Summary & Conclusions
SUMMARY AND CONCLUSIONS

*Picrorhiza kurroa* is a perennial herb endemic in North-Western Himalayas at altitudes of 3000-4300 metres. It is a rich source of hepatoprotective picrosides, Picroside-I and Picroside-II and other medicinal metabolites like Picroside-III, Picroside-IV, Apocynin, Catechol, Kutkoside, etc. The roots and rhizomes of *P. kurroa* have been extracted recklessly from its natural habitat thus putting it in the category of endangered plant species, which warrants the development of tissue culture technology for its rapid multiplication and sustainable conservation as well as for the production of medicinal compounds. Plants are capable of synthesizing an overwhelming variety of secondary metabolites usually with unique and complex structures. 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 with the advantage that a continuous and reliable source of plant pharmaceuticals is made available. For this purpose axillary shoot tips cultured on MS media modified with different combinations of growth hormones resulted in the identification of MS +IBA (2mg/l) + KN (3mg/l) + sucrose 3% (w/v) + agar-agar 0.8% (w/v) as the best medium for multiple shoot formation with 86.3% shoot apices forming multiple shoots after incubation of cultures at 25±1°C. In order to reduce the cost of media components for commercial production of planting material of *P. kurroa*, sucrose was replaced with table sugar and agar-agar was omitted completely. Out of 6 low-cost media combinations tested, MS liquid medium supplemented with IBA (2mg/l) + KN (3mg/l) + table sugar 3% (w/v) was found to be the best with 27 shoots/explant. Seventy per cent shoots formed roots on half strength MS salts supplemented with IBA (3mg/l) + table sugar 3% (w/v) + agar-agar with an average of 5.6 roots per shoot. The substitution of sucrose with table sugar and omission of agar-agar from the medium resulted in reduction of cost of medium required for production of one plantlet to the tune of 1/30th of the cost of standard nutrient medium. The *in vitro* regenerated plantlets were hardened in potting mixture of vermiculite:sand:soil (1:1:1) and subsequently transferred to the field conditions.
*Picrorhiza kurroa* being pharmacologically important and listed as an endangered herb, optimization of *in vitro* conditions for callusing and regeneration is of paramount importance not only for the selection of cell lines with enhanced content of phytopharmaceuticals or in the genetic transformation of *P. kurroa* but the cell cultures hold a great promise in the *in vitro* production of metabolites. Callus cultures were established from different explants such as leaf discs, nodal and root segments of *P. kurroa*. Callus induction was highest (70%) in root segments followed by leaf discs (56.3%) and nodal segments (38.3%) on MS medium supplemented with 2, 4-D (2 mg/l) + IBA (0.5 mg/l) + sucrose 3% (w/v) + agar-agar 0.8% (w/v). The callus cultures derived from different explants were differentiated into multiple shoots on MS medium containing different concentrations and combinations of BA, KN and IBA. Regeneration was highest in the calli derived from root segments and leaf discs on MS + BA (2 mg/l) + KN (3 mg/l) + sucrose 3% (w/v) + agar-agar 0.8% (w/v) with 76.7% and 72.2% per cent calli forming shoot primordia, respectively. Most of the nodal segment and root derived calli got differentiated into roots rather shoots. The rooted plantlets were acclimatized to the external environment through hardening and eventually transferred to pots.

A commercial formulation named as Picroliv has been prepared from *P. kurroa* extracts. Picroside-1 is the main ingredient of Picroliv. Experiments on optimization of tissue culture conditions for rapid multiplication of *P. kurroa* as well as for induction of callusing and regeneration from different explants were carried out at 25±1°C, which has been reported suitable for tissue culture of different plant species. Estimation of Picroside-I and Picroside-II in shoot and callus cultures formed at 25±1°C showed negligible amount of these medicinal compounds, thereby reconitaring to optimize cell culture parameters for their production. Moreover the shoots formed at 25±1°C were very thin and slender with poor survivability in the field conditions. Further modifications in the media composition did not help to obtain vigourous shoots so that the same can be sucessfully transferred to the field conditions. For this purpose, culturing of shoot apices of *P. kurroa* on nutrient medium, MS + KN (3mg/l) + IBA (2mg/l) + sucrose 3% (w/v) + agar-agar 0.8% (w/v) and incubation at four temperature regimes (10±1°C, 15±1°C, 25±1°C, 30±1°C) showed that the growth and proliferation of shoots was highest in
cultures incubated at 15±1°C. The total shoot biomass yield was highest with 3.73g fresh shoot wt. at 15±1°C compared to 2.01g and 1.33g fresh shoot wt. in shoots formed at
25±1°C and 10±1°C, respectively. The accumulation of Picroside-1 was maximum (2.03 mg/g fresh shoot wt.) in shoots formed at 15±1°C compared to 0.12 mg/g and 1.38 mg/g
in shoots formed at 25±1°C and 10±1°C, respectively. The incubation of shoot cultures
for 6 weeks at 15±1°C resulted in maximum accumulation of Picroside-1, whereas the
accumulation of Picroside-1 was negligible in shoots cultured at 25±1°C and 30±1°C
even after 8 weeks of incubation. The combination of growth hormones KN (3mg/l) and
IBA(2mg/l), which was found suitable for in vitro shoot formation and proliferation at
15±1°C also showed the highest accumulation of Picroside-1 (2.00 mg/g fresh shoot wt)
compared to all other test concentrations and combinations of auxins and cytokinins. The
supplementation of nutrient medium with methyl jasmonate resulted in four fold increase
in the biosynthesis and accumulation of Picroside-1. The current study thus provide a
tissue culture process for in vitro production of Picroside-1 in shoot cultures of P. kurroa
so as to relieve pressure from its natural habitat and thus provides a sustainable strategy
for the conservation of this endangered plant species.

No information is available on the molecular biology of the production of
Picroside-1 and Picroside-II in P. kurroa. The biosynthesis and accumulation of
Picroside-I in shoot cultures of P. kurroa with almost negligible accumulation in roots
and callus cultures and also negligible accumulation of Picroside-II in shoots, roots and
callus cultures prompted us to investigate systematically the biosynthesis and
accumulation of Picroside-I and Picroside-II vis-à-vis different developmental stages in
tissue cultures of P. kurroa. Establishment of callus cultures and plant regeneration from
different explants coupled with estimation of Picrosides in morphogenetically different
developmental stages showed that Picroside-1 accumulates in shoot cultures of P. kurroa
with no detection of Picroside-II. The Picroside -1 content was 1.9 mg/g , 1.5 mg/g,
and 1.01mg/g in leaf, nodal and root segments, respectively. The Picroside-1 content
gradually declined to almost non- detectable levels in callus cultures derived from all
three explants. The biosynthesis and accumulation of Picroside-1 started again in callus
cultures differentiating into shoot primordia and reached to the concentrations
comparable to original explants of leaf and nodal segments in fully developed shoots with contents of 2.00mg/g and 1.50 mg/g in shoots from leaf and nodal derived callus cultures, respectively. The shoots formed from root-derived callus cultures were relatively slow in growth as well as the amount of Picroside-1 content was low (1.01mg/g) compared to shoots derived from callus cultures of leaf and nodal segments, respectively. Negligible amount of Picroside-I (0.01 mg/g fresh explants wt.) was detected in root explants and callus cultures derived from root segments compared to shoots regenerated from root derived calli, which showed accumulation of Picroside-1. There was no change in the biosynthesis and accumulation of Picroside-II, which remained negligible(0.001mg/g fresh wt.) in all tissue culture stages of P. kurroa. The current study concludes that the biosynthesis and accumulation of Picroside-1 is developmentally regulated in different morphogenetic stages of P. kurroa tissue cultures.

P. kurroa being an endangered herb leaves with the alternative of identification of genetically superior strains with high content of Picrosides so as to reclaim its populations in the nature. There is no information as of today on identification of genetically superior strains of P. kurroa neither from its natural populations nor through tissue cultures. For generating somaclonal variants, callus cultures were established from different explants (leaf, nodal and root segments) of P. kurroa on MS + 2,4-D(2.0 mg/l) + IBA (0.5 mg/l) + sucrose (3.0% w/v) + agar-agar (0.8% w/v) and maintained in callus phase for 6 months at 15±1°C rather at 25±1°C, because the former temperature has been found optimum for biosynthesis and accumulation of Picroside-I. The callus cultures were differentiated into shoots on MS + BA (2.0mg/l) + KN (3.0mg/l) + sucrose (3.0% w/v) + agar-agar (0.8% w/v) and a total of 120 somaclones were regenerated. Individual shoots regenerated from callus cultures were considered as one somaclone and these were further multiplied to obtain sufficient tissue for HPLC analysis and DNA fingerprinting. The HPLC analysis of somaclones revealed that most of the plants showed picroside content of ~2.0 mg/g fresh wt., which was comparable to the source tissue culture plants. However, two somaclonal variants, 4 & 16 showed significantly higher Picroside-I content with 3.3 mg/g and 4.6 mg/g fresh wt., respectively. Four somaclonal variants (1, 6, 10, 15) showed low amount of Picroside-I content (0.52 mg/g fresh wt.). The RAPD
analysis of 18 somaclonal variants, consisting of high, low and normal picroside-1 content with 14 random primers showed that OPD 20 primer detected maximum polymorphisms. The high Picroside-1 content somaclones showed common RAPD fingerprinting pattern. The phylogenetic analysis of RAPD profile grouped 18 somaclonal variants into 5 groups, thereby showing that significant genetic variations had occurred during tissue cultures of *P. kurroa*.

The present study has thus optimized a tissue culture process wherein different *in vitro* parameters have been optimized for increased shoot biomass yield coupled with Picroside-1 accumulation in *P. kurroa*. The process provides a platform for its up-scaling to a commercial scale for year round production of a medicinal compound, which is not otherwise feasible through conventional agricultural methods/practices because the biosynthesis and accumulation of medicinal compounds occur in *P. kurroa* only at high altitudes of Himalayas and that too during a particular time of the year. The study thus provides a sustainable alternative to field-grown source of Picroside-1, thereby, relieving pressure from the natural habitat of the target plant species, which is classified as an endangered to extinction. In tissue culture, the plants are completely protected from insects, diseases, and adverse weather conditions, which are otherwise common biotic and abiotic constraints to which plants are exposed when grown in the field conditions using agricultural practices. Most of these concerns or technical constraints are taken care of in a tissue culture process optimized for the accumulation of medicinal compounds *in vitro*. The process developed in the current study holds great potential in the industry because the cost of Picroside-1 available with the ChromaDex Inc. is Rs. 30,000/5 mg, which puts Picroside-1 in the category of high value phytochemicals. Also, the Picroside-1 being a major ingredient of a hepatoprotective formulation Picroliv provides a viable alternative for the exclusive production of medicinal compound. The *in vitro* production of Picroside-1 under controlled environmental conditions also provides a suitable system for understanding the molecular biology of biosynthesis of picrosides in *P. kurroa*, which is lacking as of today. The study also identifies dynamics of the biosynthesis and accumulation of picrosides *vis-à-vis* morphogenetic tissue culture stages of *P. kurroa*. The identification of a optimum incubation temperature of 15±1°C not only for profuse
growth of shoots of *P. kurroa* but also suitable for biosynthesis and accumulation of Picropside-I provides a suitable biological system to go for thorough investigation of the molecular biology of the biosynthesis of picrosides in *P. kurroa*, which is otherwise completely lacking as of today.