Introduction
Field grown Picrorhiza kurroa plant
Although modern synthetic drugs are in common usage, yet the use of herbal drugs is well accepted, and a continuously high demand for plant material and natural products has been observed. Hence, the approaches of medicinal plant biotechnology currently focus more on distinct natural products and biosynthetic pathways. It is not only plants that are of great interest to the pharmaceutical industry, but also defined natural products. This situation is supported by the fact that ~25% of all drugs dispensed during the 1970s in the USA contained compounds obtained from higher plants (Farnsworth et al. 1976). Moreover, 11% of the 252 drugs considered to be basic and essential by the WHO are isolated and used directly from plant sources (Rates 2001). In addition, approximately 40% of pharmaceutical lead compounds for the synthetic drugs used today are derived from natural sources, including plants.

Today, only 10% of all medicinal plant species used are cultivated, with by far the larger majority being obtained from wild collections. Harvesting from the wild has, however, become problematic, as was seen in the case of Podophyllum and Taxus species, Piper methysticum, Cimicifuga racemosa, Arctostaphylos uva-ursa, and P. kurroa whereby both loss of genetic diversity and habitat destruction have occurred.

From this background the question arises as to whether there is a need for biotechnology and gene technology for medicinal plants. With regard to medicinal plants, biotechnology could be described as a method for enhancing the formation and accumulation of desirable natural products, with the possibilities of their modifications. Biotechnological tools are also important in order to select, multiply and conserve the critical genotypes of medicinal plants.

Today, natural products from plants provide better templates for the design of potential chemotherapeutic agents than synthetic drugs. Paclitaxel (Taxol), podophyllotoxin and camptothecin are some lead molecules which have proved to be Nature’s boon in the treatment of cancer. To meet ever-increasing demands, biotechnological methods offer an excellent alternative, but the economy of such a production is the major hurdle to be
overcome. The successful industrial application of plant cell cultivation for the production of these therapeutic compounds has triggered further research on other promising plant-based chemotherapeutics. As the initial hurdles for large-scale cultivation of plant cells have been overcome, the new areas of concern to produce desired products are synergistic product enhancement strategies, along with in-depth knowledge of the biosynthetic pathway. Understanding of biochemistry, enzymology, physiology, bioreactor design and the application of proteomics and genomics are other areas on which to focus. Several points in a given metabolic pathway can be controlled simultaneously, either by overexpressing and/or suppressing several enzymes, or through the use of transcriptional regulators to control endogenous genes. However, identification of biological pathways and their components remains a challenge for medicinal plants.

It is not an easy task to produce these compounds economically by extraction from intact plants and meet the ever-increasing demand. This may be due to very low concentrations of these active compounds in plants, the slow growth rate of plants, complex accumulation patterns, and high susceptibility to geographical and environmental conditions. Other possible reasons are the non-availability of uniform and unadulterated quality plant material in quantities sufficient for industrial production and uneconomical chemical synthesis, particularly for large complex molecules. Therefore, biotechnological methods offer an excellent alternative for production of such compounds. The most difficult problems encountered in paclitaxel production are supply limitation arising from its very low concentration in the bark of the T. brevifolia (ca. 0.01% dry weight), and the very slow growth of the yew tree (Cragg et al. 1999). In order to isolate 1 kg of taxol, 10,000 kg of dried bark from 3000 T. brevifolia trees must be extracted, whilst almost 2 g of taxol is needed to treat one patient. On the other hand, certain secondary metabolites such as camptothecin are accumulated only after a certain age or maturity of the plant. Hence, it is difficult to increase the area under plantation. To overcome all these hurdles, the industry requires alternative methods of supply of uniform material throughout the year. Plant cell culture technology is undoubtedly one of the appropriate approaches to solve the above-described problems. However, before implementing this approach, the cost of the product and its demand should justify production by biotechnological means. For commercialization,
one must consider the economic aspects and development of feasible bioprocess technologies and relevant reactor design and mode of operations for the production of these plant secondary metabolites. The goal should therefore be to improve the productivity of cells in order to make the production of these compounds commercially viable. Commonly, no single growth-enhancing strategy can produce such a large increase, but in many cases the simultaneous application of multiple strategies results in synergistic interaction and thus an improvement of the yield.

The plant kingdom provides a wide variety of natural products with diverse chemical structures and a vast array of biological activities. Over the years, many of these compounds have found applications in the health sciences such that today, medicinal plants are very important to the global economy (Srivastava et al. 1995), with approximately 85% of traditional medicine preparations involving the use of plant or plant extracts. Much of the traditional healthcare industry is highly dependent on wild populations to supply raw materials for the extraction of medicinally important compounds. The isolation of active compounds from plants is sometimes very difficult due to their extremely low concentrations. Furthermore, when the raw material is scarce or its chemical production is too low, the industry – at present – lacks viable methods to produce the desired plant-derived active compounds. In addition, some active compounds are available only in endemic or extremely rare plants. Consequently, there is great interest in developing alternatives for the production of bioactive secondary metabolites of commercial importance. Biotechnological approaches such as plant cell cultures offer rapid and efficient methods for the production of these high-value medicinal compounds in cultured cells (Oksman-Caldentey and Inze 2004). The potential of plant tissue culture techniques to produce useful secondary metabolites, especially, for drug development, were first perceived during the late 1960s. The major advantages of cell culture systems over the conventional cultivation of whole plants are:

1. Useful compounds can be produced under controlled conditions independent of climatic changes or soil conditions.
2. Cultured cells would be free of microbes and insects.
3. The cells of any plants, whether tropical or alpine, could be easily multiplied to yield their specific metabolites.

4. Automated control of cell growth and rational regulation of metabolite processes would contribute to reductions in labor costs and improvements in productivity.

5. It should be possible to achieve the synthesis of a broad spectrum of secondary metabolites with complex chemical structures using plant tissue culture technology.

In addition, the novel compounds which have not been found previously in plants or have never been synthesized chemically could also be produced by cell cultures. In this respect, many research groups have demonstrated the outstanding metabolic capacities of plant cell cultures, and highlighted the variability of plant cell biosynthetic capacity. Moreover, this variability could be exploited to identify high-yielding cultures for use on an industrial scale (Tabata et al. 1976; Bisaria and Panda 1991; Yamada 1984). Ongoing research in plant cell culture is largely focused on identifying the rate-limiting steps in biosynthetic pathways, while other approaches such as elicitation, precursor feeding, cell immobilization, *in situ* product removal and bioreactor design have led to enhancements in production (Sivakumar et al. 2005).

Plants are capable of synthesizing an overwhelming variety of organic compounds, termed as secondary metabolites usually with unique and complex structures. The evolving commercial importance of the secondary metabolites has during the recent years resulted in great interest in secondary metabolism, and particularly in the possibility of altering the production of bioactive metabolites by means of cell culture technology. Advances in biotechnology, most notably in methods of culturing plant cells and tissues, have resulted in new approaches to the commercial production of rare and endangered plants and the bioactive compounds that they produce.

*P. kurroa* Royel ex Benth (Family: Scrophulariaceae) is a perennial herb also known as kutki or karu mainly found in the North-Western Himalayas at altitudes of 3000-4300 meters. *P. kurroa* is a well-known herb in the Ayurvedic system of medicine and has traditionally been used to treat disorders of the liver and upper respiratory tract, reduce
fevers, and to treat dyspepsia, chronic diarrhea, scorpion sting, etc. The active constituents are obtained from the dried roots and rhizomes. The pharmacological importance of *P. kurroa* has been demonstrated to be due to rich source of hepatoprotective picrosides, Picroside-I and Picroside-II and other metabolites like Picroside-III, Picroside-IV, Apocynin, Androsin, Catechol, Kutkoside, etc (Weings et al. 1972, Stuppner et al. 1989). Powder, decoction, infusion, confection, and alcoholic extract of the drug are prescribed in Ayurveda and Homeopathy. *P. kurroa* has a long, creeping rootstock that is bitter in taste, and grows in rock crevices and moist, sandy soil. The leaves of the plant are flat, oval, and sharply serrated. The flowers, which appear in June through August, are white or pale purple and borne on a tall spike; manual harvesting of the plant takes place October through December.

The pharmacological properties of *P. kurroa* have been demonstrated and validated in modern system of medicine like hepatoprotective (Chander et al. 1990), antioxidant (particularly in liver) (Ansari et al. 1988), antiallergic and antiasthamatic (Dorch et al. 1991), anticancerous activity particularly in liver (Joy et al. 2000) and immunomodulatory (Gupta et al. 2006). A commercial formulation named as Picroliv prepared from *P. kurroa* extracts containing Picroside-1 and kutkoside was launched as a hepatoprotective drug after clinical testing (Ansari et al. 1991). Picroliv has also been shown to have immunostimulating effect in hamsters and helps to prevent infections (Puri et al. 1992; Gupta et al. 2006).

The medicinal herb, *P. kurroa* is self-regenerating in nature, however, unregulated over-harvesting of plants has reduced its populations in the natural habitat to a level that it has been categorised as a threatened plant species nearing to extinction (Kala 2000). The endangered status of *P. kurroa* coupled with its high medicinal value in pharmaceutical and biotechnology industries warrants that alternative means be developed for producing its tissue biomass so that the medicinally important metabolites can be produced under controlled conditions. Tissue culture in particular the micropropagation of *P. kurroa* has been reported (Lal et al. 1988; Upadhyay et al. 1989; Trivedi et al. 2006; Chandra et al. 2006), however, none of those studies have attempted at *in vitro* production of medicinal compounds. Moreover, in most of the previous tissue culture studies on *P. kurroa*, the
shoots were very thin and slender (Lal et al. 1988) with a problem of vitrification (Uppadhay et al. 1989) and poor survival rate of tissue cultured plants in the field conditions.

There are reports on tissue culture of other plant species, wherein the components of tissue culture media have been modified or replaced with low cost substitutes such as sucrose with table sugar (Kaur et al. 2005), omission of agar-agar (Mehrotra et al. 2007) and use of sunlight and tubular skylight (Kodym et al. 2007; Sharma and Singh 1995). The use of shake cultures utilizing liquid culture medium alone (Weathers and Giles 1988) or in combination with solid culture medium (Debergh and Maene 1981; Aitken-Christie and Jones 1987) has also been tried as low-cost alternatives (Earle and Langhans 1975; Takayama and Misawa 1981; Takayama 1991; Paque et al. 1992; Chu et al. 1993).

The identification of genetically superior strains of medicinal plants is another research area of high priority. However, there is no report on the identification of high content strains of *P. kurroa* neither from the natural habitat nor induced through *in vitro* cell cultures. High content strains can be developed through either by induction of variants in callus cultures from different explants or by genetic manipulation of metabolic pathways. Both these approaches require the standardization of *in vitro* conditions for establishment of high frequency callusing and regeneration in *P. kurroa* and the later approach requires thorough understanding about the molecular biology of picrosides biosynthesis, which is completely lacking. The identification of *in vitro* conditions for high frequency callusing and regeneration will also provide suitable starting material for induction of somatic embryogenesis for rapid multiplication and conservation of *P. kurroa*. This study reports high frequency callusing and regeneration from different explants of *P. kurroa*.

Tissue culture has been demonstrated to be a sustainable alternative for the production of medicinally or industrially important metabolites in callus, cell suspension, hairy roots or shoot cultures in various plant species (Vanishree et al. 2004; Vongpaseuth and Roberts 2007). From among these studies, the production of berberine from *Coptis japonica* by Mitsui Petrochemicals (Morimoto et al. 1988), production of vincristine and vinblastine from *Catharanthus roseus* (Zhao et al. 2001), production of ginesinosides from...
*Panax ginseng* (Sivakumar et al. 2005) and production of paclitaxel from different species of *Taxus* (Tabata et al. 2004) are notable case studies wherein the tissue culture processes have been upscaled to a commercial level.

Picroside-I is the major ingredient of Picroliv and, therefore, makes this compound a highly valued secondary metabolite of *P. kurroa*. The only source of Picroside-I at present is from the shoots, roots and rhizomes of *P. kurroa*, which involves extensive isolation and purification steps because the rhizomes not only contain Picroside-I but higher amounts of Picroside-II and other metabolites. There is thus an urgent need of optimizing cell culture conditions in *P. kurroa* for exclusive production of Picroside-I. The only way to overcome pressure from natural habitat of *P. kurroa* is to optimize cell cultures conditions for large-scale production of its metabolites *in vitro* and to conserve quality germplasm. However, the production of metabolites through tissue cultures of *P. kurroa* requires thorough understanding of their biosynthesis and accumulation in different morphogenetic tissue culture stages so as to identify a particular stage which is most suitable and amenable for *in vitro* cultures coupled with biosynthesis and accumulation of Picrosides.

The biosynthesis and accumulation of medicinally important metabolites has been reported to occur in different tissues and organs of plants and is largely influenced by the developmental stage of a particular organ/tissue as well as in response to external stimuli (Campos-Tamayo et al. 2008). The biosynthesis of Picroside-I and Picroside-II is reported to occur differentially in shoots and roots of *P. kurroa* wherein the Picroside-I accumulates preferentially in shoots and Picroside-II in the roots of field grown plants of *P. kurroa* (Chauhan & Sood unpublished). The differential accumulation of Picroside-I and Picroside-II in shoots and roots of field grown plants indicates that the biosynthesis of both these metabolites occurs in specialized cell types. However, what determines the biosynthesis of Picroside-I in the shoots and that of Picroside-II in the roots is not known. Moreover, the biosynthesis and accumulation of Picroside-I and Picroside-II occur in *P. kurroa* at high altitudes and that too during a particular time of a season, which complicates the process of understanding biology of their biosynthesis. The cell cultures offer a suitable biological system with a controlled environment wherein the morphogenetic events can be regulated by
manipulating the levels of growth hormones in the nutrient medium resulting in rapid production of plant metabolites of pharmaceutical importance (Ray and Jha 2001; Tanaka et al. 1995). Tissue cultures of *P. kurroa* have been done for its rapid multiplication (Lal et al. 1988; Uppadhay et al. 1989). However, there is no information pertaining to biosynthesis and accumulation of Picroside-I and Picroside-II in tissue cultures of *P. kurroa*. The identification of developmental events in the tissue cultures of *P. kurroa* starting from original explant such as leaf, nodal and root segments, passing through different morphogenetic stages such as de-differentiation of original explants into a callus mass and then re-differentiation of callus into shoot primordia and fully developed shoots and roots needs to be undertaken so as to look at the dynamics of Picroside-I and Picroside-II biosynthesis and accumulation in morphogenetically different tissue culture stages of *P. kurroa*.

The endangered status of *P. kurroa* also demands multiplication of genetically superior strains, either identified from the nature or developed through cell culture. Tissue culture induced variation known as somaclonal variation has been recognized as a tool for increasing yield, conferring resistances and improving other agricultural traits in different plant species (Larkin and Scowcroft 1981). Somaclonal variation occurs due to epigenetic and genetic changes in plants that become apparent either during or after *in vitro* culture of plant cells, callus or organs (Skirvin et al. 1993; Kaeppler et al. 2000). During tissue culture phase, particularly through callus phase, changes of interest to plant breeders occur, which are heritable and result from changes in the plastid or nuclear genome. The introduction of variation may be either undesirable or useful for horticulturists and plant breeders, and may occur in high frequency during adventitious plant regeneration from long-term callus cultures (Kaeppler et al. 2000). Morphological and cytological analysis such as, karyotypic analysis of metaphase chromosomes and isozyme analysis have been used to determine genetic variations in somaclones and to identify parental hybrids and cultivars (Brown et al. 1993). Numerous investigators have successfully employed RAPD and AFLP molecular markers for genetic analysis of regenerated plants (Toyler et al. 1995) and genotypic screening (Chunuongase et al. 1993). Tissue culture studies have been done in *P. kurroa* (Lal et al. 1988; Upadyay et al. 1989; Trivedi et al. 2007 and Chander et al. 2006), however,
all these studies have focused mainly on development of micropropagation conditions that too with limited success.

The cell cultures also offer a suitable biological system with a controlled environment towards understanding the biosynthesis of picrosides as well as their production. There is a need of investigating the morphogentic control of picrosides biosynthesis in tissue culture of *P. kurroa* so as to enable in not only identifying a particular tissue culture stage which accumulates maximum amount of picrosides but also provides a suitable system to investigate the molecular biology of their biosynthesis. The current study has been undertaken to address the problem of endangered status of *P. kurroa* with the overall goal of optimizing cell culture conditions for rapid multiplication as well as *in vitro* production of medicinally important metabolites with the following objectives:

**OBJECTIVES**

1) Development of a low-cost micropropagation technology for *P. kurroa*

2) Identification of a suitable explant with high frequency callusing and plantlet regeneration

3) Optimization of tissue culture conditions for *in vitro* production of Picroside-I & Picroside-II

4) Understanding biosynthesis and accumulation of picrosides vis-à-vis morphogenetic developments in tissue cultures of *P. kurroa*

5) Generation of somaclonal variants with increased picrosides content and their characterization through DNA fingerprinting