and Murashige, 1977). However, many worker have commonly used formulation described by Murashige and Skoog (1962) with or without minor changes (Chand et al., 1997; Jha and Sen, 1985; Ravishankar and Venkatraman, 1988; Rout et al., 1999; Zhou et al., 1994).

Components of plant tissue culture media contains some or all of the following components: macronutrients, micronutrients, vitamins, amino acids or nitrogen supplements, source of carbon, undefined organic supplements, growth regulators and solidifying agents.

The elements in concentration greater than 0.5mM$^{-1}$ are defined as macronutrients by International Association of Plant Physiology (de Fossard, 1976).

Nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulphur (S) are essential elements other than Carbon (C), Hydrogen (H) and Oxygen (O) in plant tissue culture media.

The elements in concentration less than 0.5mM$^{-1}$ are defined as micronutrients by International Association of Plant Physiology (de Fossard, 1976). The micronutrients include iron (Fe), manganese (Mn), zinc (Zn), boron (B), copper (Cu) and molybdenum (Mo).

Nitrogen is one of the important regulators of growth and development as it is known for its role in regulating protein expression through mechanism affecting
transcription (Sugiharto and Sugiyama, 1992). Nitrogen is also important for synthesis of alkaloids (Zhong, 2001), anthocyanin and shikonin production from cell suspension cultures (Kim and Chang, 1990). In addition, ammonium/nitrate (NH$_4^+$ to NO$_3^-$) ratio in tissue culture media affects growth of plant cells (Veliky and Rose, 1973) as well as secondary metabolite production (Smetanska, 2008). High nitrate/ammonium (NO$_3^-$/NH$_4^+$) ratio increased production of betacyanin in *Phytolauca Americana* and its lower concentrations decreased production of berberine in *Thalictrum minus* & ubiquinone in *Nicotiana tabacum* (Nakagawa et al., 1984; Ikeda, 1977). The ammonium/nitrate ratio controls pH of the growth media, stimulates morphogenesis and embryogenesis in woody plant cultures (Lee et al., 2011).

In plant culture media sucrose is generally used as carbon source. The sucrose which acts as morphogenetic trigger in formation of auxiliary buds found better for growth when autoclaved than filter sterilized. Autoclaving hydrolyze sucrose into more efficiently utilizable sugars such as fructose (Vinterhalter and Vinterhalter, 1997).

Vitamins are limiting factors for cell growth and differentiation for *in vitro* development of plant cells or tissues as they are required by plants to catalyse various metabolic processes. Thiamin (B1), nicotinic acid and pyridoxine (B6) are mostly used vitamins in plant tissue culture media. Thiamin is necessarily required by all cells (Ohira et al., 1976). Myo-inositol which is a carbohydrate is also added to plant tissue culture medium to stimulate cell growth (Vasil and Thorpe, 1994). Amino acids are also part of tissue culture medium, they are important in providing a source of nitrogen that is easily assimilated by tissues and cells faster than inorganic nitrogen sources.

The most important constituent of plant tissue culture media are plant growth regulators for their vital role in tropism, apical dominance, stem elongation etc. Plant growth regulators are classified in; auxins, Cytokinins, gibberellins and abscisic acid. Success of any plant tissue culture experiment depends upon levels and type of plant growth regulator added to medium.

Proportion of auxin to Cytokinins is deciding factor for type of organogenesis in plant cell cultures (Skoog and Miller, 1957). Both Cytokinins and auxins are part of regulation of cell cycle but the role of auxin is in regulation of DNA replication whereas cytokininins regulate the process leading to mitosis which corresponds to cell
growth and morphogenesis in *in vitro* cultures (Hartig et al., 2008; Pesternak et al., 2000)

Tissue culture would be a good means for understanding factors responsible for cell differentiation and organ formation (Evans *et al.*, 1984; Razdan, 1995; Gamborg and Phillipes, 1998).

Plant propagation through tissue culture can be divided into three broad categories. First is Micro-propagation, in this; organized meristems like shoot tips or axillary buds are isolated and induced to produce complete new plant. Second is induction of adventitious shoots, in this approach adventitious shoots are initiated on root, stem, leaf explants directly or on callus derived from those organs. The induced shoots are then excised and inoculated on other medium for root development leading to formation of complete plant. Third system comprises somatic embryogenesis, where somatic embryos are induced on tissue directly or in callus cultures (Rout *et al.*, 2000).

Callus consist of loosely arranged amorphous mass of parenchymatous cells arising from the proliferating cells of cultured explants (Dodds *et al.*, 1985) callus formation is observed in angiosperms, gymnosperms, pteridophytes and bryophytes (Yeoman and Macleod, 1977).

A process in which a single somatic cell or group of somatic cells form embryo is known as somatic embryogenesis. The somatic cells when are induced *in vitro*; generate embryogenic cells which after series of biochemical and morphological changes results in the formation of somatic embryo (Quiroz-Figueroa, 2006). These embryos shows similarities to zygotic embryos in having bipolar structures i.e. it show root and shoot meristems (Gaj, 2004) and they do not show vascular connections with original tissue (Thorpe, 1981). So, Rout *et al.* (1991) consider somatic embryogenesis provides integrated and resilient material as compared to organ culture developed material.

Generally, two main phases are attributed to the process of development of somatic embryogenesis viz. acquaintance of embryogenic competence to proliferate as embryogenic cells; also known as phase O’ (Komamine *et al.*, 1992), determination phase (Rao, 1996) or induction phase (Dodeman *et al.*, 1997)

In the process of somatic embryogenesis not all cells show conversion to somatic embryo but it is restricted to certain type of cells which shows competence to develop into somatic embryos. This competence depends upon sensitivity of these
cells to different plant growth regulators (Dudits et al., 1995; Von Arnold et al., 2002; Rai et al., 2007). According to Feher et al. (2003) various agents ranging from plant hormones to stress treatment are used to induce in vitro somatic embryogenesis in somatic cells of plants. These strong signals evoke activation of large chromatin regions (Feher, 2006).

So, in response to this; Feher, 2008 mention that somatic embryogenesis may occur if genes responsible for embryogenic developmental program are released from chromatin mediated gene silencing in vegetative cells.

In many cases, development of somatic embryo proceeds after induction under auxin free conditions (Dudits et al., 1991) indicating that cell become capable of auxin synthesis. Auxin synthesis along with its polar transport is key stage in the formation of meristem underlying embryo development (Nawy et al., 2008).

Somatic embryogenesis is a fastest way to propagate plants (Naing, 2013) and also a valuable tool used to enhance the genetic improvement of commercial crop species (Stagolla, 2003).

Plants prove as a promising source for the production of secondary metabolites of therapeutic importance.

Secondary metabolites are the metabolites not involved in synthesis of primary metabolites. They are derived biosynthetically from metabolism of primary products.

Production of secondary metabolites in in vitro cultures face many obstacles; strategies like use of elicitors, signal compounds and abiotic stresses can be employed to enhance production of secondary metabolites (Yukimune et al., 1996). However, even though such strategies promote the production of wide Range of metabolites; it fails to meet the need of industrial application. Production of Shikonin by Lithospermum erythroethizon cell cultures and taxol by Taxus cell cultures has been proved successful commercially. In the recent years most of the research on production of secondary metabolites is focused on increased production using various enhancement strategies mentioned above (Zhong et al., 2001), understanding signal transduction pathways involving such strategies, genetic manipulation of regulator genes to increase production of secondary metabolites (Memelink et al., 2001), cloning of genes responsible for such increased production (Verpoorte and Memelink, 2002).

Plant tissue culture techniques are being helpful for the enhancement of secondary metabolite production using cell/organ cultures in vitro. In other words,
useful metabolite production from tissue culture has created new methodology for their commercialization (Sarin, 2005).

The high yield commercial production of useful secondary metabolites; various efforts have been made focusing isolation of biosynthetic activities of cultured cells through optimizing cultural conditions, selecting high yield strains, precursor feeding, transformation methods and immobilization techniques. (DiCosmo and Misawa, 1995).

The research has been proved successful in producing wide range of valuable secondary metabolites in callus or cell suspension cultures. However, many cases require organ culture especially when metabolite of interest is produced only in specialized plant tissues or glands (Davioud, 1989).

In nature, the production of secondary metabolites is a defense mechanism against pathogen attack or as response to various biotic and abiotic stress (Ramakrishna and Ravishankar, 2011). These various conditions are named Elicitors by Dornenburg and Knorr (1995). According to Radman (2003) elicitors are compounds stimulating any type of plant defense. Elicitors which are of fungal, bacterial and yeast origin as well as salts of heavy metals are known to induce over production of secondary metabolites (Ramchandra Rao et al., 1996; Rajendran et al., 1994; Liu et al., 2002; Furze et al., 1992; Robbins et al., 1991; Dicosmo et al., 1987, Funk et al., 1987).

Biotic elicitors are recognized by binding to specific receptor protein on plasma membrane followed by inhibition of plasma membrane ATPase, which leads to reduction of protein electrochemical gradient across the membrane (Hagendoorn et al., 1991)

In addition to this, other signaling molecules such as methyl jasmonate, salicylic acid are also used in cell and organ culture for same purpose of secondary metabolite enhancement (Kim et al., 2003; Dong et al., 2010). Various factors contribute to elicitation strategy for the enhancement of secondary metabolites. These factors are elicitor concentration, duration of exposure, age of culture at time of elicitor treatment (Murthy et al., 2014).

Cell proliferation and differentiation determine the production of secondary metabolite (George, 2008) and the quantity of secondary metabolites accumulation is determined by concentration of growth regulator used for tissue culture (Deus and
Accumulation of secondary metabolites in tissue cultures depends on the type of culture as well as culture stage (Lystvan et al., 2010).

Various media optimization conditions like type of culture medium, salt strength of the medium, type and level of carbohydrate, nitrate levels, phosphate levels and growth regulator levels could also influence synthesis of secondary metabolites in plant cell and organ cultures. According to Zenk et al. (1977) high auxin levels in media decreases secondary metabolite production and type of auxin and its concentration plays important role to alter other metabolic activities of cell (Dougall, 1987).

Precursor feeding is one of the popular strategies to increase secondary metabolite production in plant cell cultures. This works on idea that a chemical compound present at beginning, end or intermediate position on biosynthetic route of secondary metabolite would trigger its final production (Ramachandra Rao and Ravishankar, 2002).

The genus *Ephedra* belongs to gymnosperm family Ephedraceae. *Ephedra* is known as soma plant in traditional ancient Indian Ayurvedic system. In addition, it is being used as Traditional Chinese medicine, where it is popularly known as Ma huang.

Some reports indicated that the ancient Aryans discovered *Ephedra*/soma as energizer cum euphoriant. Consuming juice made from *Ephedra* plant was part of ancient Indian Aryan’s customs mentioned in Rigveda. The same tradition was followed by ancient Romans (Mahdihassan, 1981; Mahdihassan and Mehli, 1989). Soma is the name of a drink in Rigveda which confers immortality (Mahdihassan, 1987).

Worldwide there are 68 species of *Ephedra* (Sharma and Dhiman, 2010) which are adapted to semiarid or desert conditions.

According to Sahni (1990) in India there are nine species of genus *Ephedra* viz. *E. foliata*, *E. gerardiana*, *E. intermedia*, *E. nebrodensis*, *E. regeliana*, *E. saxatilis*, *E. pachyelada*, *E. przenalskii*. However, recently Sharma & Uniyal (2008) and Sharma et al. (2010) discovered *E. sumlingensis*, *E. kardangensis* and *E. khurikensis* from Sumling (Spiti district), Kardang (Lahul district), Khurik (Spiti district) respectively in Himachal Pradesh.

*Ephedra* has been used for over five thousand years in Traditional Chinese Medicine for the treatment of allergies, bronchial asthma, chills, cold, cough, edema,
fever, flu, headaches and nasal congestion (Zhu, 1998). Hot tea prepared from dried stems is generally administered to patients according to traditional system.

The therapeutic activities of *Ephedra* are attributed to its alkaloidal contents (Parsaeimehr *et al.*, 2010a 2010b) and the principal alkaloid is ephedrine (Hoffmon, 2009). The action of ephedrine is more prolonged than that of adrenaline, being an active vasoconstrictor; alkaloids from *Ephedra* can be used to elevate blood pressure and respiratory rate (Gilman *et al.*, 1990). Mark and Penny (1995) reports that it can also be used as thermogenic and for weightloss.

Ephedrine’s pharmacological efficiency can be related to it being act both as direct and indirect sympathetic actions. In direct effect it acts as sympathomimetic agonist at both α- and β- adrenergic receptors. Whereas, indirect action is marked by enhanced release of norepinephrine from sympathetic neurons. The α- and β-adrenergic receptor stimulation induce effects such as enhancement of cardiac rate, vasoconstriction, bronchodilation and central nervous system stimulation. These effects explains role of *Ephedra* being used in traditional system of medicine. The weight loss promoting effect of ephedrine is by increasing metabolic rate of adipose tissue (Murray, 1995). Ephedrine is also used during anesthesia, prescribed during allergy and countering overdose of depressants (Lewis and Elvin-Lewis, 1977).

The pseudoephedrine is most popularly used in flu medications to relieve nasal decongestion due to its anti-inflammatory effect (Hikino *et al.*, 1980) and due to vasoconstrictive effect (Hoffman and Lefkowitz, 1996).

In addition, other minor compounds like Ephedroxane shows anti-inflammatory activity (Konno *et al.*, 1979; Kasahara *et al.*, 1985). Ephedrannine A and maokonine shows hypotensive activity (Tamada *et al.*, 1978). The compounds showing hypotensive activity are present only in roots of *Ephedra*. This confirms Chinese belief that Ma huang gen, is constituent of roots that produce opposite effects to those of aerial parts. However, maokonine an L-tyrosine betaine obtained from roots shows hypertensive activity but the overall effect of root extract shows hypotensive activity (Tamada *et al.*, 1978). Konno *et al.*, (1985) reports hypoglycemic activity of Ephedrans A, B, C, D and E.

According to Hegnauer (1986) who has classified alkaloids according to their biogenetic patheway, l-ephedrine, nor-ephedrine, d-pseudoephedrine and nor-pseudoephedrine can be classified as proto alkaloid.
The total contents and relative amounts of ephedrine alkaloids vary from species to species of Ephedra. This is because of other factors such as geographical distribution, growing conditions (Tanaka et al., 1995; Kondo et al., 1999).

Aerial parts of different Ephedra species contain from 0.02% to 3.4 % of alkaloids concentrated in Internodes (Leung and Foster, 1996).

The physiological effects of Ephedra have been attributed to six ephedrine alkaloids which include (-)-ephrine, (+)-pseudoephedrine, (-)-N-methylephedrine, (-)-N-methylpseudoephedrine, (-)norephedrine, (+)-norpseudoephedrine (Caveney et al., 2001).

Ephedra foliata which is known as Unth Phog in India; is a single gymnosperm species of Thar Desert (Bhandari, 1990). It is dioecious woody climber. Ramawat (1978) and O’Dowd et al. (1993) reported that E. foliata contains trace amount of ephedrine and pseudoephedrine alkaloids.

The conventional way to propagate this plant species is through seeds. However, Singh (2004) and Lodha et al. (2014a) reported that this plant species has high ratio of male over female plants. So, proper seed setting is a major problem. Khan et al. (2003) and Singh (2004) mention that this endemic plant has now become rare and endangered species.

Taking into consideration all the facts, male Ephedra foliata plant is selected in the present investigation for its propagation and enhanced production of alkaloids and to check its potential to prove as an alternative source of ephedrine alkaloids.
Aims and Objectives:

1. To establish the *in-vitro* propagation protocol.
2. To establish callus and cell suspension culture.
3. To check the secondary metabolites in *in vitro* raised biomass.
4. To study effect of elicitors on production of ephedrine alkaloids.