Chapter I
GENERAL INTRODUCTION

1.1. Importance of medicinal plants

Knowledge of plants and knowledge of healing have been closely linked from the time of man’s earliest social and cultural groupings. The medicine man was usually an accomplished botanist. In ancient times, botany and medicine were considered to be virtually one and the same discipline. About 1500 A.D. the two began to separate from their close association, to the advantage of both sciences. Plants are the most important source of medicine. The application of plants as medicine dates back to prehistoric period. The early civilization reveals that a considerable number of drugs used in modern medicine have figured in ancient manuscripts such as the Rigveda, the Bible, the Quran, the Iliad, the Odyssey and the History of Herodotus. The ancient Chinese were the first to use the natural vegetation as medicine for the past 6000 years. In India, the Ayurvedic systems of medicine have been in use for over three thousand years. Charaka and Susruta, two of the earliest Indian scientists had sufficient knowledge of the properties of the Indian medicinal plants. The Charaka Samhita and the Susruta Samhita are esteemed medicinal works and considered even today as the treasures of literature on indigenous medicines (Khan and Khanum, 1998).

Plants synthesize a bewildering array of chemical compounds with a variety of physiological roles, starting from air, water, minerals and sunlight as the energy source. Various compounds produced by plants can be broadly grouped into two categories namely, “Primary Metabolites” and “Secondary
Metabolites”. The secondary metabolites are also referred to as “Natural Products”. It is believed that more than 100,000 different structures of secondary metabolites are synthesized by organisms, to a tune of $10^9$ tons per year (Bowles and Leyser, 1994). Out of this, more than 80% are found in plants (Harborne, 1993). They are used either as medicines/pharmaceuticals, foods, neutraceuticals (foods as well as medicines used for preventive and curative treatments), flavours, colours, spices or fragrances by humans. Although secondary metabolism was first recognized in 1873 (Sachs, 1873), its function was elucidated only in 1888 (Stahl, 1888). But upto 1950s, secondary metabolites were regarded as end products of deluxe metabolism and relegated to the rank of ‘waste products’. It was only during 1960s, when eco-relations were discovered (Kurz and Constabel, 1998).

Medicinal plants have their (secondary metabolites) values in the substance or substances present in various tissues. These produce specific physiological action in the human body. The more important of these substances are alkaloids and flavonoids, compounds of carbon, hydrogen, oxygen and nitrogen. Besides these substances, glucosides, essential and fatty oils, resins, gums, mucilage, tannins, etc. are also have large use. These active principles may be present in the storage organs of the plant, viz: roots, seeds, leaves, etc (Khan and Khanum, 1998).

Medicinal plants are the most exclusive source of life saving drugs for the majority of the world’s population. Medicinal plants have been the subject of man’s curiosity since time immemorial (Constable, 1990). Almost every civilization has a history of medicinal plant use (Ensminger et al., 1983). Approximately 80% of the people in the world’s developing countries rely on
traditional medicine for their primary health care needs, and about 85% of traditional medicine involves the use of plant extracts (Vieira and Skorupa, 1993). World Health Organization (WHO) reported that 21,000 plant species have medicinal values in the world. India is considered to be a storehouse of medicinal plants and it has a record of more than 10,000 species, of which 1,800 medicinal plants are used in ayurvedha, 4,700 in traditional medicinal practice, 1,100 in Siddha medical system, 750 in unani, 300 in homeopathy, 300 in chinese system of medicine and 100 in allopathic system (Ameerjahan, 2001). Plants are probably the best cell factories on this planet from which a diversity of >100,000 low molecular secondary metabolites have been isolated, with the estimated total number of plants exceeding 500,000 (Hadacek, 2002).

In recent years, traditional system of medicine has become a topic of global importance. Although modern medicines are available in developed countries, herbal medicines (phytopharmaceuticals) have often maintained popularity for historical and cultural reasons. Many of the plant species that are considered as medicinal herbs have been scientifically evaluated for their possible medical applications.

The resurgence of public interest in plant based medicine coupled with rapid expansion of pharmaceutical industries have necessitated an increased demand for medicinal plants, leading to over-exploitation that threatens the survival of many rare species. Also, many medicinal plant species are disappearing at an alarming rate due to rapid agricultural and urban development, uncontrolled deforestation and indiscriminate collection. Consequently, in vitro culture techniques are imperative for conservation of the
rare medicinal plants. The present investigation involves some in vitro experimental studies of an important medicinal plant viz., *Psoralea corylifolia* L.

1.2. Taxonomic position and botanical description of *Psoralea corylifolia* L.

*Psoralea corylifolia* L. (synonyms - *Cullen corylifolia* (L.) Medik.) commonly known as “Babchi”, or “Bukchi” in Hindi (Yadava and Verma, 2005) and in Tamil – Karpogam or Karpo-karishi (Sharma *et al.*, 2001) belongs to the family Leguminosae, sub family – Papilionaceae or Fabaceae (Bentham and Hookers, 1862-1883). It is an erect herbaceous annual, 60-120 cm height with grooved and gland-dotted stems and branches. Leaves simple, broadly elliptic, rounded and mucronate at apex, clothed with white hairs on both surface, main nerves 5, originating from base. Flowers bluish purple, in dense, axillary, 10-30 flowered racemes. Pods 5 mm long, subglobose, slightly compressed, closely pitted, black, beaked without hairs. Seeds oblong flattened, dark brown with an agreeable aromatic odour and taste.

1.3. Distribution of *Psoralea corylifolia* L.

*Psoralea corylifolia* L. is an endangered and rare herbaceous medicinal plant distributed in the tropical regions of the world (Jain, 1994). It is also distributed in most of the plain districts of India, Pakistan, Sri Lanka, Burma, China, Yunnan, Arabia and Socotra. It is found throughout India, especially in Tamil Nadu, Himalayas, Dehra Dun, Oudh, Bundelkhand, Bengal, (some valley in) Bihar, Madhya Pradesh, Uttar Pradesh, Rajasthan, Deccan, Gujarat, Punjab, Karnataka and Andhra Pradesh (Bhattacharjee, 1998; Sharma *et al.*, 2001).
1.4. Magnitude of *Psoralea corylifolia* L.

*Psoralea corylifolia* L. is an important medicinal plant, the powdered seeds of this plant have been used since ancient time, in the early Ayurvedic system of medicine, for the treatment of skin depigmentation (also called vitiligo or leucoderma). The basis of this therapy was reported as far back as 2000 B.C. in a sacred book called Atharva Veda, in which the cure, practiced by ancient Hindus, was described (Fitzpatrick and Pathak, 1959). The plant is native to India, used in Folk, Siddha and Ayurvedic system of medicine (Jain, 1994; Bourgaud *et al.*, 1995). The plant is well recognized in Indian and Chinese folkloric medicine (Sahrawat and Chand, 2001). This plant has been used traditionally as medicine in India and China and recommended for certain skin diseases (Jiangninga *et al.*, 2005; Yadava and Verma, 2005).

1.4.1. Chemical constituents

Chemical studies by various workers revealed a number of compounds belonging to different chemical groups such as furanocoumarins, coumestrol group, chalcones, and flavones. A number of chemical constituents including coumarins, flavonoids and meroterpene phenols were isolated from this plant (Ji and Xu, 1995). The plant contains major compounds like coumarins *viz.*, psoralen, isopsoralen (Jois *et al.*, 1933; Jois and Manjunath, 1934), psoralidin (Chakravarti *et al.*, 1948), isopsoralidin (Siddappa and Sathyabhamma Devi, 1957), raffinose (Bhattacharji, 1961), bakuchiol (Gupta *et al.*, 1979), bavachin, angelicin, daidzein (Gupta *et al.*, 1980; Bouque *et al.*, 1998). The plant also consists of bavachinin, 4-o-methylbavachalcone, isobavachalcone, neobavachalcone, bakuchalcone, isoneobavachalcone, stigmasterol, psoralone, isopsoralone, limonene, geranylacetate, corylinal and neobavaisoflavone including the methyl ethers of the two compound, psoralenol, 5'-formyl-2',
4-dihydroxy-4\textquotesingle methoxychalcone, and bavachromanol. Mainly, psoralen and isopsoralen (angelicin) are present in the seeds, roots and leaves and daidzein, sitosterol, coumesterol and trilaurin in roots (Sharma et al., 2001). The plant species is also characterized by the presence of essential oil, terpenoids and resin (Jois et al., 1933; Gupta et al., 1979).

1.4.2. Pharmacological task

Psoralea corylifolia L. has been specially recommended in the treatment of stomachic, deobstruent, anthelmintic, diuretic, vitiligo and also for certain skin diseases like psoriasis and leprosy (Kotiyal and Sharma, 1992; Sharma et al., 2001) and prescribed both for oral administration and external application in the form of a paste or ointment (Orient Longman, 1996). It is also used in indigenous medicine as laxative, aphrodisiac, anthelmintic, diuretic and diaphoretic in febrile conditions (Anand et al., 1978; Rastogi and Mehrotra, 1993). Psoralen and isopsoralen were being investigated against several diseases including AIDS (Bhattacharjee, 1998). Some of the chemical constituents of the plant exhibited antibacterial, antitumour, broadening coronary artery and estrogen-like activities (George and Pandalai, 1949; Anand et al., 1978; Kotiyal and Sharma, 1992; Ji and Xu, 1995; Latha and Panikkar, 1998; Citarasu et al., 2003). There were also several reports on antioxidative (Jiangninga et al., 2005) and antiplatelet properties (Tsai et al., 1996). Quite recently, a simple flavonoid, 4-o-methoxy flavone, from this plant was reported to display antifungal activities (Kotiyal and Sharma, 1992; Rajendra et al., 2004) and a few coumarins of this plant showed antibacterial activities (Khatune et al., 2004; Yin et al., 2004). Recently, High-Performance Liquid Chromatography (HPLC) was developed for fingerprint analysis of Psoralea corylifolia (Zhao et al., 2005).
1.4.3. Importance of psoralen and its pharmaceutical interest

Furanocoumarins are important secondary metabolites, class of natural compounds and are mainly derivatives of the linear furanocoumarin psoralen (7H-furo[3,2-g]chromen-7-one) or its angular isomer angelicin (2H-furo[2,3-h]chromen-2-one). Furanocoumarins (psoralens) are of pharmaceutical interest because of their innumerable biological activities. In reference to the traditional use against vitiligo, these molecules can strongly activate melanogenesis. Therefore, apart from their use against depigmentation diseases, they are commonly employed in cosmetics and suntan preparations (Lane-Brown, 1981; Kligman and Forlot, 1989). Another remarkable activity of the furanocoumarins is their capacity to bind covalently with the pyrimidic bases of DNA or RNA (Rodighiero and Dall Acqua, 1976). Consequently, these molecules exhibit antiproliferative properties, against the living cell, by preventing DNA replication. The binding is light dependent, as the addition of psoralens on nucleic acids needs long-wavelength UV. This photo activity of furanocoumarins is being increasingly used in dermatology for photochemotherapy of skin diseases like vitiligo and psoriasis (Fitzpatrick et al., 1974; Scott et al., 1976; Pathak and Fitzpatrick, 1992).

Psoralen is a group of naturally occurring or synthetic compounds with interesting photosensitizing, photobiological and phototherapeutic properties. Psoralen is an interesting compound from the pharmaceutical viewpoint, since it is known to induce skin photosensitization followed by hyperpigmentation. These activities are used cosmetically in tanning products and dermatologically for the photochemotherapy of vitiligo and skin diseases such as psoriasis, mycosis fungoides and eczema (Pathak and Fitzpatrick, 1992; Diawara et al., 2003). Their biological activity increases strongly under the action of ultraviolet
radiation (PUVA), which allows them for the treatment of psoriasis and other skin diseases (Potapenko and Kulkosky, 1988). The medicinal use of the psoralen has been, however, associated with an increased incidence of skin cancer (Sternberg, 1997). The psoralen proved to be both mutagenic and carcinogenic (Young, 1990). The United States National Toxicology Program (NTP/NIEHS) tested 8-methoxypsoralen (xanthotoxin or methoxsalen) for sub chronic toxicity in rats using oral administration (Stern et al., 1997). Psoralen was also found to exhibit anti-HIV activity (Zhou et al., 2000; Shikishima et al., 2001). Psoralen photochemotherapy (PUVA) and narrowband UVB phototherapy had beneficial effects in the treatment of psoriasis (Yones et al., 2005).

1.4.4. Strategy of *Psoralea corylifolia* L. in Indian pharmaceutical industries

Many Indian pharmaceutical industries are using *Psoralea corylifolia* as raw material for producing medicines and some of them are (1) Research Drugs and Pharmaceuticals (Gujarat) Private Limited, Ahmedabad, India (2) Pioneer Enterprise, Mumbai, India and (3) Dr. J. R. K's Siddha Research and Pharmaceuticals Private Limited, Chennai, India etc.

1.5. Choice of in vitro culture techniques for medicinal plant

In recent years, there has been an increased interest in *in vitro* culture techniques which offer a viable tool for mass multiplication, quick regeneration, germplasm conservation of rare, endangered, threatened, important medicinal plants and secondary metabolite production (Saxena et al., 1997; Sahoo and Chand, 1998; Prakash et al., 1999; Fraternale et al., 2002; Baskaran and Jayabalan, 2005). In the search for alternatives to the production of desirable
medicinal compounds from plants, biotechnological approaches, specially, plant tissue cultures, are found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites (Ramachandra Rao and Ravishankar, 2002). *In vitro* cultures provide a potential alternative to the mass harvesting of plants for the purpose of obtaining crude drug extracts (Kang, 2004). Secondary metabolites normally accumulate in *in vitro* at the later stages of the growth cycle when growth slows down or reaches a plateau (Biondi *et al*., 2004).

Research in the area of plant tissue culture technology has resulted in the production of many pharmaceutical substances for new therapeutics. Plant tissue culture techniques have been reported for conservation and multiplication of several medicinal plants (Bhojwani, 1980; Balachandran *et al*., 1990; Bhat *et al*., 1992) and the biosynthesis of secondary metabolites, particularly on plants of pharmaceutical significance, holds an interesting alternative for controlled production of plant constituents (Chadha and Rajendra Gupta, 1995). Plant tissue culture is an alternative method of propagation (George and Sherrington, 1984) and is being used widely for the commercial propagation of a large number of plant species, including many medicinal plants (Rout *et al*., 2000). The conditions for the enhanced production of secondary products in plant tissue cultures have been extensively analyzed (Collin, 2001).

1.5.1. *In vitro* studies and secondary metabolite production

Micropropagation or direct regeneration (absence of an intermediate callus phase) is one of the useful alternative techniques over the conventional methods of vegetative propagation. The plants are regenerated from existing meristems or from non-meristematic tissue using both seedling (Das *et al*., 1996) and mature
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plant (Reddy et al., 1998). The effective and reproducible protocol of micropropagation depends on plant growth regulators (Pattnaik and Debata, 1996; Mohamed et al., 1999), type of explant, complex extracts and amino acids (Lu et al. 1995). It is a suitable method for obtaining a large quantity of genetically homogenous and healthy plant material which can be used for planting (Pierik, 1987).

The induction of callus growth and subsequent differentiation and organogenesis is accomplished by the differential application of growth regulators and the control of conditions in the culture medium. With the stimulation of endogenous growth substances or by addition of exogenous growth regulators to the nutrient medium, cell division, cell growth and tissue differentiation are induced (Tripathi and Tripathi, 2003). The accumulation of secondary metabolites is affected by the degree of cellular differentiation and organization of the tissue (Verpoorte and Memelink, 2002). Also, the contents and distribution of secondary metabolites rely on the developmental stage and physiological condition of the plant (Kang et al., 2004). Moreover, in several studies, the secondary metabolite accumulation was highly proficient in callus cultures (Nigra et al., 1987; Jha et al., 1988). Based on these studies, regenerated plants (from calli derived) were successfully utilized for psoralen analysis.

Research is going on for the application of plant transformation and genetic modification using A. rhizogenes, in order to boost the production of secondary metabolites. Genetic transformation would be a powerful tool for the production of secondary metabolites because of their stable and high productivity in hormone-free culture conditions. Different wild strains of
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Agrobacterium rhizogenes were evaluated for transformation efficiency and secondary products from hairy roots (Pradel et al., 1997; Erkan and Taskin, 1999; Giri et al., 2001). A number of plant species including many medicinal plants have been successfully transformed with Agrobacterium rhizogenes and secondary metabolites obtained in hairy roots (Nussbaumer et al., 1998; Argolo et al., 2000; Souret et al., 2002; Shi and Kintzios, 2003; Jacob and Malpathak, 2005; Li et al., 2005; Kuzma et al., 2006).

However, pharmaceutical companies largely depend upon materials procured from naturally occurring stands which are being depleted rapidly, raising concern about possible extinction. So in vitro propagation, genetic transformation techniques and secondary metabolite production is immediately needed for one of the important medicinal plants of India, Psoralea corylifolia L. Therefore, the present investigation was undertaken with the following objectives to bring about conservation and secondary metabolite production.

 ✓ To standardize an efficient and rapid plant regeneration via micropropagation of P. corylifolia using various explants obtained from in vivo grown plants, culture medium and different concentrations of plant growth regulators.

 ✓ To find out the optimization of the additives and amino acids and their concentration and combination for shoot proliferation.

 ✓ To standardize a reproducible protocol for plant regeneration via direct organogenesis from hypocotyl explants of P. corylifolia.
To standardize a proficient protocol for plant regeneration via indirect organogenesis from leaf, petiole, stem, cotyledon, hypocotyl and TCL hypocotyl explants of *P. corylifolia*.

To develop an efficient protocol for somatic embryogenesis from leaf, hypocotyl and TCL hypocotyl explants through solid and suspension culture.

To standardize a competent protocol for root culture propagation from leaf and hypocotyl explants through solid and liquid culture.

To standardize a suitable transformation protocol for *Agrobacterium rhizogenes* (wild strains) from hypocotyl explants through solid and suspension culture.

To assess the content of psoralen in *in vivo, in vitro* and *ex vitro* tissues of *Psoralea corylifolia* L.