Plants interact with their environment by producing a diverse array of secondary metabolites (Harborne, 1996), which are low molecular-weight organic compounds, usually with unique and complex structures. These compounds are formed from glucose metabolism intermediated by the shikimic acid, acetate and amino acid pathways (Geissman and Crout, 1969). A range of physiological and ecological functions have been reported for these natural products, such as hormone regulation, organogenesis, plant defence against biotic and abiotic agents, chemical signaling to guide pollinators or fruit disperser and plant – microorganism symbiotic interaction (Curir et al., 1990; Van Tunen and Mol, 1991; Vogt et al., 1994; Turlings et al., 1995). Many of these compounds are valued for their pharmacological activities and industrial or agricultural properties, which increase the commercial value of crops (Bingham et al., 1998; Paganga et al., 1999; Bingham et al., 1998). However, despite great efforts by the chemical industry to mimic and synthesis specific plant secondary metabolites, little success has been achieved and plants still remain the major source of many vital medicinal compounds (Wink, 1990). As most of these compounds originate from plants, any factor (e.g. climatic, political, etc.) Which affects the continued supply of these molecules will endanger world supply. Presently, 100,000 such compounds have been isolated from higher plants (Verpoorte, 2000). Some economically important plant derived metabolites is represented in table 2. Although secondary metabolism offers attractive targets for plant breeding, the enormous biosynthetic potential of plant cells is still not being exploited. In sharp contrast, metabolism of microorganisms has been successfully engineered for increased production of pharmaceuticals or novel compounds (Hutchinson, 1994, Chartrain et al., 2000). Several ethnobotanical and phytochemical research projects have been conducted on medicinal plants from Suriname over the past three decades. Despite a few decades of research, plant secondary metabolism remains poorly characterized (Verpoorte and Mensink, 2002). Genetic maps of biosynthetic pathways are still far from complete, whereas knowledge on the regulation of these pathways is practically nonexistent. However, such knowledge is of crucial importance to bypass the low product yield of various secondary metabolites in plants or plant cell cultures.
In drug discovery, the major secondary metabolites (terpenoids, phenolics, and alkaloids) are of potential medicinal interest. Secondary metabolites are synthesized by the plant during latter stage of plant development and are time, tissue and organ specific. They can be induced by biotic and abiotic factors. In contrast to primary metabolites, they are not present in all plant cells and not essential to sustain growth. Many aromatic secondary metabolites are phenylpropanoids, e.g. flavonoids, phytoalexins, agents that protect against ultra-violet light and, most importantly, lignin (Mabry and Ulubelen, 1980). Expression of the phenylpropanoid biosynthetic pathway is precisely regulated in response to developmental signals, nutrient status and environmental stimuli such as light, heat and pathogen attack. Compounds that are constitutive in one plant species or tissue can be induced by various stresses in another species or in another tissue of the same plant (Beggs et al., 1987; Christie et al., 1994).

2.2 Secondary metabolites and abiotic stress

The role of abiotic stress in plant cell organization and its relation to the induction of different secondary metabolites has been the subject of extensive investigations (Santosh et al., 1993; Lummerzhein et al., 1995). Biochemical studies have revealed similarities in processes induced by stress that lead to accumulated metabolites in vascular and non-vascular plants, algae fungi and bacteria (Csonka, 1989; Galinski, 1993; Potts, 1994). Biotic and abiotic stress activates complex defence mechanism in plants cells, altering both primary and secondary metabolism in a coordinated ways (Marques and Brodelius, 1988; Dalkin et al., 1990). However little is known about the influence of abiotic elicitors on specific pathways of secondary metabolite biosynthesis. Based on biological activities in vitro and on the correlation between rates of accumulation and expression of resistance in vivo, it is indicated that aromatic compounds such as isoprenoid and phenylpropanoid play an important role in the induced and constitutive response of plants to biotic and abiotic agents (Dixon and Paiva, 1995). In most systems studied, the induction of isoprenoid and phenylpropanoid synthesis under conditions of stress is the result of increased transcription of genes encoding the corresponding biosynthetic enzymes (Fahrendorf and Dixon, 1993; Uhmann and Ebel, 1993; He et al., 1998; Alex et al., 2000). Coordinate transcriptional control of biosynthetic genes is the major mechanism dictating the final level of secondary metabolites in plant cells. This regulation of biosynthetic pathways is achieved by specific transcription factors.
Transcription factors are sequence specific DNA-binding proteins that interact with the promoter regions of target genes, and modulate the rate of initiation of mRNA synthesis by RNA polymerase II. These proteins regulate gene transcription depending on tissue type and/or in response to internal signals, for example plant hormones, and to external signals such as microbial elicitors or UV light. External signals may induce production of internal signals. For several plant species, it has been shown that elicitor-induced accumulation of secondary metabolites is mediated by the stress hormone jasmonic acid (Memelink et al., 2001). Transcription factors have been isolated and characterized for two plant metabolic pathways, leading to biosynthesis of flavonoids and terpenoid indole alkaloids (TIA), respectively (Vander Fits and Memelink, 2000; Memelink et al., 2001).

The activation of xanthophylls cycle has been detected in several plants subjected to drought stress (Foyer 2001). Tannins and flavonoids are amongst the main phenolic substances induced in plant's defence response to abiotic stress (Van Tunen and Mol, 1991; Booker et al., 1996). Murphy et al (2002) have identified several treatments that affect the accumulation of phenolics, including osmotic stress by addition of PEG or mannitol to the culture medium and hormone treatments simulating biotic stress (ethylene and methyl jasmonate). These treatments affect accumulation of secondary metabolites differently. Osmotic stress increases the concentration of flavonoids, particularly the anthocyanin. Copper sulphate ions act as an elicitor and cause in an increase in PAL in palisade cells while phenolic compounds increase in spongy cells, giving rise to necrotic punctuate region in Phyllanthus tenellus plants. This elicitor may be a useful tool in the understanding of the regulation of biosynthetic phenolic pathways in Phyllanthus tenellus and diversity of plant defence response against plant biotic and abiotic stress (Santiago et al., 2000). There are reports that therapeutically active compounds phyllanthin and hypophyllanthin, enhanced at certain levels of Cd due to abiotic stress (Varica Rai et al., 2005). Quite frequently a relatively low yield of active components and difficulties in standardization are bottlenecks in medicinal plant exploitation. Modern analytical methods, biotechnology approaches, genomics, proteomics and metabolomics are now a day applied in medicinal plant research and contribute to the advancement of the field. Molecular studies have revealed that a wide variety of species express a common set of genes and similar proteins (Skriver and Mundy, 1990; Vilardell et al., 1994), which get induced upon exposure of plant to various kinds of biotic (insects and pathogen) and abiotic (salinity, drought, low and high temperature etc.) stresses. These genes can be modified and over expressed into the
medicinal plants enabling them to withstand various kinds of stresses and accumulate secondary metabolites at high levels.

Recent research shows that the applications of metabolic engineering are technically possible in plants at the experimental level: increasing vitamin A content (Ye et al., 2000); increasing essential oil production (Mahmoud and Croteau, 2001); decreasing lignin deposition (Abbott et al., 2002); stimulating the bioconversion of secondary metabolites to medicinally important alkaloids (Vander Fits and Memelink, 2000); improving tomato flavor (Wang et al., 2001) and producing biodegradable plastics in plants (Bohmert et al., 2000). Several of these products are now in development phase and are likely to be coming forward for regulatory approval over the next several years. However, despite the increasing number of proteins isolated from plant species there is a lack of genes encoding key secondary metabolism enzyme. Functional genomics to identify novel genes encoding key secondary metabolism enzyme is the need of the hour. Functional genomics approaches are powerful tools to accelerate comprehensive investigations of cellular metabolism in specialized tissues or whole organisms (Dixon, 2001). Yet, related to plant secondary metabolism, only a few reports have been published on such studies, which include the use of comparative quantitative trait loci mapping (Kliebenstein et al., 2001), 2D gel electrophoresis-based proteomics (Decker et al., 2000) or transcript analysis tools, such as differential display (Schoendorf et al., 2001 Yamazaki and Saito, 2002), EST databases (Lange et al., 2000, Shelton et al., 2002, Suzuki et al., 2002) and micro arrays (Aharoni et al., 2000, Guterman et al., 2002). Nevertheless, still little is known about the genetics that control quantitatively and qualitatively natural variation in secondary metabolism. Because of the lack of extensive genomic data for the vast majority of medicinal plants, it is difficult to use the commonly used microarray-based approach for transcriptome analysis in these plant systems. Such an approach requires prior development of large EST or cDNA clone collections (Aharoni et al., 2000 Guterman et al., 2002). As such, the cDNA-amplified fragment length polymorphism (AFLP) technology (Breyne and Zabeau 2001; Breyne, et al., 2002, Breyne et al., 2003) offers an attractive alternative to identify genes involved in plant secondary metabolism. This method provides quantitative expression profiles, does not require prior sequence information, and, most importantly, allows the identification of novel genes. Here, we applied this technology, in combination with targeted metabolite analysis, on jasmonate-elicited tobacco bright yellow 2 (BY-2) cell cultures. A transcriptome of nearly 600 jasmonate-modulated genes could be composed that was

Jamia Hamdard
compared with the observed jasmonate-induced shifts in biosynthesis of tobacco metabolites and similar transcriptome analyses previously reported for tobacco and *Arabidopsis* plants.

2.3 Importance of aromatic plant phenols in biological systems

Plants have almost limitless ability to synthesis aromatic substances, most of which are phenols or their oxygen–substituted derivatives (Geissman, 1963, Harborne 1995, 1998). Phenolic compounds are aromatic secondary metabolites formed essentially via the shikimic and/or melodic pathways (Geissman and crout, 1969). Modifications in their basic structure such as oxidation, glycosylation and methylation have lead to a large range of phenolic substances with different biological functions (Gottlieb, 1982; Van Tunen and Mol, 1991; Nicholson and Hammerschmidt, 1992). The first reaction in the phenylpropanoid pathway is catalysed by phenylalanine ammonia-lyase (PAL), the branch point enzyme between primary Shikimate pathway (Harrmann, 1995) and secondary (phenyl propanoid) metabolism (Halibrock and Scheel, 1989; Lewis and Yamamoto 1990) converting L Phenylalanine to trans-cinnamic acid (Hanson and Havir, 1981).

Natural phenolic compounds make a considerable contribution to the nutritional quality of fruits and fruit products, which play an important role in the daily diet. They also play a key role in anti-oxidative defence mechanism in biological system and they may have inhibitory effect on mutagenesis and carcinogenesis. Natural phenolic acids belong to two different classes, hydroxybenzoic acid (HBA) and hydroxycinnamic acid (HCA). They are derived from two non-phenolic molecule, benzoic and cinnamic acids, respectively. Phenolic acids are widely represented in fruits, although their distribution may vary considerably according to species, cultivar and physiological stage. Phenolic acids are of great interest to man because they contribute to the sensory and nutritional qualities. Most natural antioxidant posses a polyphenolic structure and a large number of reviews have been published concerning their origin and role (Ho, 1992a; Shahidi et al., 1992; Harborne 1994; Meltzer and Maltzerud, 1997; Haslam, 1998). Phenolic acids can act as endogenous precursor for many of other phenolic molecules founds in plants.

The phenylpropanoid Pathway in vascular plants ultimately leads to important natural products such as the lignins, lignans, and flavonoids (Croteau et al., 2000). Flavonoids have a range of biological effects in a great number mammalian cell systems, *in vitro* as well as *in vivo* flavonoids have been shown to possess antiviral and endocrine effects, activity on mammalian enzymes, effects on the modulation of immune and inflammatory
Table 2. Economically important plant-derived metabolites.

<table>
<thead>
<tr>
<th>Species</th>
<th>Metabolites</th>
<th>Use/Potential Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artemesia annua</td>
<td>artesinin</td>
<td>antimalarial</td>
</tr>
<tr>
<td>Atropa belladonna</td>
<td>atropine/ hyoscyamine</td>
<td>parasympatholytic</td>
</tr>
<tr>
<td>Buddleia globosa</td>
<td>verbascoside</td>
<td>antihypertotoxic</td>
</tr>
<tr>
<td>Catharanthus roseus</td>
<td>vincristine</td>
<td>anticancer</td>
</tr>
<tr>
<td>Chondrodendron tomentosum</td>
<td>d-tubetanine</td>
<td>muscle relaxant</td>
</tr>
<tr>
<td>Coleus blumei</td>
<td>rosmarinic acid</td>
<td>anti-inflammatory</td>
</tr>
<tr>
<td>Colchicum autumnale</td>
<td>colchicine</td>
<td>anticancer</td>
</tr>
<tr>
<td>Digitalis purpurea</td>
<td>digitoxin/digoxin</td>
<td>cardiotonic glycoside</td>
</tr>
<tr>
<td>Dioscorea spp</td>
<td>diosgenin</td>
<td>oral contraceptive</td>
</tr>
<tr>
<td>Forsythia suspensa</td>
<td>suspensaside</td>
<td>antasthmatic</td>
</tr>
<tr>
<td>Lithospermum spp.</td>
<td>lithospermic acid</td>
<td>antigonadotropic</td>
</tr>
<tr>
<td>Origanum vulgare</td>
<td>galangin</td>
<td>antimutagen</td>
</tr>
<tr>
<td>Podocarpus jaborandi</td>
<td>imidazole alkaloids</td>
<td>glaucoma</td>
</tr>
<tr>
<td>Rauwolfia serpentina</td>
<td>reserpine</td>
<td>antihypertensive</td>
</tr>
<tr>
<td>Rheum officinale</td>
<td>anthrones</td>
<td>cathartic</td>
</tr>
<tr>
<td>Salvia miltiorrhiza</td>
<td>salvianolic acid A</td>
<td>antilulcer</td>
</tr>
<tr>
<td>Thymus vulgaris</td>
<td>thymol</td>
<td>anticaries/ antibacterial</td>
</tr>
<tr>
<td>Zingiber cassumunar</td>
<td>curcuminoids</td>
<td>anti-inflammatory</td>
</tr>
</tbody>
</table>
cell functions, effects on smooth muscle, and effects on lipid peroxidation and oxyradical production (Harborne, 1994; Formica and Regelson, 1995; Meltzer and Malterud, 1997). Lignans, a group of relatively simple di phenol, consist of two cinnamooyl alcohol monomers derived from the general phenylpropanoid pathway which shares the same molecular origins as the much more complex polymeric lignins. However, the biosynthetic pathway to the lignans diverges from lignin at the early stage of the phenylpropanols, primarily coniferyl and sinapyl alcohols, which are formed within the general phenylpropanoid pathway. Substantial evidence now indicates that the biosynthetic pathways to the lignans and lignins are fully independent after this stage (Lewis et al., 1998). Phenolic substances have different biological functions, as defence chemicals lignin and lignan protect the cell wall from attack by insects, microorganisms, and even other plants by imparting rigidification to the cell wall. (Nicholson and Hammerschmidt, 1992; Ayer and Loike, 1990). The diverse antimicrobial phenolics such as daidzein, genistein and psoralen synthesized and accumulated during phytopathogen invasion (Graham, 1991; Nicholson and Hammerschmidt, 1992) in plants. The activation of the Rhizobium nod genes involved in root nodulation is mediated by nutritional stresses for example, low nitrogen induce flavonoids such as 7,4-dihydroxyflavone (Coronado et al., 1995) and isoflavonoids may act as signal molecules during plant–mycorrhiza interaction (Paiva et al., 1994). The specific location of anthocyanin in the plant epidermal layer may protect internal cell layers against UV-B radiation (Braun and Tevini, 1993) and an increase in furanocoumarins leads to plant protection against high levels of ozone (Eckey–Kaltenbach et al., 1994).

Besides their importance in plant biology, many secondary metabolites of phenolic nature have been employed in human phytotherapy. For example semi synthetic cytotoxic lignans derived from podophyllotoxin (etoposide, teniposide) are used in cancer therapy and show antiviral properties (Smollny et al., 1998). Phenolic compounds synthesized by Phyllanthus L., Species are potent antieplastic and inhibitors of the DNA polymerase of the hepatities B virus (Venkateswaran et al., 1987; Blumberg et al., 1989). The phenolic compounds of Phyllanthus emblica are inhibitors of immuno-deficiency virus replication with only a few side effects (Pettit et al., 1990; El-Mekkawy et al., 1995). Also methanolic extracts of some species have been shown to be potent antiphedic and analgesic agents (Kuster et al., 1994; Santos et al., 1994; Calixto et al., 1997). Among various phytopharmaceuticals, plant phenolics are an important group of
secondary metabolites having diverse food processing, dietary and medicinal applications.

2.4 *Phyllanthus amarus* as Phytomedicine

The use of plants, plant extracts and plant-derived chemicals in the treatment of diseases, in supplementing foods and in making cosmetics is firmly rooted in the past and still developing. In recent years, researchers have examined the effects of plants used traditionally by indigenous healers and herbalists to support liver function and treat diseases of the liver. In most cases, research has borne out the traditional experience and wisdom by discovering the mechanisms and modes of action of these plants, as well as confirming the therapeutic effectiveness of certain plants or plant extracts in clinical studies.

Plants of the genus *Phyllanthus* have been widely used against various liver diseases, stomach ailments like diarrhoea, dysentery, dropsy, urinogenital problems and also as external application for edematous swelling and inflammation by traditional medicinal practitioner over the years. An increasing interest in these plants has been provoked more recently by reports demonstrating that the aqueous extract of some species of *Phyllanthus* exhibit potent *in vitro* as well *in vivo* inhibition against hepatitis B virus (Venkateswaran *et al.*, 1987; Thyagarajan *et al.*, 1988; Blumberg *et al.*, 1989; Thyagarajan *et al.*, 1990; Shead *et al.*, 1992), and its wide usage in traditional medicine, suggesting it might possess other biological effects as well (Unander and Blumberg, 1991; Unander, Webster, and Blumberg 1991). *Phyllanthus* has been intensively studied clinically for its antiviral effects. A systematic review of 22 randomized clinical trial showed that *Phyllanthus* species have positive effect on antiviral activity and show positive effects on liver biochemistry in chronic hepatitis B virus infection (Calixto *et al.*, 1998, Liu *et al.*, 2001). Disruption of hepatitis B virus polymerase activity, mRNA transcription and replication (Lee *et al.*, 1996), antiviral activity against hepatitis B virus and possibly against the reverse transcriptase of retroviruses supports its role as an antiviral agent (Unander, 1998). The anti hepetities B virus property of *P. amarus* at cellular level and its use in the treatment of acute and chronic hepetities B were reported by Thyagarajan (1996),Jayaram *et al.*, (1997). Bhattacharya *et al.* (2003) reported antiviral activity of *P. amarus* root extracts against the HCV surrogate virus BVDV. Herbal preparations containing *P. amarus* for various liver disorders have been proved to have anti hepatotoxic activity (Ram, 2001). CCl4 -induced hepatotoxicity in the liver of rats, as judged by the raised serum enzymes, glutamate oxaloacetate transaminase and glutamate
pyruvate transaminase, was prevented by pretreatment with the extracts of *Phyllanthus niruri*, demonstrating its hepatoprotective action (Harish and Shivanandappa, 2006). This is an important ingredient in many ayurvedic preparations, especially for the treatment of jaundice. The major lignans, phyllanthin and hypophyllanthin which were reported from *P. niruri* (Row et al., 1966; Ajaneyulu et al., 1973) and also present in *P. amarus* were shown to be antihepatotoxic against carbon tetrachloride and galactosomine induced hepatotoxicity in primary cultured rat hepatocytes (Syamasunder et al., 1988). Antiviral activity, expressed as the inhibition *in vitro* viral DNA, was present in extracts of both roots and shoots, as well as in extracts from plants ranging in age from a month to about six month old (under et al., 1991). These results suggested the inherent presence of the active compound(s) and raised the possibility that active principle would be produced in a callus culture. The antihepatotoxic activity of *P. amarus* and other *Phyllanthus* species *in vivo* has also been reported (Sane et al., 1995, Sane and Kuber, 1993). Phyllanthin and hypophyllanthin are reported as the major active principles present in the whole plant of *P. amarus* (Row et al., 1966, Sharma and Singh, 1993), hence this plant could be standardized with reference to these markers.

The lignans niranthin, phyltetralin and nirtetralin isolated from aerial parts of *P. amarus* exhibited marked anti-inflammatory properties and suggest that these lignans are the main active principles responsible for the traditional application of this plant for anti-inflammatory properties (Kassuya et al., 2005). Screening of plant extracts for the treatment of malaria on Java showed strong antimalarial and antibabesial activity (Trimurningsih et al., 2005, Subeki et al., 2005). Aqueous extracts inhibited the HIV-key enzymes e.g. integrase, reverse transcriptase and protease (Calixto et al., 1998, Notka et al., 2004). Furthermore, the plant has been reported to possess antibacterial, antifungal and antiviral properties (Verpoorte and Dihal, 1987; Macre et al., 1988). *Phyllanthus* species have long been used in folk medicine to treat a broad spectrum of disorder, and there are numerous references to controlled assays. Unander et al. (1990, 1991, 1992, and 1995) published an extensive, four-part survey of the usage of bioassays in the genus *Phyllanthus*. In Brazil and many other countries, infusion of leaves, stems and roots of several *Phyllanthus* species (e.g., *P. coroliniensis*) have been used in folk medicine for thousands of year for treatment of intestinal infections, diabetes, hepatitis B virus and disturbances of the kidney and urinary bladder (Morton, 1981; Olive-Bever, 1983; Calixto et al., 1998). Phytosterolss, quercetin, gallic acid ethyl ester and geraniin were identified in *P. coroliniensis* and pharmacological studies with quercetin, gallic acid

Jamia Hamdard
ethyl ester and some flavonoids have shown antinociceptive action in mice (Cechinel Filho et al., 1996). *P. amarus* extract could also increase the antioxidant defence mechanism in mice and thereby protect the animals from radiation induced cellular damage (Kumar et al., 2004). Aqueous extracts of *p. amarus* has reportedly shown to have effect on blood glucose in non-insulin dependent diabetic patients (Moshi et al., 2001). Species like *Phyllanthus tenellus* have been used in kidney disease as a diuretic and to dissolve renal stones (Pio-Correa 1974). The studies on callus and root extract of these different species have shown the presence of Phyllemblin, a tannin which has antimicrobial activity, hydrolysable tannin which inhibit DNA and RT, genanin and its derivatives which showed high activity in the inhibition of HIV RT and angiotensin-converting enzyme involved in diabetic complications (Ueno et al., 1988; Ogata et al., 1992; Unander, 1996). The methanolic extracts of callus culture of *P. niruri, P. corcovadensis* and *P. tenellus* exhibit potent analgesic properties against neurogenic and inflammatory pain in mice (Santosh et al., 1994). Hindu Physician considered the plant deobstruent, diuretic, astringent and cooling agent. They prescribed the dry powder or fresh juice for jaundice. The plant was also used in skin diseases like scabby, offensive sores and bruises. In western part of India it was used as diuretic in gonorrhoea and acidity of urine. The root with rice water was a remedy for menorrhagia. In chronic dysentery, the plant along with fenugreek was given. The centre for indigenous knowledge of Indian herbal resources (CIKIHR) is collecting information on the use of herbs for treating jaundice and other liver dysfunctions. Already, many locally used herbs have been identified as belonging to the genus *Phyllanthus*. In north India the species used to treat jaundice are *Phyllanthus fraternus* and *Phyllanthus amarus*, and in Bengal *Phyllanthus debilis* is used. Hydrolysable tannins viz., phyllanthusin D, amarin, amarulone and amaromic acid; alkaloids viz norsecurinine sobubbialine, epibubbialine, diarylbutane, nyrphyllin and anecolignan, phyllninurin were reported as minor compounds from *P. amarus* (Murali et al., 2001). Murali et al. (2001) also reported 98% recovery of phyllanthin and hypophyllanthin from *Phyllanthus amarus* following HPLC method. Kale et al. (2001) also estimated phyllanthin and hypophyllanthin from *Phyllanthus niruri*. High performance thin layer chromatographic method (HPTLC) were described for the determination of the bioactive lignans, phyllanthin and hypophyllanthin content in dried sample of *P. amarus* (Sane et al., 1997). Srividya et al. (1995) reported *P. amarus* as potential diuretic, hypotensive and hypoglycemic drug for human. Four new phytocconstituents phyllanterpenyl ester (diterpene ester) pentaconsaayl ester and
heptacosanoic acid (hydrocarbon) phyllanthusone (tetraterpene ketone) have been isolated from the root of Phyllanthus fraternus (Gupta et al., 1999). Amarin, novel hydrolysable tannin together with geraniin, corilagin, 1, 6-diglloylglucopyranoside as well rutin and quercetin-3-0-glucopyranoside were isolated from the polar fraction of P. amarus (Foo, 1993)

2.5 Tissue culture

Practically any plant transformation experiment relies at some point on tissue culture. There are some exceptions to this generalization but the ability to regenerate plants from isolated cells or tissues in vitro underpins most plant transformation systems, and can often prove to be the most challenging aspect of a plant transformation protocol. Key to success in integrating plant tissue culture into plant transformation strategies is the realization that a quick (to avoid too many deleterious effects from somaclonal variation) and efficient regeneration system must be developed. However, this system must also allow high transformation efficiencies from whichever transformation technique is adopted.

Two concepts, plasticity and totipotency, are central to understanding plant cell culture and regeneration. Plants, due to their sessile nature and long life span, have developed a greater ability to endure extreme conditions and predation than have animals. Many of the processes involved in plant growth and development adapt to environmental conditions. This plasticity allows plants to alter their metabolism growth and development to best suit their environment. Particularly important aspects of this adaptation, as far as plant tissue culture and regeneration are concerned, are the abilities to initiate cell division from almost any tissue of the plant and to regenerate lost organs or undergo different developmental pathways in response to particular stimuli. When plant cells and tissues are cultured in vitro they generally exhibit a very high degree of plasticity, which allows one type of tissue or organ to be initiated from another type. In this way, whole plants can be subsequently regenerated. This regeneration of whole organisms depends upon the concept that all plant cells can, given the correct stimuli, express the total genetic potential of the parent plant. This maintenance of genetic potential is called ‘totipotency’. Plant cell culture and regeneration do, in fact, provide the most compelling evidence for totipotency. Realization of regeneration capacity in vitro depends on knowledge of requirements for stimulation of the morphogenic response. Unfortunately genetic, epigenic and physiological status of the explant is still limited and in practice the general approach is to find out the most appropriate chemical
or physical stimuli to provoke totipotency of the cell. Laibach (1925, 1929), Gautheret (1934), White (1934, 1937), Van Overbeck et al (1941), Skoog (1944), Loo (1945), and Murashige and Skoog (1962) have contributed in establishing a strong foundation for the application of this versatile technology. The important morphological application of plant tissue culture is micropropagation. The use of micropropagation is of much importance in medicinal plants. The important medicinal plants are otherwise becomes endangered due to heavy exploitation and low propagation response. Micropropagation includes auxiliary budding, induction of adventitious and somatic embryogenesis. When propagated by conventional methods, take a long time for multiplication, have low rates of fruit set, poor seed germination, and are often under protection or threatened with extinction. The pollution of the human environment brings the risk that collecting plants from natural environments may not be safe for health. The alternative to this situation is the rapid multiplication of plants and their cultivation under special condition. The efficacy of medicinal herbs is affected by different environmental factors. Temperature, rainfall, day length and soil characteristics are some of the factors which affects the potency of the medicinal plants. A plant may grow well in different situations, but fail to produce the same constituents. The production of crude drugs is subject to the vagaries of the climate, to crop disease, to varying methods of collection and drying which influence quality, and to the inherent variation of active constituents arising from plants of the same species having different genetic characteristics. To overcome this problem, recently, one of the rapidly expanding areas of pharmacognosy has involved the application of tissue culture of plant cells, tissues and organs in the study of medicinal plants.

This technology offers advantages over conventional methods of propagation for a rapid and large-scale multiplication of productive plants under in vitro condition, irrespective of season, with conservation of space and time (Nehra and Kartha, 1994; Rao et al., 1996a). Micropropagation of medicinal plants and the problems involved has been discussed by many (Murashige, 1978; Evans et al., 1981 a, b; Ammirato, 1983; Flick et al., 1983; Hussey, 1983; Murashige, 1984; Bajaj, 1986; Hussey, 1986; Withers and Anderson, 1986). These establish the authenticity/efficacy of in vitro technology and its profitability.

2.5.1 In Vitro Propagation Techniques

The biotechnological tools are important to select, multiply and conserve the critical genotypes of medicinal plants by adopting techniques such as micropropagation, creation

Jamia Hamdard
of somacional variations and genetic transformations. *In vitro* propagation involves cell culture systems of a range of ex-plant tissues and mostly micropropagation is achieved from organised tissues by multiplication of meristems and auxiliary buds. In many cases it provides an opportunity to maintain type-to type plant species and the propagation system can produce a large number of plants from a single clone. Plant regeneration from shoot and stem meristem has yielded encouraging results in medicinal plants like *Catharanthus roseus*, *Cinchona ledgeriana* and *Digitalis spp.* (Furmanowa et al., 1986, Luckner et al., 1984). The production of tropane alkaloids by hairy root culture has been reported in several medicinal plants like Atropa, Datura and *Hyoscyamus*. Different response and development pathways have been observed in *in vitro* grown medicinal plant depending on various external and internal factors. In broad terms, two methods of plant regeneration are widely used in plant transformation studies, i.e. somatic embryogenesis and organogenesis.

In somatic (asexual) embryogenesis, embryo-like structures, which can develop into whole plants in a way analogous to zygotic embryos, are formed from somatic tissues. These somatic embryos can be produced either directly or indirectly. In direct somatic embryogenesis, the embryo is formed directly from a cell or small group of cells without the production of an intervening callus. Though common from some tissues (usually reproductive tissues such as the nucellus, styles or pollen), direct somatic embryogenesis is generally rare in comparison with indirect somatic embryogenesis. In indirect somatic embryogenesis, callus is first produced from the explant. Embryos can then be produced from the callus tissue or from a cell suspension produced from that callus. Organogenesis relies on the production of organs, either directly from an explant or from a callus culture. There are three methods of plant regeneration via organogenesis. The first two methods depend on adventitious organs arising either from a callus culture or directly from an explant. Alternatively, auxiliary bud formation and growth can also be used to regenerate whole plants from some types of tissue culture. Organogenesis relies on the inherent plasticity of plant tissues, and is regulated by altering the components of the medium. Regeneration of medicinal plant from shoot tip, auxiliary bud, leaf, stem segments, shoot bud, hypocotyls, cotyledons, roots anthers, and seedlings has been established. Regeneration via embryogenesis has also been reported in medicinal plant. Multiple shoot induction from shoot tip explant, auxiliary bud region in nodal segment, internodal explant in various plant on MS or B5 medium supplemented with cytokinin BAP has been reported by various groups. Regeneration through shoot tip is reported
from Coriendrum sativum (Sehgal, 1972), Mentha viridis (Codaccioni, 1978), Arctostaphylos uva-ursi (Linebrink and krans, 1982), Poeniculum capillaceum (Furmanowa et al., 1981), Salvia officinalis (Sarnetekii and Meszceryakova, 1983), Artemisia umbelliformis (Ledder et al., 1984), Digitalis lanata (Luckner et al., 1984), Catharanthus roseus (Furmanowa et al., 1986). Induction of multiple shoots was achieved from auxillary region of single node stem segments on MS medium with BAP and NAA (Reddy et al 1998). Auxillary bud proliferation in nodal segments was also reported from Hedeoma multilorum, Centella asiatica (L.) with BAP and NAA (Koroch et al., 1997; Tiwari et al., 2000). Regeneration via shoot bud in nodal segment of Dioscorea detioidea (Furmanowa et al., 1985), Dendronix regia (Gupta et al., 1996), Morus Alba (Hassanein et al., 2003), and Cunila galoikes (Fracaroet al, 2001) were also reported. Direct organogenesis in in vitro culture of shoot internodes were reported by Rakousky and Matousek (1994), Becker (2000).

Several workers have reported tissue culture of herbaceous medicinal plant genus Phyllanthus. Rajasubramaniam et al. (1997) first reported shoot tip and nodal culture in Phyllanthus fraternus using BAP or kinetin in Gamborg medium (B2). Auxillary shoot proliferation of Phyllanthus caroliniensis using nodal segments was developed on Murashige and skoog’s medium (MS) or Anderson Rhododendron medium (AR) supplemented with BA, Kinetin or 2ip (Catapan et al, 2000), Shoot tip culture of Phyllanthus amarus in Murashige and skoog’s medium supplemented with kinetin /BAP singly or in combination with IAA has been reported (Bhattacharyya et al., 2001). Multiple shoots were also reported from Phyllanthus amarus shoot tip, nodal and internodal explant when explants were cultured on Murashige and skoog’s medium supplemented with different concentration of BAP and Kinetin (Ghanti et al., 2004). Cytokinins played a predominant role in multiple shoot regeneration. The effect is even more pronounced when two types of cytokinins were used in combination. Maximum shoot multiplication response was reported in Gossypium hirsutum L. cultivar CIM-443 when both BAP and Kinetin was used in 1.0 mg /L concentration (Ali et al, 2004). Best results for multiple shoot formation in Citrus aurantifolia (lime) were obtained with 1mg /L BAP and 0.5mg/L kinetin (Al-Khayri et al., 2001). The studies available on callus cultures of Phyllanthus species are on P.emblica, P. urinaria, P. amarus, P abnormis, P.caroliniensis, P.tehelius, P. fraternus and P.niruri and on transformed root cultures of P.niruri (Unander, 1991; Ishimaro et al., 1992; Santos et al., 1994; Rajasubramaniam et al., 1994; Catapan et al., 2000). Khanna and staba (1968) described induction and growth
of callus in the woody *P. emblica* on MS medium containing 2,4-D. Induction of callus from stem and branch pieces of *Phyllanthus amarus*, *P. urinaria* and *P. abnormis* on MS medium in presence of 2,4-D or IBA alone and in combination was reported by Under (1991). High frequency callus initiation and growth was achieved when nodal segments of *P. carolinensis* were placed on MS medium supplemented with 2,4-D (Catapan et al., 2000). *In vitro* morphogenesis via organogenesis was achieved from callus culture of diverse plant species from various explant type. Callus induction and *in vitro* plantlet regeneration was optimized on root, hypocotyl, cotyledon and leaf explant of *Saussurea obvallata* (DC) and best results were obtained using leaf explant in MS medium supplemented with BA and NAA (Dhar et al., 2005). Fast growing calli were reported from hypocotyls and root explant of *Paspalum simplex* on MS medium containing 2,4-D and kinetin but plant regeneration was achieved when medium was supplied with BAP and NAA (Molinari et al., 2003). Vengadesan et al., (2000) reported induction of callus on MS medium supplemented with BAP and 2,4-D from hypocotyls explant and regeneration of adventitious bud in *Acacia sinuata* on same medium containing BAP and IAA along with coconut milk. Hypocotyls explant of *P. amarus* showed adventitious shoot differentiation on B5 medium supplemented with BAP in combination with 2,4-D, profuse rooting occurred from the hypocotyls explant on medium supplemented with BAP and NAA (Rajasubramaniam et al., 1994). Thus a role of cytokinin and auxin can be observed in callus induction and plantlet regeneration. Thu et al. (2003) reported induction of differentiating callus from cotyledonary region of germinating seedlings of *pegionpea* grown on B5 medium supplemented with BAP (1mg/L). Shoot induction was obtained when calli were transferred on the same medium with lower BAP (0.2 mg/l) concentration. *Emblica Officinalis* (Euphorbiaceae) formed a continuously growing callus on MS medium supplemented with an auxin (2, 4-D or IAA) and a cytokinin (K or BAP). Subculture of callus on MS with BAP (0.2 mg/l) and IAA (0.1 mg/l) resulted in formation of shoots and embryo-like structures in 50% and 8% cultures, respectively. Regeneration of shoots was more frequent when both BAP (0.2 mg/l) and IAA (0.1 mg/l) were present than on BAP (0.2 mg/l) alone (Sehgal et al., 1985). Incubation period also play a important role in regeneration of callus. Callus subcultured beyond 3 weeks failed to regenerate (Kumar et al., 1983), but George and Eapen (1994) reported retention of regeneration potential of callus even after 2 years.
2.6 Stress protein

In plant, stress protein include a great diversity of heat shock proteins and proteins that accumulate in response to cellular dehydration (Bohnert et al., 1995, Ingram et al., 1996), osmotin, like protein among them (Zhu et al., 1995). A space-filling model of tobacco osmotin is represented by using Ras Mol software (Figure 1). Osmotin is a stress responsive multifunctional protein adapted to NaCl and desiccation. Singh et al., (1985) studied the proteins in tobacco var. Wisconsin 38 and gave the name to a basic 24 KDa protein that accumulates in cells on osmotic stress adaptation. Barthakur et al. (2001) reported that osmotin could be involved in osmotic adjustment of plants under stress either by facilitating the accumulation or compartmentation of compatible solutes. Further they suggested that osmotin is a proline rich protein and its degradation could also possibly lead to increased accumulation of proline, at least under conditions when the protein is over produced. Stines et al. (1999) reported similar results where it has been shown that proline accumulation in developing grapevine fruits is independent of and not associated with either increases in steady--state levels of proline dehydrogenase protein. The function of osmotin gene through transgenic approach is well documented in providing protection against fungal infection. First evidence of osmotin protein activity as an antifungal agent came by over expressing the tobacco osmotin gene in transgenic potato plants (Liu et al., 1994). Sense and antisense expression of the osmotin gene further confirmed the antifungal nature of the protein and the sense transgenics showed increased tolerance to late blight caused by Phytophthora infestans (Zhu et al., 1996). Transcription of an osmotin gene is induced by at least 10 signals namely ABA, ethylene auxin, infection by TMV, salinity, lack of water, cold, UV light, wounding, and fungal infection. Osmotin shares several characteristics with many PR proteins, including mRNA induction by TMV and wounding, the presence of an n-terminal signal peptide, and vacuolar targeting of the basic isoform. Such similarities have lead to the inclusion of osmotin in PR-5 protein family (LaRosa et al., 1992) whose members are homologous to the sweet–testing protein thaumatin isolated from the fruits of Thamnotococcus donilli, present in the salt adapted cells (Bressan et al., 1987). Eleven PR proteins groups have been isolated and characterized in various plants (Fritig et al., 1998). Most PR members are induced by phytopathogens or abiotic stresses and have antifungal activity in in vitro assay (Vigers et al., 1991). Wołoszuk et al. (1991) and Vigers et al. (1991) demonstrated in-vitro that osmotin has anti-fungal activity against a variety of fungi including P. infestans, Candida albicans, Neurospora crassa and
Fig 1: Space filling model of Tobacco
(Nicotiana tobaccum cv. Wiscosin 98)
Osmotin
Tricoderma reesi. PR-5 proteins are divided into three subgroups: acidic (PR-s), basic (osmotin), and neutral proteins (osmotin like protein, OLP). OLP has been identified in tobacco, potato, tomato, and Arabidopsis (King et al., 1988, Capelli et al., 1997). Three OLP cDNAs isolated from potato responded to a variety of abiotic stimuli and pathogenic fungus (Zhu et al., 1993). High levels of osmotin like protein gene expression were detected in the roots and flowers, which is typical of other PR proteins including osmotin, B-1, 3-glucanase and chitinase (Larosa et al., 1985; Meeks et al., 1989; Neale et al., 1990). It is possible that PR genes play important role in plant growth and development and later evolved to have additional function in plant defense. Two clear inducer of osmotin gene, ABA (abscisic acid) and ethylene are the plant hormones most related to both osmotic and pathogen stresses. ABA modulates gene expression in several osmotic stresses such as freezing, drought, and high salt (skriver et al., 1990) and has been shown to activate a number of plant defense-related genes encoding PR-5 osmotin (Larosa et al., 1985; Singh et al., 1989). The role of dehydrins, late embryogenesis proteins and related proteins, which accumulate in seeds and water-stressed vegetative tissues, has been reviewed by Close, (1997). Transgenic rice expressing HVAAl, a gene encoding a late embryogenesis abundant protein from barley, has increased tolerance to drought and NaCl as shown by simple growth analysis (Xu et al., 1996). Aquaporins (water channel proteins) are clearly involved in controlling water movement between cells (Maural, 1997) and may be a target for manipulating water flow through the plant with potential for improving water relations and water use efficiency. There is evidence for a nitrogen-activated protein kinase type system in plants analogous to that involved in yeast osmoregulation. In support of such a system, a protein kinase is rapidly activated in maize roots exposed to low water potential (Conley 1997).

2.7 Bioengineering of medicinal plants

Plant genetic engineering is a young science. It was only in 1983 that gene was first expressed in genetically transformed plant tissue (Bevan et al., 1983). This science has made a giant leap in the past 20 years of its growth as is evident from the fact that already genetically engineered plants resistant to insect pests and herbicides have been produced. Transgenic technology has emerged as a boon for improving agronomic traits for different applications in recent years (Galun and Breiman, 1997). Globally, the area under cultivation of transgenic crops has increased from 1.7 m ha in 1996 to 44.2 m ha in
Advances in the technology have been due to the development of a range of Agrobacterium mediated and direct DNA delivery techniques, along with the appropriate tissue culture techniques for regenerating whole plants from plant cells or tissues in a large number of species.

Recombinant DNA technology offers new opportunities to directly modify plant secondary product synthesis through metabolic engineering, and the availability of cloned genes encoding terminal enzymes of isoprenoid or phenylpropanoid compounds may provide the means to assess product function by using reverse genetics (i.e. antisense or sense suppression) in species that are amenable to genetic transformation. Establishment of hairy root cultures following infection with Agrobacterium rhizogenes displayed enhanced production of secondary metabolites which occur naturally in untransformed roots, resulting in amounts of secondary compounds comparable or even higher than those present in intact roots (e.g. Sharp and Doran, 1990; Zarate, 1999). Over the last 10-15 years, the successful genetic transformation of plants has been reported in about 200 species including agricultural crops, trees, ornamentals, fruits and vegetables. Such genetic modification has improved specific crop traits, such as resistance to pathogens, to herbicides and to various environmental factors including drought and floods (Bajaj, 1999). Following on from this success genetic transformation of medicinal plants has been attempted, primarily to enhance the production of various pharmaceuticals, flavooids, and pigments. Transgenic cultures and plants have been reported for about 70 of these species (Bajaj, 1999), and interesting results are already appearing. In (1991) Bailey suggested that metabolic engineering of biosynthetic networks might be achieved by application of recombinant DNA methods, but also suggested how complex cellular responses to genetic perturbation could complicate the predictive metabolic design

2.7.1 Gene Transfer Technology in Plants Ti Plasmid-Based Transformation

Workers in the late 1970’s including Eugene Nester and Mary Dell-Chilton discovered that the formation of tumors depends on the presence of a large plasmid in the Agrobacterium cells. This plasmid was named the Ti (tumor inducing) plasmid. The Ti plasmids contain genes that are required for formation and maintenance of the tumor as well as genes for the synthesis and utilization of opines. Genes required for opine utilization and T-DNA transfer are located outside the T DNA region. Genes required for
tumor maintenance; Roi, (root induction, involved in IAA synthesis [tryptophan mono-oxygenase]) and Shi (shoot induction, involved in cytokinin synthesis [Isopentenyl transferase]) are located within the T-DNA along with OCS (octopine synthase) or NOS (nopaline synthase) genes. None of these genes is required for T-DNA transfer. Genes involved in T-DNA transfer are located outside the T-DNA and are collectively called the Vir region. This region includes at least 6 loci called virA-G. The virulence area containing different silent vir genes does not enter the plant genome but are required for T-DNA transfer. These genes are activated by wound tissue metabolites, such as lignin precursors and acetylsyringone (Melchers et al., 1989), which may explain why tissue wounding appears to be a pre requisite for efficient infection. Upon infection of a host plant a small portion of the Ti plasmid, the T-DNA is actually transferred to the plant cells which are stably integrated in to the chromosome of the plant (Rogers et al., 1986; Schell, 1987; Weising et al., 1988; Zambryski, 1988).

Several gene transfer system have potential for plant transformation. Currently, the most widely used method for the production of transgenic plants is the Agrobacterium mediated gene transfer system (Cramer and Radin, 1990; Klcc et al., 1987; Kuhlemeier et al., 1987; Weising et al., 1988; Zambryski, 1988). This procedure is an indirect method of gene transfer because gene must first be transferred in to Agrobacterium. Agrobacterium tumefaciens and the related species Agrobacterium rhizogenes are gram-negative soil bacterium. These species, which belong to the Rhizobiaceae, are natural metabolic engineers able to transform or modify mainly dicotyledonous plants (Tepfter, 1990), although there are reports on the infection of monocotyledonous plants (Hiei et al., 1994; Ishida et al., 1996). Infection results in the formation of a tumor of usually undifferentiated tissues (callus) at a wound site and hairy root respectively. Strains of Agrobacterium can cause different symptoms and form tumors that have different characteristics. One distinguishing characteristic is the production of opines which are novel derivatives of arginine. Agrobacterium that can induce tumors can utilize opines as their sole source of carbon and nitrogen. Another species called Agrobacterium rhizogenes that carry a Ri plasmid cause a “hairy root” disease and usually produce agropine.

2.7.1.1 T-DNA transfer

The critical feature of the T-DNA is the presence of direct repeats that demarcate the left at right borders. The right border is critical for T-DNA transfer; however, the left border is less necessary. Other sequences within the borders are not required for T-DNA
transfer. The virD gene products recognize the right border sequences and introduce a nick in the right repeat. New DNA synthesis occurs, displacing the old strand, which is packaged by the virE product (ssDNA binding protein). The transfer continues until it reaches a second nick near the left border. How the DNA is transported to the plant cell, becomes double stranded and is incorporated is not understood. Incorporation into the plant genome appears to occur randomly although A: T rich regions are preferred (this is similar to some transposons) no defined target sequence has been recognized. Incorporation at the right border is precise (occurs at second base of the repeat ±1 base). The left side is more variable but usually occurs <100 bases from the left repeat. Single or multiple copies can be integrated, multiple copies can be present as tandem repeats but how this occurs is unknown. Another sequence of interest is called the overdrive sequence located immediately adjacent to the right border. Overdrive enhances the transfer process; it functions in cis and is position independent. VirC1 and possibly virC2 seem to interact with the overdrive sequence. Anything located between the right and left borders of the T-DNA can be transferred to plant cells. The process of gene transfer from Agrobacterium tumefaciens to plant cells implies several essential steps 1) bacterial colonization 2) induction of bacterial virulence system 3) generation of T-DNA transfer complex 4) T-DNA transfer and 5) integration of T-DNA into plant genome. However, efficient infection normally requires wounding and/or a rapidly dividing cell suspension culture. Attachment occurs at a cell wall surface of a wounded plant. Two plant cell wall proteins have been proposed to mediate bacterial attachment, a vitronectin-like protein (Wanger and Matthysse, 1992) and a rhicadhesin-binding protein (Swart et al., 1994). However, the possible role of these plant proteins in bacterial attachment has not been shown by genetic analysis. Agrobacterium attaches to plant cells in a popular manner in a two-step process. The first step is likely mediated by a cell-associated acetylated, acidic capsular polysaccharide (Reeuws et al., 1997). The second step in attachment involves the elaboration of cellulose fibrils by the bacterium, which enmeshes large numbers of bacteria at the wound surface (Deasey and Matthysse, 1984; Matthysse et al., 1981, 1982). Agrobacterium can inefficiently infect plant cells in the absence of a wound site, most likely by entry through stomata (Escudero and Hohn, 1997). Successful genetic transformation of plants using Agrobacterium relies on optimization of a combination of several factors including plant tissue harvesting and manipulation, bacterial colonization and infection, T-DNA transfer, and post-transformation culture conditions. The importance and advantage of using the Agrobacteria system is that by genetic
engineering and DNA recombinant technology it is feasible to remove most of the genes in the T-DNAs of both *A. tumefaciens* and *A. rhizogenes*. Subsequent cloning of the desired foreign gene(s) follows, which can then be co-transformed and integrated into the host genome after infection where it can encode specific enzymes dealing with the formation of wanted metabolites or other goals. The defined integration of transgenes, potentially low copy number and preferential integration into transcriptionally active regions of the chromosomes are some important features. In conclusion *A. tumefaciens* has been used directly to genetically modify many plants, and in most cases this requires the tedious and time consuming process of *in vitro* culture and plant regeneration resulting in low levels of transformation in certain plant species. In addition, two other major problems encountered when using *Agrobacterium* are susceptibility and hypersensitivity of some plant cells or tissues. The latter leads to tissue necrosis and cell death, although the use of antioxidants such as polyvinylpolypyrrolidone and dithiothreitol can restore plant viability and inhibit necrosis (Perl et al., 1996). To increase transformation efficiencies in these species, much effort has been placed on understanding the molecular mechanisms of T-DNA transfer (Holford et al., 1992; Fullner et al., 1996) with the goal of manipulating and controlling the transfer process. Although these studies have been useful and have led to enhanced transformation rates in many plants, there have been few studies on the dynamics and optimization of agrobacterial colonization and infection, the initial events upon which gene transfer between plant and host are ultimately dependent.

### 2.7.1.2 Direct gene transfer

Monocot plant species, including the most important crops species, are typically resistant to infection by *Agrobacterium* and so are recalcitrant to Ti plasmid-based gene transfer methods. Recently, however, ‘super virulent’ Ti vectors that have duplicated *vir* regions have been developed that can be used to infect some monocot species including rice and maize. However, typical methods for monocot transformation have used a variety of methods to directly introduce DNA into the plant cells. Direct gene transfer technique include chemical treatment, electroporation, microinjection, Micro particle bombardment, lasers (Weber et al., 1988), ‘whisker’ of silicon carbide (Kaeppler et al., 1990), the pollen tube pathway, sonication, liposome mediated method. Direct gene transfer technique delivers foreign DNA directly into the plant cell.
For both chemical treatment and electroporation, cell walls of the cells to be transformed are removed by digestion with a mixture of hydrolases. The resulting protoplasts can easily be transformed by chemical treatments such as PEG (Farely et al., 1986; Potrykus, 1988; Weising et al., 1988). For electroporation of plant protoplast or germinating pollen cells are exposed to high-voltage electric charge for short time. Cell membrane develops large pores through which DNA moves inside the cell and can be incorporated into the plant genome (Zimmermann et al., 1984; Farely et al., 1986; Saunders et al., 1989; Weising et al., 1988). Unfortunately, with the exception of rice, protoplasts of most monocots lose their ability to regenerate. Therefore, methods to introduce DNA through an intact cell wall had to be developed. The breakthrough in this area was the development of the so called "gene gun". The gene gun is part of a method called the biolistic (also known as bio ballistic) method, in which, the explant is subjected to the shower of high-velocity particles (usually tungsten or gold) that are coated with DNA (Klein et al., 1988; McCabe et al., 1988; Schell et al., 1987). By accelerating this DNA-particle complex in a partial vacuum and placing the target tissue within the acceleration path, DNA is effectively introduced (Gan, 1989). DNA from the particles could reach the nucleus and the genes were expressed transiently. If a selectable marker gene was included in the plasmid, it was possible to select transformed cells with stable DNA insertions. If the target tissue is able to regenerate, whole transgenic plants could then be produced. Uncoated metal particles could also be shot through a solution containing DNA surrounding the cell thus picking up the genetic material and proceeding into the living cell. A perforated plate stops the shell cartridge but allows the slivers of metal to pass through and into the living cells on the other side. The cells that take up the desired DNA, identified through the use of a marker gene (in plants the use of GUS is most common), are then cultured to replicate the gene and possibly cloned. The biolistic method is most useful for inserting genes into plant cells such as pesticide or herbicide resistance. Different methods have been used to accelerate the particles: these include pneumatic devices; instruments utilizing a mechanical impulse or macroprojectile; centripetal, magnetic or electrostatic forces; spray or vaccination guns; and apparatus based on acceleration by shock wave, such as electric discharge (Christou and McCabe, 1992). Microinjection is achieved by placing a small needle into the cell and injecting DNA directly into the nucleus (Fraley et al., 1986; Potrykus, 1988).
Enhancement in transformation frequency was observed when cells were mechanically injured by sonication prior to Agrobacterium infection (Trick and Finer, 1997, 1998). This technique is known as Sonication Assisted Agrobacterium Transformation (SAAT). More recently however, there has been a tendency to modify the transformation protocols by avoiding sophisticated tissue culture steps (Brar et al., 1994; Chowriria et al., 1995, 1996; McKently et al., 1995). In planta transformation is very successful in Arabidopsis. Thousands of transformed lines were produced in a few years by applying Agrobacterium culture to Arabidopsis seeds (Feldmann and Marks, 1987; Feldmann, 1992). Chang et al. (1994) used another approach called “clip ‘n Squirt” method for in planta transformation for Arabidopsis. Transformed plants were recovered by “vacuum infiltration” method. Plants at early stages of flowering were uprooted and submersed in a fresh culture of Agrobacterium and the bacteria are introduced into plants by vacuum infiltration. Treated plants were then grown and selfed and allowed to set seeds. Successful in planta transformation with high transformation rate (2.9% to 76%) in forage legume Medicago truncatula by Agrobacterium vacuum infiltration has been reported (Trieu et al., 2000). Transgenic plants were obtained only when seedlings were subjected to 4°C/14d vernalization treatments.

2.7.1.3 Bioengineering of medicinal plants

Plants are subjected to several harsh environmental stresses like drought, salinity, low and high temperatures etc that limits the productivity of plants and crops. Drought, high salinity and freezing impose osmotic stress on plants. Although plant species vary in their sensitivity and response to the decreased in water potential caused by drought, low temperature or high salinity, it may be assumed that all plants have encoded capability for stress perception, signaling and response. Stresses trigger a wide range of plant responses at molecular, cellular and whole plant level viz., altered gene expression, cellular metabolism, changes in growth rates and crop yields. The metabolism of carbohydrates, fats, proteins, and nucleic acids is adversely affected upon exposure of tissues to stress conditions. Effects of stresses have been noted with respect to almost all major physiological processes including photosynthesis, nitrogen fixation, nitrogen metabolism and respiration. Different developmental stage of the plant growth including seed germination, seedling growth, vegetative growth, reproductive growth, seed maturation and senescence are differentially affected in response to stress conditions.
Many plants and other organisms cope with osmotic stress by synthesizing and accumulating some compatible solutes, which are termed as osmoprotectants or osmolytes. The solutes that accumulate during the osmotic adjustment fall into three major groups: amino acids (e.g. proline), quaternary amines (e.g. glycine betaine, dimethylsulfoniopropionate) and polyol/sugars (e.g. mannitol, trehalose) (Taniura et al., 2003). Many eubacteria, algae, and higher plants accumulate free proline in response to osmotic stresses (Delauney and Verma, 1993), which can increase the tolerance of plants to abiotic stresses (Hong et al., 2000). The primary function of compatible solutes is to maintain cell turgor and thus the driving gradient for water uptake. Recent studies indicate that compatible solutes can also act as free-radical scavengers or chemical chaperones by directly stabilizing membranes and/or proteins (Lee et al., 1997; Hare et al., 1998; Bohnert and Shen, 1999; McNeil et al., 1999; Diamant et al., 2001). Over expression of compatible solutes in transgenic plants can result in improved stress tolerance. Proline is synthesized from glutamate via glutamic- semialdehyde (GSA) and D1-pyrroline-5-carboxylate (P5C). P5C synthase (P5CS) catalyzes the conversion of glutamate to P5C, followed by P5C reductase (P5CR), which reduces P5C, to proline. In the reverse reaction, proline is metabolized to glutamate in a feed-back manner, via P5C and GSA with the aid of proline dehydrogenase (ProDH) followed by P5C dehydrogenase (P5CDH). Proline biosynthetic pathway is represented (Figure 2). Transgenic tobacco (Nicotiana tabacum) over expressing the P5CS gene that encodes P5CS produced 10- to 18-fold more proline and exhibited better performance under salt stress (Kishor et al., 1995). Freezing tolerance was achieved by transforming tobacco with the same gene (p5cs; Konstantinova et al., 2002). The elevated proline also reduced free-radical levels in response to osmotic stress and significantly improved the ability of the transgenic seedlings to grow in a medium containing up to 200 mM NaCl (Hong et al., 2000). Betaines are quaternary ammonium compounds, i.e. amino acid derivatives in which the nitrogen atom is fully methylated. Many important crops, such as rice, potato and tomato, do not accumulate glycine betaine and are therefore potential candidates for the engineering of betaine biosynthesis (McCue and Hanson, 1990). Genetic engineering for glycine betaine biosynthesis in non-accumulating plants has been extensively reported (Lilius et al., 1996; Hayashi et al., 1997; Alia et al., 1998a, 1998b; Sakamoto et al., 1998, 2000; Sakamoto and Murata, 2002). A number of “sugar alcohols” (mannitol, trehalose, myo-inositol and sorbitol) have been targeted for the engineering of
Fig 2: Proline biosynthetic pathway
compatible-solute overproduction. Tarzynski et al. (1993) introduced a bacterial gene that encodes mannitol 1-phosphate dehydrogenase into tobacco plants, resulting in mannitol accumulation and enhanced tolerance to salinity. In addition, transgenic tobacco plants carrying a cDNA encoding myo-inositol O-methyltransferase (IMT1) accumulated D-mannitol and, as a result, acquired enhanced photosynthesis protection and increased recovery under drought and salt stress (Sheveleva et al., 1997). Engineered overproduction of these compatible solutes provides an opportunity to generate more tolerant plants. But genetic manipulations of compatible solutes do not always lead to a significant accumulation of the compound (except some cases of proline overproduction; Chen and Murata, 2002), suggesting that the function of compatible solutes is not restricted to osmotic adjustment, only accumulation of compatible solutes may also protect plants against damage by scavenging of reactive oxygen species, and by their chaperone-like activities in maintaining protein structures and functions. Incorrect gene expression of compatible solutes often causes pleiotropic effects (e.g. necrosis and growth retardation) due to disturbance in endogenous pathways of primary metabolisms. To minimize the pleiotropic effects, the over-production of compatible solutes should be stress-inducible and/or tissue specific (Garg et al., 2002). Tolerance to abiotic stresses so far has mainly been achieved through engineering for increased cellular levels of osmotically active solutes. In general, the rate-limiting step in developing modified plants through biotechnological means is the isolation of the relevant genes. Gene isolation and cloning through molecular biology research can be based on mRNA or protein expression, differential screening, differential display technique, DNA insertions such as transposon or T-DNA insertions, map based cloning and method of random cDNA sequencing and genome sequencing (46 and 47). Differential hybridization technique has been exploited for isolation of a number of stress-responsive genes (48 and 49). Genomic wave in the recent years has witnessed complete sequencing of the genomes of several prokaryotic and eukaryotic systems. Arabidopsis thaliana is the first higher plant for which complete nucleotide sequence has been determined (Arabidopsis thaliana genome initiative 2001). The genomic science forms an important input for identifying genes linked to different metabolic reactions, including those associated with abiotic stress biology. Functional genomics has the potential for random sequencing of the stress induced cDNA libraries as well as isolation and sequencing of the differentially expressed cDNA clones. The discovery of novel genes, determination of their expression patterns in response to abiotic stress, and an improved understanding of their
roles in stress adaptation (obtained by the use of functional genomics) will provide the basis of effective engineering strategies leading to greater stress tolerance. Proteomics science is a step further for development of protein maps of stress-induced metabolic alterations. A large number of abiotic stress tolerant transgenic plants has been produced in recent years. This success has been achieved by increasing the cellular levels of proteins that control levels (such as rate limiting enzymes in proline and glycine betaine synthesis) and transport functions (such as vacuolar antiporter proteins like H^+/ATPase and H^+/PPi ases) or regulate gene activation (i.e., transcription factors and signaling components). The engineered over expression of genes for enzymes that increase putative osmoprotectant compounds such as proline, polyols, or fructans (Delauney and Verma, 1993; Tarczynski et al., 1992; Pilon-Smits et al., 1995) can protect plants to greater extent from stress, even when they are present at low and osmotically insignificant level (Sakamoto and Murata, 2002). Research on the role of proline oxidation-linked regulation of the pentose phosphate pathway and how this could regulate metabolite flow to purine and aromatic pathways for producing novel plant metabolites are carried out by Shethy, Professor of Food Science, University of Massachusetts. Kavi et al. (1995) developed transgenic tobacco plant using pyrroline carboxylate synthetase and reported increase biomass production and enhance flower development under salinity stress. Zhu et al. (1998) developed transgenic rice using same gene and reported increase proline accumulation and thereby counteract salinity stress. Kim et al. (2002) reported cloning and characterization of pHOSM, the gene encoding osmotin protein and demonstrated that its expression is associated primarily with development and wound signals related to plant defence. Barthakur et al. (2001) over-expressed the osmotin gene under the control of constitutive CaMV 35S promoter in primary transgenic tobacco (cv. Harrison Special) plants and demonstrated increase in free proline content (2-6 fold increase) compared to the wild type plants during water stress and after recovery from stress.