Humans have used phyto-compounds for treating diseases since the beginning of human race. Over 50,000 plant species are currently being in use for therapeutic purposes (http://www.fao.org). Analgesic (morphine), antitussive (codeine), antihypertensive (reserpine), cardiotonic (digoxin), antimalarial (artemisinin), antineoplastic (vinblastine and paclitaxel), active compounds, are some of the important products obtained from medicinal plants (Nessler 1994). Over 25% of the new drugs approved in the last 30 years are based on a molecule of plant origin (Terryn et al. 2006) and seven plant-derived compounds with anticancer activity have received FDA approval for clinical use. These are Taxol/Paclitaxel obtained from *Taxus brevifolia*, vinblastine and vincristine from *Catharanthus roseus*, topotecan and irinotecan from *Camptotheca acuminata* and etoposide and teniposide derived from *Podophyllum peltatum*. Thus, plants are the basic source of production of numerous valuable substances (Georgiev et al. 2007) including a huge variety of secondary metabolites (Sharafzadeh and Alizadeh 2012). These substances evolutionary developed by plants to protect themselves against pathogens and predators or to attract pollinators, exhibit wound-healing, anti-inflammatory, anti-microbial or psycho-active properties (Wink 1988). Many of these compounds are of high economic value such as drugs, flavours, dyes etc. These compounds usually play a role in the interaction of plants with its environment (Verpoorte 1998).

Biotechnological methods have lately been used to produce therapeutics in a controlled and predictable manner encompasses cell cultures, initiated from medicinal plants of interest (Boehm 2006). For some plant species, which are difficult to cultivate and rare in nature, biotechnological methods are perhaps the only possibilities for larger production of the substances of interest. The successful examples of compounds are alkaloids (Zong 2002), shikonin, fragrances and insecticides (Yazaki et al. 1999).

### 1.1 Plant *in vitro* cultures

The production systems using *in vitro* plant cultures are not used at a commercial level so far, but several of them are under development at academic level (Hellwig et al. 2004). Plant cell cultures are more promising to mammalian cell cultures from the view of biochemical engineering in terms of cost due to simple media with cheaper medium components (mostly inorganic salts and sucrose) and the lack of human pathogenic particles that have to be eliminated in the downstream processing (Boehm 2006). The callus tissue, which may be a source of alkaloids itself, commonly provides a starting material for establishing suspension
culture or for plant organ induction like shoots and roots, the two good sources for several phyto-compounds. In alkaloid enrichment programme, the following two approaches have currently been adopted: (1) the optimization of cultural conditions / factors that control synthesis and (2) metabolic engineering and / or over expression of key enzymes (van der Heijden et al. 2004). In some practices, plant cell cultures are elicited with combination of various carbohydrates, lipids for the production of phytoalexins and secondary metabolites (Yamaguchi et al. 2002). Elicitation is one of the most effective ways to promote secondary metabolites production in plant cell cultures (Zhang et al. 2000). These compounds have been successfully used in cell cultures of different species such as *Thalictrum rugosum* (Brodelius et al. 1989), *Tagetes patula* (Buitelaar et al. 1992), *Catharanthus roseus* (Vazquez–Flota et al. 1994), *Taxus* sp. (Ciddi et al. 1995) and many others. The use of elicitors from pathogenic microorganism and other external factors has been used for improving the productivity of useful secondary metabolites (Li et al. 2002). In such culture, the isolated cells from the whole plant or plant parts are cultivated under appropriate physiological conditions and the desired product is extracted from the cultured cells. The recent developments in plant tissue culture techniques and their processing have shown promising results to improve the productivity by many folds (Chattopadhyay et al. 2002). Beside callus and suspension culture, the cultivation of other plant parts, i.e. shoot, roots, hairy root, zygotic and somatic embryo at different stages have also been exploited for *in vitro* biosynthesis of secondary metabolites (Pietrosiuk et al. 2007; Aslam et al. 2009).

1.2 Somatic embryogenesis

In nature, the fusion of one spermatic unit with the egg cell takes place and gives rise to the zygote and initiates the embryogenesis process, called zygotic embryogenesis. Alternatively, embryogenesis can also occur without the involvement of fertilization or gamete fusion (Karami et al. 2009). The origin of such asexual embryos is quite diverse e.g., apomictic embryos are derived from an unfertilized egg cell or from maternal tissues (Nogler 1984). It is also possible to obtain *in vitro* “androgenic” embryos from microspores and pollen grains (Raghavan 2000), while in somatic embryogenesis, embryos are originated from somatic or vegetative cells without any fertilization event (Bhojwani and Razdan 1983).

The process can be divided into two phases: induction and expression. During the induction phase, differentiated somatic cells acquire embryogenic competence and proliferate as embryogenic cells. In the expression phase, the embryogenic cells display their embryogenic
competence and differentiate to form somatic embryos (Jimenez 2001). The two phases were suggested to be independent of each other and influenced by different factors (Jimenez 2001). The term ‘embryogenic cells’ is restricted to those cells that have completed their transition from a somatic state to one in which no further exogenous stimuli, such as the application of plant growth regulators (PGRs) are necessary to produce the somatic embryo (Komamine et al. 2005). The cells in a transitional state that still require only minimal exogenously applied stimuli to become embryogenic are defined as competent cells (Toonen et al. 1994).

1.2.1 Direct and indirect somatic embryogenesis

The cells in culture can be induced to go through a direct or indirect somatic embryogenesis by modulating tissue culture conditions. In the first, embryos develop directly on the surface of organized tissue such as a leaf, stem segment, zygotic embryo, young inflorescence, on protoplasts and on microspores (Williams and Maheswaran 1986). Alternatively, indirect somatic embryogenesis can occur in tissues in which there is an intermediary step of callus formation or cell suspension culture (Gamborg 2002). The induction of somatic embryos in different species and cultivars are usually made by trial and error and or by analysing the effects of different culture conditions (Henry et al. 1994). The cultural conditions on somatic embryogenesis are PGR balance, osmotic conditions, changing pH, amino acid, salt concentrations, heat shock and treatment with various chemical substances (Namasivayam 2007). Other than auxin (Feher et al. 2002), the presence of PGRs like cytokinin (Sagare et al. 2000), abscisic acid (ABA) (Nishiwaki et al. 2000) and also in the absence of PGRs (Choi et al. 1998) embryogenesis has also been reported.

1.2.2 Somatic embryo formation and development

An embryogenesis system follows a certain programmed developmental path, which occurs in succession: initiation of embryogenic callus from vegetative tissues or cells, maintenance and multiplication of embryogenic cell lines, somatic embryo formation and maturation and finally conversion (germination) of somatic embryos into viable plantlets (Zegzouti et al. 2001). Initiation of embryogenic cultures is done by culturing the primary explant on medium supplemented with PGRs, mainly with auxin alone or with cytokinin (Bellarosa et al. 1992). Since most somatic cells are not naturally embryogenic, an induction phase is required for the cells to acquire embryogenic competence (Dodeman et al. 1997). The frequency of embryo induction process does not only depend on culture conditions, but it also depend on factors like the genotype, tissue and physiological state of donor material (Carman 1990), as well as
the levels of endogenous PGRs in the explant (Jimenez 2001). The range of possible induction treatments suggests that it is not possible always that a single inducing molecule is responsible for embryogenic competence (Toonen and De Vries 1996).

Once embryogenic cells are formed, the cells continue to proliferate, forming proembryogenic masses (PEMs). The proliferation of embryogenic cultures are made on solidified medium or in liquid medium supplemented with PGRs, similar to initiation. Auxin is required for proliferation of PEMs but is inhibitory for the development of PEMs into somatic embryos (Filonova et al. 2000a). However, for large scale propagation it is usually better to establish suspension cultures where proliferation rate is higher and the cultures become more synchronized (von Arnold et al. 2002).

Maturation is regarded as an important stage of embryogenesis since the frequency of plant recovery is high from mature embryos (Zegzouti et al. 2001). Embryo maturation is a culmination of the accumulated carbohydrates, lipids and protein reserves; at this time dehydration in embryo and a reduction in cellular respiration were noted (Trigiano and Gray 1996). Thus, maturation is a preparatory stage for embryos for effective germination. Etienne et al. (1993) earlier observed that the embryo maturation is a transitory, frequently indispensable stage between embryo development and embryo germination phases, bypassing the maturation phase will result in precocious germination of embryos, causing a significant reduction in viable plantlets.

The process of somatic embryogenesis is however, quite complex in which the quality of final product, i.e. the survival and growth of regenerated plants, depends on the conditions provided at earlier stages, where mature somatic embryos are formed and germinated. Only mature embryos with a normal morphology, which have accumulated enough storage materials and acquired desiccation tolerance during maturation develop into normal plants (von Arnold et al. 2002).

1.2.3 Morphology versus embryogenic capacity

Although totipotency is an important characteristic of plant cells, not all cells express it at specific conditions and even when we assume that all cells are equally totipotent, the number of embryos formed in culture is minimal compared to the total number of cells in a cellular aggregate (Francisco et al. 2006). Actually, the ability of a given tissue to generate embryos is often a characteristic and confined to:
• A limited fraction of the cell population (Nomura and Komamine 1985).
• A discrete zone of the cell cluster (Nabors et al. 1983).
• The cell wall participates in the growth and differentiation of cells during embryogenesis as plants cell walls of embryogenic cells contained arabinogalactan proteins (AGP’s) while non-embryogenic cells did not (Majewska–Sawka and Nothnagel 2000).

1.2.4 Plant growth regulators (PGRs)

PGRs play an important role in controlling the way in which plants grow and develops while metabolism provides the power and building blocks for plant life (Davies 1995). PGRs regulate the speed of growth of the individual parts, integrate these parts to produce the form that we recognize as a plant and they play a controlling role in the processes of reproduction (Skoog and Miller 1957). PGRs (auxin and cytokinin) are synergistically required to induce cell division and growth in plant tissue cultures. It has not yet been established that a PGR might operate a specific function by itself. On the contrary, there are several potential mutual interacting points between PGRs (Coenen and Lomax 1997), depending upon the plant species and tissue type (Schmulling et al. 1997).

Signal transduction is a recently defined focus of research in plant biology in which plant cells show responses to certain type of signals (Trewavas 2000). PGRs, in general sense, are certainly among the external stimuli or signals. Auxins and cytokinins were originally considered to produce growth responses at distances from their sites of synthesis, thus fitted the definition of transported chemical messengers (Gasper et al. 2003). It is now clear that none of the officially recognized five classes of PGRs (auxins, cytokinins, gibberellins, abscisic acid and ethylene) fulfill the requirements of a PGRs in the mammalian sense (Gasper et al. 2003). So the synthesized PGRs may be transported and have specific action at a distance, but it is far from being the general case; the PGRs also act in the tissue or even within the cell in which they are synthesized (Gasper et al. 2003).

The PGRs and their use in plant culture media have been developed from initial observations made in the 1950s. It has been considerably difficult to predict the effects of PGRs due to the great differences in culture response between species, cultivars and even plants of the same cultivar grown under different conditions. Auxins and cytokinins are the most widely used PGRs in plant tissue culture and are usually used together (Figure a), the ratio of auxin and
cytokinin determines the success of culture to be established or regenerated (Schmulling et al. 1997). A high auxin to cytokinin ratio generally favours root formation, whereas a high cytokinin to auxin ratio favours shoot formation and an intermediate ratio favours callus production (Gasper et al. 2003).

1.2.4.1 Auxins

Beside the above role, auxins also promote both cell division and cell growth (Table a). IAA (indole-3-acetic acid) is the most important naturally occurring auxin but its use in plant tissue culture media is limited because its unstablity to heat and light. 2, 4-Dichlorophenoxyacetic acid (2, 4-D) is the most commonly used auxin and is extremely effective in most circumstances (Lakshmanan and Taj 2000). Indole-3-butyric acid (IBA) is root-inducing synthetic auxins. All auxins are used in the concentration range $10^{-7}$ to $10^{-5}$ M. Auxin has been implicated in diverse physiological processes of which some are related to development, notably apical dominance, vascular patterning and lateral root formation.

1.2.4.2 Embryogenesis with PGR’s association

Auxin is considered to be the most important PGR in regulating somatic embryogenesis in vitro (Cooke et al. 1993). The regulation probably occurs through the establishment of an auxin gradient or in establishing cell polarity, which is essential for initiating bilateral symmetry during somatic and zygotic embryogenesis in dicotyledons and monocotyledons (Fischer and Neuhaus 1996; Davies 1995). For this gradient to be established, relatively high levels of IAA in the competent tissues may be necessary. It was noted that the establishment and maintenance of embryogenic cultures of nearly all species has primarily relied on the manipulation of PGRs (Smith and Krikorian 1990b). The most commonly used way for initiation of embryogenesis involves the induction of callus in an auxin-supplemented medium and somatic embryogenesis upon transfer of callus to a medium containing low concentration of PGRs (Gray 1992). The presence of auxin promoted callus proliferation and inhibited differentiation while the removal or decrease in auxin favours somatic embryo development to progress (Dudits et al. 1995). Upon transferring callus to an auxin-free medium several morphogenetic changes were observed (Zimmerman 1993). It appears that the removal of auxin from the medium provides the signal for the callus cells to develop into an organized pattern of growth. The fact that embryogenesis can occur upon withdrawal of growth regulators suggests that in the presence of auxin, the proembryogenic masses (PEMs) within the culture system may already be destined to complete the globular stage of
embryogenesis; in some observations, it was noted that the PEMs may also contain products inhibitory to the progress of the embryogenesis programme (Filonova et al. 2000a). As auxin was proved to be the principal agents responsible for cell polarity it won’t be unfair to say that it somehow control the efficiency of somatic embryogenesis in culture (Deo et al. 2010).

Table a: Commonly used auxins, their abbreviations and chemical name:

<table>
<thead>
<tr>
<th>Abbreviation/ name</th>
<th>Chemical name</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-D</td>
<td>2, 4-dichlorophenoxyacetic acid</td>
</tr>
<tr>
<td>2,4,5- T</td>
<td>2,4,5-trichlorophenoxyacetic acid</td>
</tr>
<tr>
<td>Dicamba</td>
<td>2-methoxy-3,6-dichlorobenzoic acid</td>
</tr>
<tr>
<td>IAA</td>
<td>Indole-3-acetic acid</td>
</tr>
<tr>
<td>IBA</td>
<td>Indole-3-butyric acid</td>
</tr>
<tr>
<td>MCPA</td>
<td>2-methyl-4-chlorophenoxyacetic acid</td>
</tr>
<tr>
<td>NAA</td>
<td>1-naphthylacetic acid</td>
</tr>
<tr>
<td>NOA</td>
<td>2-naphthoxyacetic acid</td>
</tr>
<tr>
<td>Picloram</td>
<td>4-amino-2,5,6-trichloropicolinic acid</td>
</tr>
</tbody>
</table>

1.2.4.3 Cytokinins

Cytokinins promote cell division. Of the naturally occurring cytokinins, only zeatin and 2iP (2-isopentyl adenine) have some use in plant tissue culture media. The synthetic analogues, kinetin and BA (benzyladenine) are used more frequently (Haliloglu 2006). Non-purine based chemicals, such as substituted phenylureas are also used as cytokinins in plant tissue culture media. Cytokinins are known to stimulate cells and, as such, they are also suitable candidates for induction of somatic embryogenesis and caulogenesis (Dodeman et al. 1997). Cytokinins are now fully recognized as one of the major groups of endogenous PGRs. Several cytokinin are adenine derivative and occur in plants as nucleosides and nucleotides. The following compounds belonging to cytokinin are of special importance in plant tissue culture (Table b).
### Table b: Commonly used cytokinins, their abbreviations and chemical name:

<table>
<thead>
<tr>
<th>Abbreviation/name</th>
<th>Chemical name</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAP&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6-benzylaminopurine</td>
</tr>
<tr>
<td>2ip (IPA) &lt;sup&gt;b&lt;/sup&gt;</td>
<td>[N&lt;sup&gt;b&lt;/sup&gt;-&lt;sub&gt;6&lt;/sub&gt;-(2-isopentyl)adenine]</td>
</tr>
<tr>
<td>Kinetin&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6-furfurylaminopurine</td>
</tr>
<tr>
<td>Thidiazuron&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea</td>
</tr>
<tr>
<td>Zeatin&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4-hydroxy-3-methyl-trans-2-enylaminopurine</td>
</tr>
</tbody>
</table>

<sup>a</sup>Synthetic analogues  
<sup>b</sup>naturally occurring cytokinins  
<sup>c</sup>A substituted phenylurea-type cytokinin

**Figure a:** Different combinations of auxins and cytokinins resulted into different tissues.

**1.2.4.4 Gibberellins**

Gibberellins are involved in regulating cell elongation, in determining plant height and in fruit-set. Gibberellins are also used in tissue culture studies but are not obligatory (Shannon et
al. 1999). Of the 90 or so designated gibberellins, which are structurally different and biological active, GA$_3$ forms an important, naturally occurring PGR in higher plants. These are a large family of cyclic diterpene acids freely soluble in water.

1.2.4.5 Abscisic acid (ABA)

ABA occurs naturally in plant tissues and its synthesis is noted to be within the plastids (Salisbury and Ross 1992). It is used in plant tissue culture to promote distinct developmental pathways such as somatic embryogenesis (Raz et al. 2001).

1.2.4.6 Ethylene

Ethylene (ethane, C$_2$H$_4$), is one of the most unusual gaseous hormones synthesized in cultured cells, fungi and bacteria (Perovic et al. 2001). Flower development and ripening of fruits are known to be due to the presence of small amounts of ethylene (Chang 1996). Ethylene is associated with controlling fruit ripening in climacteric fruits, but its use in plant tissue culture is not that widespread (Wang et al. 2007). Some plant cell cultures produce ethylene, if the level is sufficient it inhibits the growth and development of the culture (Ohme-Takagi and Shinshi, 1995). The type of culture vessel and its means of closure affected gaseous exchange between the vessel and the outside atmosphere and thus the levels of ethylene is more in vessel that influenced cultural growth (Jackson 2003).

1.2.5 Significance of somatic embryogenesis

Somatic embryogenesis is an important tool in plant biotechnology and can be used in the following ways (Saiprasad 2001):

- It provides an alternative approach to conventional method of clonal propagation of elite cultivars on large scale.
- Synthetic (artificial) seed can be developed from somatic embryos, potentially facilitating large number of direct seeding of important cultivars or providing a means of moving germplasm in a less fragile form than in vitro plantlets.
- Embryogenesis via callus or secondary embryogenesis may assist in the application of gene transfer techniques for further genetic improvements.
- Somatic embryogenesis systems offer potential models for studying molecular, regulatory and morphogenetic events in plant embryogenesis.
• During regeneration, root and shoot formation is simultaneous thus eliminating the need for a root induction phase as with conventional micropropagation.
• The mode of culture permits easy scale-up and subculture with low labour inputs.
• Somatic embryogenesis provides material for protoplast isolation (Assani et al. 2002).
• Cultures can be manipulated such that embryo formation and germination can be synchronized maximizing plant output while minimizing labour inputs.
• As with zygotic embryos, somatic embryos dormancy can be induced, hence long-term storage is possible.

1.2.6 Limitation of somatic embryogenesis

Somatic embryogenesis has disadvantages as well and some of them are:

• The development of somatic embryos tended to be non-synchronous, thus embryos of all stages can be present in one culture system (Zegzouti et al. 2001).
• The percentage of somatic embryos regenerated is affected by the size of cell aggregates.
• Over a period of time, the proportion of cells that enter or complete embryogenesis decreases, eventually regeneration may become impossible leading to the unstability of cell lines (Francisco et al. 2006).

1.3 Protoplast isolation study

Protoplast has several biological definitions. A protoplast is a plant, bacterial or fungal cell that has its cell wall completely or partially removed using either mechanical or enzymatic means. More generally protoplast refers to that unit of biology which is composed of a cell nucleus and the surrounding protoplasmic materials (Smith and Jarvis 1999) (Table c).

Table c: Different enzymes and enzyme mixtures used for protoplast isolation in different cells.

<table>
<thead>
<tr>
<th>Type of cell</th>
<th>Enzyme mixture used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant cells</td>
<td>Cellulase, pectinase, xylanase</td>
</tr>
<tr>
<td>Gram-positive bacteria</td>
<td>Lysozyme (+ EDTA)</td>
</tr>
<tr>
<td>Fungal cells</td>
<td>Chitinase</td>
</tr>
</tbody>
</table>
During and post cell wall digestion period, the protoplast becomes very sensitive to osmotic stress (Koster et al. 2003). This suggests that the cell wall digestion and protoplast storage must be done in an isotonic solution to prevent rupture of the plasma membrane (Koster et al. 2003).

### 1.3.1 Sources for protoplasts

The individual cell or tissue may require special conditions for successful isolation or for culturing (Saker et al. 1999). The use of embryogenic cultures was proved to be especially valuable in providing a source of regenerable protoplasts (Chang and Wong 1994). It was also noted that if plant regeneration was accomplished from cultured cells of suspension, the same regeneration was also often possible from suspension derived protoplasts (Tan et al. 1987). Leaf mesophyll cells of a wide range of plants have also been used as a protoplasts source with success (Thomas 2009).

### 1.3.2 Protoplast technology and history

The enzymatic isolation of plant protoplasts was first reported by Cocking (1960). Since then protoplasts have been isolated and cultured in a wide range of higher plant species (Davey et al. 2005a). Protoplasts may be isolated from intact plant tissues or from cultured cells (Lambert and Geelan 2010). The success of protoplasts isolation depends especially on the condition of the tissue and the combination of enzymes being used. More recently, the protoplast technique has successfully been used to introduce resistant traits including disease resistance from wild relatives to cultivated varieties (Collonnier et al. 2001). As early as 1902, Haberlandt noted that the individual nucleated plant cells could convert into entire plants, either directly or through a callus stage. This phenomenon is termed totipotency and denotes the recovery of a whole organism from a single cell, and is also applicable to protoplasts. In theory, all cells are totipotent, but in practice it depends on the past cellular environment. Usually the morphogenetic competence is retained at the unicellular stage that corresponds to a protoplast (Haicour et al. 2004).

### 1.3.3 Protoplast and tissue culture

In case of plant cells, protoplasts may be regenerated into whole plants by growing a group of protoplasts that develops into a callus first and then by regenerated into shoots using plant tissue culture methods (Thorpe 2007). Growth of protoplasts into callus and regeneration of shoots require the proper balance of PGRs in the tissue culture medium that must be
customized for each species of plant. Unlike protoplasts from vascular plants, protoplasts from mosses, such as *Physcomitrella patens*, do not need PGRs for regeneration, nor do they form a callus during regeneration. Instead, the protoplasts regenerated directly into the filamentous protonema, mimicking a germinating moss spore (Bhatla et al. 2002).

**1.3.4 Factors affecting protoplast isolation**

**1.3.4.1 Enzyme mixture**

The success in protoplast isolation is influenced by several factors including the use of enzyme mixture. The combination of enzyme solution has been an important factor on yield and viability of protoplasts as was studied in many plant species such as *Artemisia judaica* L., *Echinops spinosissimus* Turra (Pan et al. 2003), etc.

**1.3.4.2 Concentration of osmoticum**

The concentration of osmotica in the enzyme solution also significantly affects the yield and viability of the protoplasts. The various osmotica used in protoplast study are sorbitol, mannitol, sucrose, CaCl$_2$.2H$_2$O, KCl, etc. The optimum concentration of osmoticum generally varies from plant to plant.

**1.3.4.3 Incubation period**

The incubation period during which the callus or suspension is treated with enzyme mixture for digestion of cell wall and middle lamellae significantly affects the protoplast yield and viability. The viability of protoplasts decreases with prolonged incubation period. Mostly, the mixture is incubated over night (12 hours).

**1.3.4.4 Age of callus**

Fresh suspension culture of 5-8 days is often used for the isolation of protoplasts. Protoplast viability decreases with increasing age of callus or suspension (Davey et al. 2005a).

**1.3.5 Application of protoplast technology**

Isolated protoplasts may be considered as the simplest form of genetic manipulation and is useful in plant breeding as well as in technological approaches in developing genetic diversity by exploiting somatic hybridization, transformation and protoclonal variation (Kantharajah and Golegaonkar 2004).
1.3.5.1 Somatic and gameto-somatic hybridization

Somatic and gameto-somatic hybridization have been attempted in several plant genera for various reasons that are summarized below:

- The ability to regenerate plants routinely from protoplast-derived tissues is fundamental to genetic manipulation approaches involving somatic hybridization and, to a lesser extent, transformation (Davey et al. 2005a).
- Hybrid cells, derived from protoplast fusion may regenerate into plants with balanced or asymmetric nuclear genomes (Davey et al. 2000b).
- The generation of cybrids has been exploited to transfer organellar traits such as mitochondrially-encoded cytoplasmic male sterility (CMS) or chloroplast DNA-encoded herbicide resistance e.g. atrazine (Fayez and Hassanein 2000).
- Fusion of diploid with haploid protoplasts was used to generate fertile triploid plants through gameto-somatic hybridization (Davey et al. 1996b).

1.3.5.2 Transformation of plants by uptake of DNA into protoplasts

In other experiments, protoplasts have been continually used for up-taking DNA as it:

- Routinely generates transgenics in plants like sugar beet (Hall et al. 1996), grasses and cereals particularly in rice (Blechl 2004).
- The major exploitation of DNA uptake into isolated protoplasts is currently under process through short-term experiments of transient gene expression to evaluate the performance of new gene constructs, frequently involving the beta-glucuronidase (gus) or green fluorescent protein (gfp) genes (Sheen 2001).
- The use of micro injection to insert DNA into protoplasts isolated mechanically from zygotes was also noted with 55% of injected protoplasts giving embryo-like structures which developed into plants (Holm et al. 2000).

1.3.5.3 Protoclonal and somaclonal variation

Somaclonal variation can be defined as the variation in plants regenerated from cultured tissues; however, the variation in the protoplast derived plants is called as protoclonal variation. Such variation may affect a range of traits including those for plant morphology, flower colour, yield, nutritional value, production of secondary products, resistance to pathogens and tolerance to environmental conditions (Mizuhiro et al. 2001). It was also
Introduction

noted that the longer the cells are in culture, particularly at the callus stage, the greater the variation is expected to be present in protoplast derived cells and in tissues (Hao and Deng 2003). Protoclonal variation also has the ability to increase the genetic diversity, and it may bypass the sexual cycle as well (Davey et al. 2005).

1.3.5.4 Protoplasts used in other investigations

- Protoplasts provide useful single cell system for the studies of cell ultra structure and genetics (Davey et al. 2005a). Protoplasts are also suitable to study the molecular architecture of new plants generated by *in vitro* approaches.
- Pollen protoplasts have been used as experimental material in *Brassica chinensis* to investigate the regulation of potassium ion channels by external and internal protons (Fan et al. 2003).
- Isolated plant protoplasts and protoplast-derived cells have featured in plant-fungal interactions (Melayah et al. 2001).
- Antibodies can penetrate into living plant protoplasts and therefore may be used as powerful tool in evaluating protein function (Bricare et al. 2004).

1.3.6 Some limitations of protoplast isolation technology

- Naked protoplasts often die because of adverse toxic effects of enzymes, PGRs and stresses that are either present and or often added in medium.
- Condition is also unfavorable for protoplast survival because of excessive influx/efflux of required ions.
- Protoplasts often form cell wall and thick lignified layers like middle lamella.
- Protoplasts many times, do not divide at all, thus, preventing colony/callus formation.
- Cultures derived from viable protoplasts do not regenerate into shoot, root or total plantlet; remain only at cellular or tissue state.

1.4 Elicitation

An elicitor is defined as substances or signals that trigger the synthesis of compounds, used in plant defense responses (Koga et al. 2006). The use of elicitors, one of the effective strategies employed to increase the production of important alkaloids in cell and organ culture (Pitta et al. 2000). Elicitors induce ion fluxes across the plasma membrane, changes protein phosphorylation level, and cause transient accumulation of secondary signal molecules like
jasmonic acid in cultivated plant cells (Nimchuk et al. 2003). It is well known that the plants have the ability to produce secondary metabolites (phytoalexins) to defend themselves against microbial infection and these compounds accumulated at the site of infection in intact plant (Bailey and Mansfield 1982). The utilisation of tissue cultures in the study of phytoalexin is also receiving increasing attention (Brooks et al. 1986) and there are some studies on the enzymology of sesquiterpenoid phytoalexin accumulation (Alejo and Gomez 1993). Elicitors are used to induce phytoalexin accumulation in cultured plant cells (Whitehead et al. 1988). Often complex biological preparations have been used as elicitors, where the molecular structure of the active ingredients is unknown such as YE and microbial cell-wall preparations (Radman et al. 2003).

1.4.1 Yeast extract as biotic elicitor

Yeast extract (YE) is an autolysate of the cell walls of bakers yeast (*Saccharomyces cerevisiae*). YE contains several components that can elicit plant defense responses, including chitin, N-acetylglucosamine oligomers, β-glucan, glycopeptides and ergosterol (Boller 1995). In *in vitro* cultured cells, the perception of YE leads to the induction of terpenoid indole alkaloids (TIA) biosynthetic genes including those encoding strictosidine synthase (STR) and tryptophan decarboxylase (TDC) gene (Pauw et al. 2004).

1.5 Synthetic seeds

Synthetic seeds are encapsulated somatic embryos, shoot buds, cell aggregates, or any other tissues that can be used for sowing as a seed and possess the ability to regenerate into a plant under *in vitro* or *ex vitro* conditions (Capuano et al. 1998). However, the production of synthetic seeds requires efficient and quality production of somatic embryos (Nirala et al. 2010).

1.5.1 Source for synthetic seeds

Choice of the tissue to be employed also needs fundamental deliberation. There have been many studies on encapsulation of different plant materials; somatic embryos were also tried recently in several plant systems like in sugarcane (Nieves et al. 2003), *Paulownia elongate* (Ipekci and Gozukimizi 2003) and *Daucus carota* (Latif et al. 2007). However, less attention has unfortunately been paid to encapsulation of non-embryogenic vegetative propagules such as nodal segments, axillary buds, shoot tips, hairy roots or callus (Zych et al. 2005).
1.5.2 Synthetic seed development

The culture of plant tissues via somatic embryos on a large scale makes it possible to produce synthetically coated seeds (Gray and Purohit 1991). The embryogenic culture may be used to produce synthetically coated seeds to conserve superior cell lines on a large scale as was reported earlier in other groups of plants (Redenbaugh 1993). Synthetic seed technology being a low-cost-high volume propagation system has the inherent capacity to produce clonal seeds at a cost comparable to true seeds (Monika et al. 2005).

1.5.3 Significance of synthetic seed technology

- The technology may be of value in breeding programs and allows the propagation of many elite genotype-derived plants in a short time (Nieves et al. 1998).
- Encapsulation of propagules that were produced in vitro could reduce the cost of micropropagation of plantlets for commercialization and final delivery (Chu 1995).
- Germplasm storage and exchange purposes (Danso and Ford-Lloyd 2003).
- Exchange of stock cultures between laboratories or different places (Maruyama et al. 1997) without temperature fluctuations and danger of infestation with microorganisms (Ikhlaq et al. 2010)

1.5.4 Limitation of synthetic seeds

- An increase in temperature causes desiccation of synthetic seeds and decrease in conversion rate (Micheli et al. 1998).
- Hydrated synthetic seeds were difficult to store at room temperature because of depleted nutritive reservoir and lack of quiescence that lowered conversion percentage (Nieves et al. 2001).
- The decline in conversion from encapsulated embryo into plants may be related to both oxygen deficiency in the gel bead and its rapid drying (Swamy et al. 2009).

Keeping above in view, in the present study, an attempt was made to isolate protoplasts from embryogenic suspension in *Catharanthus roseus*. The growth of protoplast derived tissue was monitored after YE elicitation. The yield of vinblastine (VB) and vincristine (VC) was quantified in post protoplast derived tissues and in plantlets regenerated from protoplasts with
or without YE elicitation. Lastly, an effort was made in developing a protocol to make synthetic seeds by using *in vitro* raised somatic embryos.