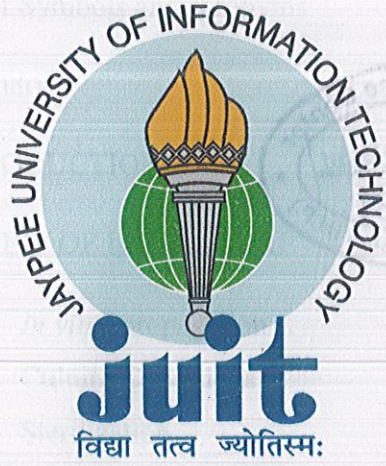
IN VITRO PROPAGATION AND ESTIMATION OF PICROSIDE AMOUNT IN DIFFERENT STRAINS OF *PICRORHIZA KURROA* COLLECTED FROM DIFFERENT GEOGRAPHICAL LOCATIONS

By:
Sanya Bakshi (071582)
Sneha Gulati (071706)

Name of Supervisor: **Dr. Hemant Sood**



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WAKNAGHAT, SOLAN-173 234, HIMACHAL PRADESH, INDIA

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Dr. Hemant Sood

Lecturer

Biotechnology & Bioinformatics

Jaypee University of

Information Technology

Waknaghat-173 215

Solan, Himachal Pradesh

CERTIFICATE

This is to certify that the thesis entitled **"In vitro propagation and estimation of picroside amount in different strains of *Picrorhiza kurroa* collected from different geographical locations"** submitted by **Ms. Sanya Bakshi and Ms. Sneha Gulati** of Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

Dr. Hemant Sood

Lecturer

Department of Biotechnology and Bioinformatics

Jaypee University of Information Technology,

Waknaghat, Dist. Solan, HP-173215

Date: 23/5/11

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Needless to say, errors and omissions are ours.

Sanya Bakshi

Date 23.5.11

Sneha Gulati

Date 23.5.11

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LIST OF ABBREVIATIONS

• KN	Kinetin
• IBA	Indole-3-butyric
• BA	Benzyl adenin
• GA	Gibberellic Acid
• BAP	Benzyl amino purine
• 2,4-D	2,4-Dichlorophenoxyacetic acid
• MS	Murashige & Skoog
• µM	Micro molar
• $HgCl_2$	Mercuric Chloride
• psi	Pound per square inch
• UV	Ultra Violet
• g	Gram
• mg	Milligram
• µg	Microgram
• l	Litre
• cm	Centimeter
• °C	Degree Celsius

SUMMARY

An efficient and rapid protocol for mass propagation of endangered medicinal herb(*Picrorhiza kurroa* Royle ex Benth) under in vitro conditions has been developed. The mother plants were obtained from different regions of Himachal Pradesh namely Lahaul spiti region, Manali region and Pangi region,their altitudes ranging from 4200m, 2500m, 5500m respectively. The motive of considering these three regions was to see the affect of altitude on the mother stock so as on the *in vitro* produced daughter or cloned plants with respect to accumulation of Picroside-I and Picroside-II both by analyzing the content in the field grown plants as well as the *in vitro* plants by using HPLC. Among the various strengths of growth regulators tested best results were recorded when shoot apices from natural plants collected from Lahaul and Pangi were cultured on MS basal solid media supplemented with IBA (3 mgl^{-1}) and KN (1 mgl^{-1}) for shoot proliferation. Results from Lahaul strain were quite significant where different combination treatments of auxins and cytokinins were tried along with gibberellins. The rooting was also optimized using auxins alone. The root induction per explants was maximum in the medium containing MS basal medium supplemented with 4 mgl^{-1} IBA. The rooted plantlets were hardened in polycups containing sterile soil, sand and vermiculite in equal ratio and were successfully acclimatized and established in soil with 75 % survival rate. Growth was better seen in 15±1°C as compared to 25±1°C.Continuously shaking liquid cultures with MS basal media supplemented with 3 mgl^{-1} IBA and 1 mgl^{-1} KN resulted in almost a four fold growth(120 rpm). Another objective was to see the change in the picroside content once *Picrorhiza kurroa* was infected with *Agrobacterium rhizogenes*. Two strains, namely Strain 15834 and MTCC – 2364 were used. The leaves, roots, stem and even the callus was infected with it. Out of which only the plants infected with Strain 15384 showed some growth and the other with MTCC -2364 strain failed to show any growth. The growth conditions were further optimized by changing its photoperiod and the antibiotic concentration.

Antibiotic Cephalaxin was used and concentration 500 $\mu g l^{-1}$ provided better results. Incubation in dark period of the infected plants showed better growth in 2 weeks. The amount of Picroside-I content in the field grown mother plant (a year old) of Lahaul strain was 9.05µg/mg whereas when *in vitro* raised plants were analyzed ,the amount of accumulation of Picroside-I was 4.85 µg/mg and no Picroside-II was found in 6 months old plantlets. The results from *Agrobacterium* infected shoots were definitely fascinating as Picroside-I (3.05 µg/mg) was found in shoots at 15°C and Picroside-II (3.08 µg/mg) was also detected in the roots of infected plantlets. Further differential results will help in interpreting various genetic and phenotypic parameters related to the metabolite production which will enable to choose a superior quality of explant for increased Picrosides production as well as help us understand if genotype is playing some role if the environmental conditions are controlled.

Sanya Bakshi

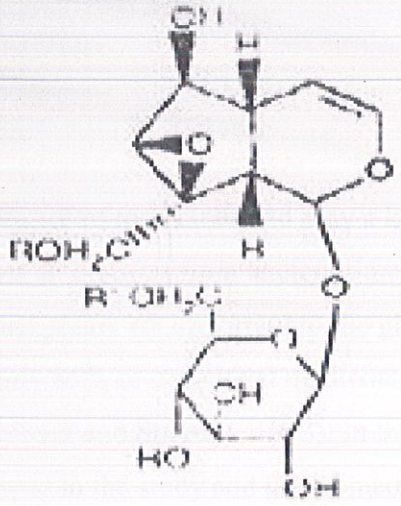
Date : 23.5.11

Dr. Hemant Sood

Date : 23/5/11

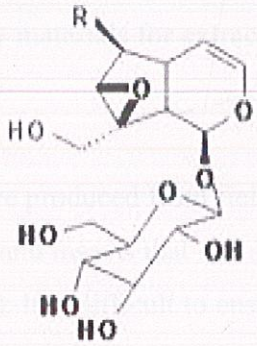
Sneha Gulati

Date : 23.5.11

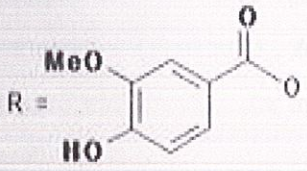


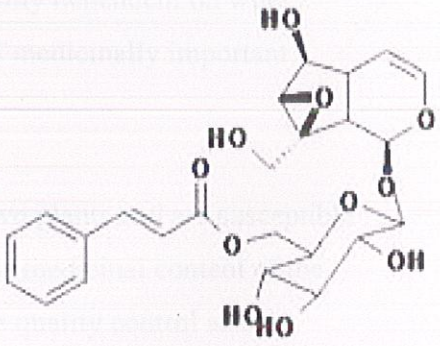
The configuration of glycosidic bond is β

R = H	R' = Cinnamoyl	Picroside 1
R = Vanilloyl	R' = H	Kutkoside

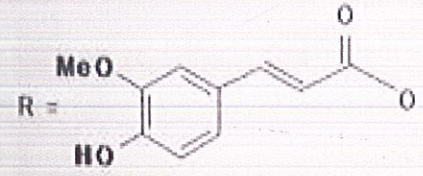


Picroside II

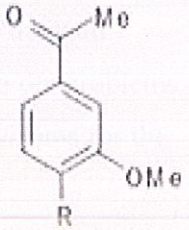




Picroside I



Picroside IV



R= OH, Apocynin
R= glucose Androsin

CHAPTER 1

INTRODUCTION

Plants are an important source of medicines and play a key role in world health (Constabel, 1990). Almost all cultures from ancient times to today have used plants as medicine. Today medicinal plants are important to the global economy (Srivastava et al., 1995), as approximately 85% of traditional medicine preparations involve the use of plants or plant extracts (Vieira and Skorupa, 1993). In the past few decades there has been a resurgence of interest in the study and use of medicinal plants in health care and in recognition of the importance of medicinal plants to the health system (Lewington, 1993; Mendelsohn and Balick, 1994; Hoareau and DaSilva, 1999). This awakening has led to a sudden rise in demand for herbal medicines, followed by a belated growth in international awareness about the dwindling supply of the world's medicinal plants (Bodeker, 2002). Most of the pharmaceutical industry is highly dependent on wild populations for the supply of raw materials for extraction of medicinally important compounds.

Generally, herbal preparations are produced from field-grown plants and are susceptible to infestation by bacteria, fungi, and insects that can alter the medicinal content of the preparations (Murch et al., 2000). It is difficult to ensure the quality control as the medicinal preparations are multi-herb preparations and it is difficult to identify and quantify the active constituents (Wen, 2000). Also, there is significant evidence to show that the supply of plants for traditional medicines is failing to satisfy the demand (Cunningham, 1993). An efficient and most suited alternative solution to the problems faced by the phyto-pharmaceutical industry is development of *in vitro* systems for the production of medicinal plants and their extracts.

The *in vitro* propagated medicinal plants furnish a ready source of uniform, sterile, and compatible plant material for biochemical characterization and identification of active constituents (Wakhlu and Bajwa, 1986; Miura et al., 1987).

In addition, compounds from tissue cultures may be more easily purified because of simple extraction procedures and absence of significant amounts of pigments, thus possibly reducing the production and processing costs (Chang et al., 1992, 1994).

Picrorhiza kurroa Royle ex. Benth commonly known as Kutki, a fast depleting medicinal value plant belonging to family Scrophulairaceae. It is endemic and grows in inner ranges of alpine Himalayas, from Kashmir to Sikkim, of Indian state at an altitude of 3,000-5,000 feet above sea level (Hooker 1885; Chopra & Ghosh 1934; Blatter 1984; Jain 1996; Agrawal 2003). It is valued as hepato-protective, anti-periodic, cholagouge, stomachic, anti-amoebic, anti-oxidant, anthelmintic, anti-inflammatory, cardio-tonic, laxative, carminative, expectorant, etc. (Chopra & Ghosh 1934; Uphof 1959; Kapoor 1990; Kapahi *et al.* 1993; Singh *et al.* 2006; Bhatt & Bhatt 1996: Gaddipati *et al.* 1999; Prajapati, 2003). Picroside I and II are the two important chemical constituents present in its rootstock which have therapeutical importance (Dutt *et al.* 2004). It is the principle source of glycosides that is Picroside-I, Picroside-II and Kutkoside. Other identified active constituents are apocynin, drosin, and nine cucurbitacin glycosides. The active constituents are obtained from the dried root and rhizome.

Picroside-I is the major ingredient of Picroliv and, therefore, makes this compound a highly valued secondary metabolite of *P. kurroa*. 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.

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*. 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. Tissue cultures of *P. kurroa* have been done for its rapid multiplication. Picrosides I and II are the active agents responsible for the medicinal effects of Kutki, and the variation in content of these compounds in plants at different altitudes is a major question to be addressed. It has also been studied that Picroside I and II accumulation depends on altitude, which could help in the selection and collection of superior genotypes with uniform effects for utilization by the pharmaceutical industry.

Plants of *P. kurroa* are perennial, herbaceous with creeping rootstock and aerial parts represented by basal leaves and flowering scape only. Such features make *P. kurroa* highly adaptable to harsh environmental conditions of the areas it inhabits. The plants spread through stolons and apparently separate looking plants at above ground level are actually joined together by stolons beneath. However, when these stolons get detached from the mother stock, the plants become independent. The simple leaves arising from the tip of the upturned stolons are present in rosettes or whorls. Cauline leaves are present only during the flowering phase.

The flowers are sessile, zygomorphic, bilipped, bisexual and purple borne in the axils of small green coloured bracts opening during June to September. Sepals persist up to capsule development stage and are of unequal in size.

It finds its place in traditional as well as in modern system of medicine as purgative, antiperiodic, brain tonic, stomachic, dyspepsia and fever. Current research has focused on its hepatoprotective, anticholestic antioxidant and immune modulating activity.

The natural resurgence of *Picrorhiza kurroa* is through rhizomes and seeds, however their cultivation rate is very poor. The poor cultivation coupled with over exploitation for pharmaceutical use has depleted the species from natural habitat. The Indian system of medicine is predominantly dependent upon the use of plant based raw materials in most of their preparations and formulations, thereby, widening the gap between demand and supply and thus putting further pressure on the species. Infact *P. kurroa* is now listed as one of the endangered plant species of India. Even after grazing the rhizome still remains seated in the soil and it regenerates when the climatic conditions are favourable. Additionally seed setting and seedling survival has reported to be poor in alpine plants(Pandey 2000).

Owing to these factors, the species is at the verge of extinction. It is essential for the conservation of *P.kurroa* to encourage *ex-situ* plantation which require large scale planting material. In view of the problems of conventional propagation and high demand of planting material the large scale multiplication of this species can only be met efficiently and economically in a sort span of time by *in vitro* propagation. Therefore, an efficient *in vitro* propagation system for producing this plant is required to further clarify its potential medicinal values and germplasm conservation.

The Production from the wild sources is about 2500 metric tons(annually) mainly from Nepal and the states of Himachal Pradesh and Assam. The Domestic consumption of the crude drug mostly by Ayurvedic pharmaceuticals and herbal drug manufactures is met from the wild sources and is estimated to be around 500 tons per year.

The Average price of the leading ayurvedic firms in India are purchasing the crude drug at the rate of Rs.150 per kg of dried herb. The average yield is 450 kg/ha and however, from high dose of forest litter treated field maximum yield of 612 kg/ha can be obtained. The rate for a kg of rhizome/roots ranges from Rs.100 to 150.The roots and rhizomes are harvested during the senescence of aerial parts in the month of September at the lower altitudes and in October at higher altitudes so as to get the maximum bioactive compounds.

In vitro propagation (Micro-propagation) is the practice of rapidly multiplying stock plant material to produce a large number of progeny plants, using modern plant tissue culture methods. Micro-propagation is used to multiply novel plants, such as those that have been genetically modified or bred through conventional plant breeding methods. It is also used to provide a sufficient number of plantlets for planting from a stock plant which does not produce seeds or does not respond well to the vegetative reproduction.

The method of Micro-propagation goes through several stages—establishment, multiplication, pretransplantation and finally transfer from culture. In the final stage, the processed plantlets are removed from the plant media and transferred to soil or more commonly to potting compost for continued growth by conventional methods. The major advantage of Micro-propagation is the production of many plants that are clones of each other. It can also be used to produce disease free plants . It produces rooted plantlets ready for growth, saving the time for the grower when seeds or cuttings are slow to establish or grow. Also, the produce is higher than those produced by conventional methods. A large number of plants produced on a comparatively smaller area.

Many strain improvement,methods for the selection of high-producing cell lines, and medium optimizations can also lead to an enhancement in secondary metabolite production. However, most often trials with plant cell cultures fail to produce the desired products. In such cases, strategies to improve the production of secondary metabolites must be considered.

Among the *in vitro* culture systems, the cultured cell suspensions are not gained momentum because of their instability and non-uniformity of the product formation. Hence the differentiated organ cultures such as hairy root cultures are widely studied. Hairy root cultures are used as alternative production systems for secondary metabolites due to their tremendous potential to higher growth rate and uniform product formation. Being organized, they are amenable for scaling-up in bioreactors which is an added advantage. The metabolite pattern found in hairy roots is similar, if not always identical to that of plant roots. A major characteristic of hairy roots is the concomitant production of secondary metabolites with growth. Hence it is possible to get a continuous source of secondary metabolites from actively growing hairy roots.

Recent studies in India are aiming at:

- CSIR net work project on " Developing medicinal Plant chemotypes for enhanced marker and value added compounds". Dr. M .K. Koul and Dr. S. C. Taneja (PI's) RRL Jammu.
- "Identification of genes responsible for cold stress in *P.kurroa* and its relation of stress and glucosides contents". Dr.M.K.Koul, PI, RRL Jammu
- Studies on Reproductive Biology of *Picrorhiza kurroa.* Director National Research Centre for Medicinal and Aromatic Plants Boriavi, Anand, Gujarat.

Therefore in view of all the above stated points, the present study was carried out with following objectives :-

1. Development of *in-vitro* cultures through micro- propagation , to study the pattern of biosynthesis and accumulation of Picroside-I and Picroside-II in these different strains of *Picrorhiza kurroa* collected from Lahaul, Pangi and Manali regions of Himachal Pradesh.
2. Induction of *Agrobacterium*- mediated transformation and to see its effect on Picroside content.
3. Comparison of Picrosides accumulation in the in-vitro propagated strains and the field grown strain of *Picrorhiza kurroa*.

CHAPTER 2

REVIEW OF LITERATURE

Review is discussed in the light of available literature relevant to the research on *Picrorhiza kurroa*.

2.1 In vitro propagation

The endangered status of *P. kurroa* warrants its rapid multiplication on a large scale and subsequent plantation in its natural habitat.

There are fragmentary reports on micropropagation of *P. kurroa* by Lal *et al.*, 1988; Upadhay *et al.*, 1989; Trivedi and Pandey, 2007; Chandra *et al.*, 2006 and induction of hairy roots (Verma *et al.*, 2007). However, none of those studies were aimed at developing a low-cost micropropagation technology for *P. kurroa* which was done by Sood et al, 2009. Moreover, in previous reports the tissue culture plantlets of *P. kurroa* were very thin and slender (Lal *et al.*, 1988) with a problem of vitrification (Upadhay *et al.*, 1989), thereby reducing the survival rate of tissue cultured plants in the field conditions.

2.2 Cuture Conditions

2.2.1 Media

Sood et al cultured axillary shoot tips on MS+IBA (2 mg L^{-1})+KN (3 mg L^{-1})+sucrose 3% (w/v)+agar-agar 0.8% (w/v) was the best medium for multiple shoot formation with 86.3% shoot apices forming multiple shoots. The 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 Indole-3-Butyric Acid (IBA) (2 mg L^{-1})+kinetin (KN) (3 mg L^{-1})+table sugar 3% (w/v) was found to be the best with 27 shoots/explant.

Seventy percent shoots formed roots on half strength MS salts supplemented with IBA (3 mg L^{-1})+table sugar 3% (w/v)+agar-agar with an average of 5.6 roots per shoot. The study has resulted in the identification of a low-cost medium combination for rapid multiplication of *P. kurroa* with a potential that the technology can be up-scaled to a large-scale production.

An efficient and rapid protocol for mass propagation of *Picrorhiza kurroa* Royle ex Benth - an endangered medicinal plant- in vitro without using cytokinin has been developed by Arif Jan, Phalisteen S., G.T. Thomas and A. S. Shawl. The nodal explants were obtained from in vitro raised seedlings grown in moist cotton raised from chill treated seeds (4°C) and in vivo matured plants in natural conditions respectively. Both types of genesis, direct and indirect, of shoot and root in this species using single type of hormones viz; auxins with MS basal medium is being reported for the first time. Among the various strengths of growth regulators tested best results were recorded when nodal explants from natural plants were cultured on MS basal media supplemented with 0.25 mg^{-1} 2,4-D mgl^{-1}, 0.25 mgl^{-1} BAP ,0.2 mgl^{-1} NAA and 0.6 mgl^{-1} NAA for profuse callusing, shoot induction, indirect and direct rapid shoot proliferation respectively. Combination treatments of auxins were also testified but the results were not satisfactory as that of NAA alone. The rooting was also optimized using auxins alone or/and in combinations of two. The root induction per explants was maximum in the medium containing MS basal medium supplemented with 0.4 mgl^{-1} NAA. The rooted plantlets were hardened in polycups containing sterile soil, sand and vermiculite in equal ratio and were successfully acclimatized and established in soil with 81.5 % survival rate.

A procedure has been developed for the clonal propagation of *Picrorhiza kurroa* Royle ex Benth. through shoot tip culture by Nand Lal, Paramvir Singh Ahuja, Arun Kumar Kukreja and Banibrata Pandey. Murashige and Skoog's medium (1962) supplemented with kinetin (3.0 to 5.0 mg/l) supported rapid proliferation of multiple shoots from the explants. Addition of indole-3-acetic acid (1.0 mg/l) to the kinetin containing medium showed marked improvement in the growth of regenerated shoots.

However, presence of IAA in the medium did not alter the frequency of shoot multiplication. Rooting was readily achieved upon transferring shoots onto MS medium containing ∝-naphthaleneacetic acid (1.0 mg/l).

2.2.2 Temperature

The cultures were incubated at 15±1°C in plant tissue culture chamber with 70% relative humidity under 16 h photoperiod provided by cool fluorescent light (3000 lux). Data were collected on days to multiple shoot formation, per cent shoot apices with multiple shoots and number of shoots per explant. The data were analyzed for test of significance. The cultures were subcultured after every 4 weeks on shoot proliferation media for 2 months so as to obtain good growth.

The shoot apices were cultured on MS + KN (3 mg L^{-1}) + IBA (2 mg L^{-1}) and incubated at low temperatures (10±1, 15±1 and 25±1°C) with the same photoperiod conditions as mentioned above in order to see whether better shoot growth can be obtained. The shoot apices proliferated into multiple shoots within 5-6 days of culture with significant differences for leaf size and shoot biomass yield at 15±1°C. The leaves were ~10x longer and ~5x wider in shoots formed at 15±1°C compared to shoots at 25±1°C. Similarly the total shoot biomass yield and per cent survival were significantly higher in shoots grown at 15±1°C compared to at 25±1°C

2.2.3 Light

Duration, intensity and quality of light also influence the growth of cultures. Carlos Eduardo et al in 2010 studied that the *in vitro* physiology and carbon metabolism can be affected by the sink–source relationship. The effect of different sucrose concentrations (10, 30, and 50 g L−1), lightintensities (80 and 150 µmol m−2s−1), and CO2levels (375and 1,200 µmol mol−1) were tested during plantain micropropagation in temporary immersion bioreactors. Activities ofpyruvate kinase, phosphoenol pyruvate carboxylase, and the photosynthesis rate were recorded.

From the morphological and practical point of view, the best results were obtained when plants were cultured with 30 g L−1 sucrose, 80 µmol m−2s−1 light intensity, and 1,200 µmol mol−1 CO2 concentration. This treatment improved leaf and root development, reduced respiration during in vitro culture, and increased starch level at the end of the hardening phase. In addition to that, the number of competent plants was increased from 80.0% to 91.0% at the end of the in vitro phase and the survival percentage from 95.71% to 99.80% during ex vitro hardening.

2.3 Sterilization

An alternative protocol for *in vitro* propagation of *Picrorhiza scrophulariiflora* is described using bavistin and adenine sulphate. The explants differentiated into multiple shoot buds on MS supplemented with various concentrations of bavistin and adenine sulphate ranging from 0 - 400 mg/l either alone or in combination. Maximum number of multiple shoots were obtained on MS containing the combination of bavistin (100 mg/l) and adenine sulphate (100 mg/l). In this combination as high as 28 shoots per explant was achieved and also vetrification of the cultures were not recorded. This study also demonstrates that the bavistin has stronger cytokinin-like activity than adenine sulphate. For instance, it was observed that bavistin alone in the concentration of 300 mg/l produced as high as 24 shoots per explant, however, adenine sulphate (100 mg/l) could produce a maximum of 18 shoots per explant. Moreover, higher or lower concentration did not improve the shoot multiplication. The microshoots were separated from the multiple shoots and transferred to MS containing various concentrations of auxins. Among them, NAA (1 mg/l) produced as high as 6 roots per explant. The regenerated plantlets were hardened in plastic cups (6 x 8 cm) containing 9 : 1 virgin soil and soil at Kyongnosla nursery and acclimated for four weeks. A 90% survival rate of the plants was recorded after 60 days.

A protocol has been standardized for sterilization of nodal segments and seeds of Aconitum heterophyllum by Shrivastav et al, 2010, for its micropropagation intended for its mass propagation and conservation. Three sterilizing agents viz., HgCl2, NaOCl

and H2O2 were tested for sterilization by varying their concentration and time of exposure. 100% healthy shoots were obtained when explants were sterilized with 0.1% HgCl2 for 5 minutes, inoculated on MS basal media with appropriate hormones and observing them for 30 days, while at 7.5% concentration of H2O2, 5 minutes exposure provided 90% of aseptic seed germination. Results showed that out of three sterilizing agents HgCl2 was significantly reducing the contamination of explants and H2O2 of seeds in in-vitro, which shows that requirement of sterilization, may vary with the type tissue used for micropropagation.

2.4 *In vitro* Shooting

A reproducible *in vitro* regeneration system for Nepalese kutki (*Picrorhiza scrophulariiflora* Pennell) was developed from *in vitro* leaf derived callus by P. Bantawa, O. Saha-Roy, S. Kumar Ghosh and T. Kumar Mondal . Induction of more than seven shoot buds per explant was achieved on Woody plant medium (WPM) supplemented with 0.53 µM α-napthaleneacetic acid (NAA) and 0.23 µM kinetin (KIN). The shoots were elongated on WPM supplemented with 0.44 µM 6-benzylaminopurine (BAP) and rooted on WPM supplemented with 5.3 µM NAA within 2 weeks. The random amplified polymorphic DNA (RAPD) analysis indicated genetic uniformity of the micropropagated plants with its donor plants.

2.5 *In vitro* Rooting

Damiano (1978) reported addition of 1-2 g of activated charcoal per litre of medium promotes elongation of both shoots and roots. Comparing with IBA, IAA, NAA. Hunter et al. (1984a) found that IBA was the most desirable additive for root formation, the optimum concentration being in the range of 0.75 mg/l to 1.0 mg/l. James (1979) studied that in vitro rooting was reduced on a culture medium supplemented with phloroglucinol.

According to Moncousin (1991), during rhizogenesis high concentration of sucrose had negative effects on rooting potential. Also the ratios between nitrogen and sucrose

(Hyndman et al. 1962:Driver and Suttle, 1987), auxins and sucrose (Welander, 1976) highly influenced the rooting process. Jeong et al. (1996) reported 63 to 89 per cent of root formation frequencies in the shoots of five different cultivars of strawberry grown in liquid medium.

Kaul (1998) obtained rooting percentage (40.19) on rooting medium containing tap water and table sugar and did not differ significantly from the rooting percentage (40.74) obtained on standard rooting medium supplemented with distilled water and sucrose.

As part of a micropropagation procedure for the endangered medicinal plant *Picrorhiza kurroa* (Scrophulariaceae) by C. Wawrosch, P. Zeitlhofer, B. Grauwald, B. Kopp, the influence of the rooting conditions on establishment of the plants ex vitro has been investigated. In vitro rooting was performed on MS medium supplemented with IAA, IBA or NAA at a level of 1 μM. The percentage of rooted shoots was high on all media except for the auxin free control (70%). Root length was low when IAA or NAA were used while IBA and the control induced longer roots. Basal callus was formed on all media but was not a problem when IBA was applied. Establishment of the plantlets in the greenhouse was most successful when the shoots were rooted with IBA medium (100% survival), followed by IAA (84%) and control medium (76%). Only 24% of the plants rooted with NAA survived hardening, and there was evidence that the survival and development of the plantlets correlated with the amount of basal callusing.

2.6 Hardening

Zimmerman (1988) reported a variety of soilless media like sphagnum peat, perlite, vermiculite or mixture of two components for hardening of woody species. Shen et al. (1990) suggested that rooted microcuttings of kiwifruit were transferred to plastic containers containing mixture of sterilized perlite and vermiculite for hardening. The plantlets were grown initially under glasshouse conditions with satisfactory survival percentage of kiwifruit.

Sharma and singh (1995) successfully hardened the micropropagated plants of ginger on unsterilized potting mixtures. Sharma et al. (1992), Mahajan (1997) and Kaur et al. (1999) used cheaper mixture of FYM:sand:soil in place of expensive perlite, vermiculite and cocopeat for hardening of 'Colt' rootstock cherry, strawberry and Gerbera, respectively. Kaul (1998) obtained 85.70 per cent survival rate of micropropagated plants of kiwifruit after hardening in soilrite and vermiculite potting mixtures.

Pankaj Trivedi and Anita Pandey studied that three plant growth-promoting rhizobacteria viz. *Bacillus megaterium*, *B. subtilis* and *Pseudomonas corrugata* can be used for biological hardening of micropropagated plantlets of *Picrorhiza kurroa.* The bacterial isolates antagonized the fungal spp. postulated to cause death of micropropagated plants in plate-based assays and positively influenced survival and growth parameters in greenhouse investigation.

2.7 Altitude effect on Picroside content of different strains

In Katoch M et al.,2011 study,the picroside I and II content in various plant parts of *P. kurroa* collected from different altitudes, viz. Sonemarg (2,740 m a.s.l.), Tangmarg (2,690 m a.s.l.), and Pulwama (1,630 m a.s.l.) in the north-western Kashmir Himalayas was analyzed by HPLC. A considerable degree of variation in picroside content was observed. Picroside I and II was highest in populations collected from Sonemarg followed by Tangmarg, suggesting that picroside accumulation is directly correlated with altitudinal change. More picroside I was found in the rhizome and roots of the Pulwama population as compared to Tangmarg samples, whereas the quantity of Picroside II was reduced in plants from Pulwama compared to the Tangmarg population, suggesting that cultivation of P. kurroa at lower altitude reduces the picroside content. The quantities of picrosides also varied spatially, being highest in rhizome followed by roots, inflorescence and leaves in the populations from all three locations. The study concludes that picroside I and II accumulation depends on altitude, which could help in the selection and collection of superior genotypes with uniform effects for utilization by the pharmaceutical industry.

2.8 Medicinal Value of *Picrorhiza kurroa*

The medicinal importance of *P. kurroa* is due to its pharmacological properties like hepatoprotective (Chander et al. 1992), antioxidant (particularly in liver) (Ansari et al.1980), antiallergic and antiasthamatic (Dorch et al.1991), anticancerous activity particularly in liver (Joy et al.2000) and immunomodulatory (Gupta et al. 2006). A hepatoproctive drug formulation, Picroliv has been prepared from the extracts of *P.kurroa* (Ansari et al. 1991; Dwivedi et al. 1997). Picroliv also provides protection against other ailments such as immunostimulating effect in hamsters and prevention of infections (Puri et al. 1992; Gupta et al. 2006).

2.9 Differential accumulation of Picrosides in the plant

Sood et al. established callus cultures and plant regeneration from different explants coupled with estimation of Picrosides in morphogenetically different developmental stages showed that Picroside-I accumulates in shoot cultures of *Picrorhiza kurroa* with no detection of Picroside-II. The Picroside-I content was 1.9, 1.5, and 0.04 mg/g in leaf discs, stem and root segments, respectively. The Picroside-I content declined to almost non- detectable levels in callus cultures derived from leaf discs.

Som dutt et al. cited that during all four stages of plant growth picroside-I was the major iridoid compound present in leaves whereas picroside-II was predominant in roots. This analysis of picroside contents in leaves and roots provides the first indication of differential metabolism of Picrosides in these plant organs. This provides the foundation for comparative analysis of biosynthesis and transport between tissues.

2.10 Effect of *Agrobacterium* strains on Picroside content

Not enough literature is present in this context.

Agrobacterium rhizogenes-mediated transformation of *Picrorhiza kurroa* Royle ex Benth.: establishment and selection of superior hairy root clone (Praveen Chandra Verma, et al.,2007)

A protocol for induction and establishment of *Agrobacterium rhizogenes*-mediated hairy root cultures of *Picrorhiza kurroa* was developed through optimization of the explant type and the most suitable bacterial strain. The infection of leaf explants with the LBA9402 strain resulted in the emergence of hairy roots at 66.7% relative transformation frequency. Nine independent, opine and *TL*-positive hairy root clones were studied for their growth and specific glycoside (i.e., kutkoside and picroside I) productivities at different growth phases. The yield potentials of the 14-P clone, both in terms of biomass as well as individual glycoside contents (i.e., kutkoside and picroside I), superseded that of all other hairy root clones along with the non-transformed, in vitro-grown control roots of *P. kurroa*.

Studies on hairy root culture of *Picrorhiza kurroa* Royle ex Benth for increasing production of secondary metabolites, picrotin and picrotoxinin have been done. (Janhvi Mishra et al.2011)

CHAPTER 3

MATERIALS AND METHODS

The present experiments of Establishment of *in vitro* shoot cultures of *Picrorhiza kurroa*, regeneration of plantlets & Hardening of *in vitro* grown plantlets were carried out in the Department of Biotechnology, Jaypee University of Information Technology, Waknaghat, during 2010-2011.

The Experiments and methodology followed to carry out the above-mentioned experiments have been described under the following headlines.

3.1 Micropropagation of *Picrorhiza kurroa*

3.1.1 Collection of Plant material

3.1.2 Preparation of explants

3.1.3 Surface Sterilization

3.1.4 Media Preparation

3.1.5 Preparation of modified MS media

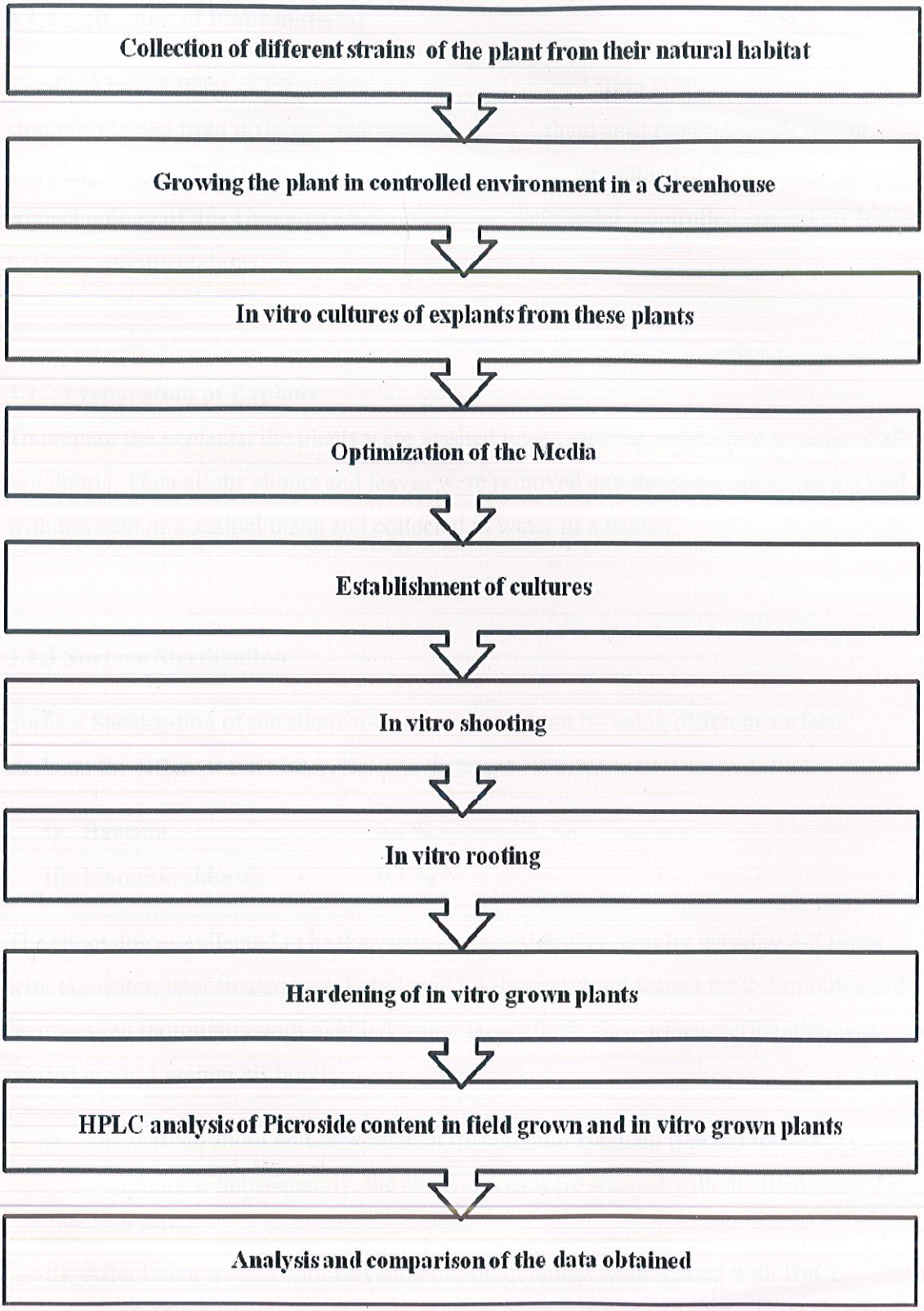
3.1.6 Cleaning of glassware

3.1.7 Culture conditions

3.1.8 Inoculation of prepared explant

3.1.9 *In vitro* shoot multiplication on prepared MS media

3.1.10 Induction of *in vitro* rooting on prepared MS media



3.1.1 Collection of Plant material

The field grown plant of *Picrorhiza kurroa* was procured from HFRI,Shimla where the strains collected from different regions of H.P.,i.e. Lahaul spiti region,Manali region and Pangi region were brought to the glasshouse of the laboratory of Department of Biotechnology of this University and maintained their under controlled conditions for further experimentation.

3.1.2 Preparation of Explant

To prepare the explants, the plants were washed under running water so as to remove all soil debris. Then all the shoots and leaves were removed and the shoot apex was excised with the help of a scalpel blade and collected in water in a beaker.

3.1.3 Surface Sterilization

Surface Sterilization of the shoot apices was carried out by using different surface sterlants for different time intervals. The different sterlants used were as follows

i) Bavistin - 0.5 %
ii) Mercuric chloride - 0.1 %

The shoot apices collected in beaker were surface sterilized first by washing 3-5 times with tap water, later treated with Labolene (2-3 drops/100 ml water) for 2-3 minutes and then washed thoroughly with distilled water. Henceforth, the surface sterilization was carried out in Laminar air hood.

i) The washed shoot apices were then treated with Bavistin (0.5 %) for 2-3 minutes. Subsequently, the shoot apices were washed with distilled water 1-2 times.
ii) After being treated with Bavistin, the shoot apices were treated with $HgCl_2$ (0.1%) for 0.5-1 minute. Finally, the shoot apices were washed with distilled water 3-4 times.

3.1.4 Media Preparation

The composition of Media was based on Murashige & Skoog (1962) medium divided into different stocks (Annexure I).The Stock Solutions were prepared and kept in the refrigerator. Each salt of the stock solution was dissolved separately in distilled water so as to avoid precipitation. The Chemicals used were of reliable grade and were obtained from reliable firms like Sigma Aldrich. Each Stock solution of MS Media was added one by one to prepare the desired medium. After addition of sucrose (30g/l), growth hormones, desired volume was made with distilled water and the pH of the medium was adjusted to 5.6-5.7 by using pH meter. Then, agar-agar (9g/l) was added and dissolved by boiling the medium till it became homogenous. The medium was finally dispensed in 250 ml culture jars for culturing. These were closed with the jar caps and autoclaved at a pressure of 15 psi, 121 °C for 20 minutes for proper sterilization of culture medium. This medium was allowed to solidify and was used after 2 days of preparation. In all 10 different combinations of media was prepared using different concentrations of appropriate growth hormones like KN, BA, IBA, NAA, 2,4-D for each of the experimental setups.

3.1.6 Cleaning of Glassware

All the glassware which were not used before were cleaned in a dilute solution of Labolene with test tube brush and rinsed thoroughly in tap water. The Glassware was then kept for drying in hot dry air oven at 80-100 °C for 2-3 hours.

The used glassware with contaminated cultures were first autoclaved and molten media was disposed off and the culture jars were cleaned.

3.1.7 Culture Conditions

All the operations were carried out aseptically in a Laminar Air Hood filled with UV Light. The Laminar Flow Chamber was thoroughly wiped with rectified spirit prior to

use and the UV Light was kept on for 15-20 minutes in Laminar Flow Chamber. Thereafter, the airflow was allowed to run and the UV Light was switched off. Before starting with aseptic manipulations, hands and arms were washed with soap and water. Hands were frequently wiped during manipulations with rectified spirit which was allowed to evaporate. All the metallic tools like scalpel blade, forceps and needles were first autoclaved and wiped with rectified spirit and flame sterilized at the time of use. The rims of tubes and jars were quickly flame sterilized before and after inoculations. Subsequently, the jars were kept in trolleys in culture room at 25 ± 2 °C under 16 hours photoperiod and 3000 flux light intensity.

3.1.8 Inoculation of prepared ex plant

For the establishment of the cultures, the surface sterilized explants were inoculated onto the MS Medium supplemented with various combinations of growth hormones. The cultures were then incubated for the proliferation of shoot apex. The proliferation of shoot apex was indicated by the emergence of micro shoots and leaflets.

Observations

Average number of micro shoots proliferated per shoot apex on prepared MS media after 15 days of inoculation was recorded.

3.1.9 *In vitro* shoot multiplication on prepared MS media

The shoots obtained from the shoot apex were subcultured on prepared MS media containing various combinations of growth hormones including BA, IBA & KN. Subculturing for multiplication was carried out at 3 weeks interval up to 5 subcultures.

Observations

Average number of shoots, shoot length after 3 -4weeks of sub culturing was recorded.

3.1.10 *In vitro* rooting on prepared MS media

Rootable shoots were excised from lavishly multiplying shoot cultures having at least 2 cm length and were transferred singly to separate culture jars containing MS Media enriched with rooting growth hormones NAA and IBA of different concentrations.

Observations

Average number of roots and root length after 3-4 weeks was recorded.

3.2 Hardening and acclimatization of *in vitro* rooted plantlets in glass house

Transplantation and hardening of *in vitro* regenerated plants was carried out in plastic pots. After growth of 5 weeks on the rooting medium, roots were well developed and the plantlets from all rooting media were taken out of culture jars. Precaution was taken to avoid any possible damage to delicate root system. The agar medium from roots was washed gently under running tap water. After removal of the adhering medium, the plantlets were kept in Bavistin solution (0.5%) for 15-20 minutes.

Plantlets were transferred to plastic pots containing pre-autoclaved mixture of sand, soil and perlite, vermiculite and coco-peat in combinations. The root portion of plantlets was then gently placed in the pots containing the various rooting mixtures and sand at the bottom. The plantlets were watered and covered with glass jars to maintain high humidity.

After a week, when the plantlets showed initial signs of establishment in pots, with the appearance of new leaves, the pots were initially uncovered for overnight. Then after 2-3 weeks of transfer to pots, the plantlets were totally uncovered.

Observations

Percentage of survived plants in different potting mixtures was recorded.

3.3 *Agrobacterium* mediated transformation

***Agrobacterium rhizogenes* grown in suspension medium**

Leaf disks ,stem,expants infected by a syringe medium (48 hrs, 25^0C ,dark)

Explant transferred to MS basal medium with antibiotic – Cephalaxin (0.05%)

Plant survived the cocultivation,growth observed at different temperatures(25^0C,15^0C)

Analysis of picroside content to see if any alterations

3.3.1 Bacterial suspension

Growth of *A. rhizogenes* in nutrient broth:-

A.rhizogenes (Strain 15834/ MTCC – 2364) was inoculated in nutrient broth and incubated at different temperatures i.e. 25°C and 37°C. The suspensions were observed between 24-48 hrs. After 36 hrs the optical density was found to be 1.2 of the culture at 37°C at 600nm. This was used to infect our explants in the below stated methods.

3.3.2 Protocol-1

The plant was picked up from the growth room and the explant was prepared by cutting small leaf discs. The leaf disc were co-cultivated with bacterial suspension in a petriplate for 24 and 48 hrs. The leaf disc was then transferred to the growth medium without auxins .The growth medium contained antibiotic (cephalaxin) at a concentration 500mg/L.

Observations

The plants did not undergo any growth, rather the quality of the infected explants deteriorated. Our main aim was to check the hairy root or any growth pattern changes.

3.3.3 Protocol 2

The media was prepared with growth hormones BAP and kinetin both with a concentration of 1mg/L. The antibiotic was filter sterilized and put into the growth media. (approx 500L)The explant was infected by wounding the site with a syringe filled with bacterial suspension in a LAF. Mid and side injury both were done to the leaves. The excess suspension was absorbed by putting the infected leaves on Whattman paper. The leaves were cultured in the medium and incubated in the growth room in both light and dark conditions.

Observations

The leaves in the dark condition showed growth of shoots in 2 weeks. Since it was a new approach to infect the whole plant instead of just the leaf our motive was to look for any growth pattern changes and hairy root induction.

3.4 Estimation of Picroside-I and Picroside-II in shoot and root cultures

The *in vitro* biosynthsis and accumulation status of Picroside-1 and Picroside –II was determined in shoot and root cultures of *P. kurroa* by subjecting fresh samples from different experiments to chemical analysis. The shoots including leaf material and roots were harvested separately while the leafy material was green, actively-growing, and non-senescent stage. The quantification of Picroside-1 and Picroside–II was carried by reverse phase High Performance Liquid Chromatography (HPLC Waters 515) through C18 (5µ) 4.6 x 250mm Waters Symmetry Column using PDA detector (Waters 2996). Fresh shoots and roots were ground separately in liquid nitrogen and suspended in 80% methanol. The sample mixture was vortexed and kept overnight at room temperature. Following day, the samples were centrifuged at 10,000 rpm for 10-15 min. and the supernatant was filtered through 0.22µ filter. The filtrate was diluted 10x and 20x and injected into above mentioned column. Two solvent systems were used for running the test samples i.e. Solvent **A** (0.05% trifluoroacetic acid) and Solvent **B** (1:1methanol/ acetonitrile mixture). Solvent **A** and **B** were used in the ratio of 70:30 (v/v). The column was eluted in the isocratic mode with a flow rate of 1.0 ml/min. Picroside-1 and Picroside-II were detected at absorbance of 270 nm wavelength in a cycle time of 30 min at 30^0C. The compounds were identified on the basis of their retention time and comparison of UV spectra with the authentic standards procured from ChromaDex Inc. The quantification was done and the data were subjected to statistical analysis.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Proliferation of shoot apex on prepared MS media

4.2 *In vitro* shoot multiplication on prepared MS media

4.3 *In vitro* rooting on prepared MS media

4.4 Hardening and acclimatization of *in vitro* rooted plantlets in glass house.

4.1. Induction of multiple shoots in shoot apices

The surface sterilized axillary shoot tips were cultured on MS media containing IBA and KN in different combinations. Out of 10 different media combinations tested for multiple shoot formation from shoot apices, MS medium containing KN (1mg/l) + IBA (3mg/l) + sucrose 3% (w/v) + agar-agar 0.8% (w/v) was found to be the best with 60 % of shoot apices proliferating into multiple shoots within 5-6 days of inoculation (Table 4.1).

The same medium was found suitable for obtaining maximum shoots (22.4) per explant. However different concentrations of IBA and KN resulted in increased shoot length and less frequency of shoots. Gibberellin supplemented media managed to develop a profused mass of shoot, increasing the shoot formation but decreasing the shoot length in about 2 weeks duration. With the addition and increase in concentration of GA3 in the medium, there was a profused growth of the shoots with no increase in the shoot length but small leaves were observed when cultures were incubated at 25±1^{0}C (Fig. 4.1). The modifications in MS media by changing the concentrations and combinations of auxins and cytokinins did not help in obtaining shoots with good growth and well developed leaves.

4.2 Proliferation of shoot apex on prepared MS media

After 3 weeks of inoculation of shoot apex, we observed 3 to 8 micro shoots emerging from each inoculated explants.

4.3 *In vitro* shoot multiplication on prepared MS media.

After 3-4 weeks of growth, the shoots thus proliferating from the shoot apex were excised under aseptic conditions and sub cultured on the prepared MS medium enriched with different concentrations of GA3, KN & IBA growth hormones (Table 1) The maximum shoot multiplication was seen in MS Media containing (MS + GA3 (1mg/l)+ KN (1mg/l) + IBA (1mg/l) +Sucrose 30g/l + agar-agar 9g/l),but the shoot length did not increase.The maximum shoot length was seen in MS media containing (MS + KN (1mg/l) + IBA (3mg/l) +Sucrose 30g/l + agar-agar 9g/l).

TABLE 1: *In vitro* shoot multiplication on different combinations and concentrations of cytokinins and auxins at 15°C

MS + Growth regulators (mg/l)			Parameters of shoot multiplication							
IBA	GA3	KN	Days to multiple shoot formation		Average leaf size(cm^2)		Average No. of Shoots per explant		Average shoot length per explant (cm)	
			Lahaul	Pangi	Lahaul	Pangi	Lahaul	Pangi	Lahaul	Pangi
0	0	0	15-20	20-25	2.6 *0.5	1.5*0.2	5.8	3.1	3.5	1.3
0	1	0	9-10	20-22	2.2 *0.4	1.3*0.2	12.3	5.2	4.8	1.5
1	1	0	9-10	18-19	2.3*0.4	1.5*0.3	13.6	5.2	3.9	1.5
1	**1**	**1**	**8-9**	**15-17**	**3.2 *0.6**	**2*0.4**	**23.6**	**9.5**	**4.3**	**2.1**
1	2	0	8-9	18-19	2.3*0.4	1.5*0.2	18.3	6.0	3.7	1.7
0	2	1	6-8	18-19	2.4*0.5	1.2*0.3	21.3	6.2	3.7	1.6
1	0	2	6-8	15-17	3.0 *0.8	1.4*0.3	20.6	6.7	5.6	2.2
2	0	1	5-6	13-14	3.3*0.8	1.4*0.3	22	7.0	5.5	2.5
2	0	2	5-6	13-14	3.3*0.9	1.5*0.3	21.6	7.8	5.9	2.6
3	0	1	6-7	11-12	4.1* 1.0	1.7*0.4	22.5	8.9	6.5	3.1

TABLE 2: *In vitro* shoot multiplication on different combinations and concentrations of cytokinins and auxins at 25°C

MS + Growth regulators (mgl)			Parameters of shoot multiplication							
IBA	GA-3	KN	Days to multiple shoot formation		Average leaf size(cm)(l*b)		Average Number of Shoots per explant		Average shoot length per explant (cm)	
			Lahaul	Pangi	Lahaul	Pangi	Lahaul	Pangi	Lahaul	Pangi
0	0	0	15-20	20-25	2.0 * 0.3	0.9*0.2	5.3	3.0	3.5	1.3
0	1	0	9-10	20-22	2.2 *0.4	1.2*0.3	11.9	4.5	3.1	1.2
1	1	0	9-10	18-19	2.2*0.4	1.0*0.3	13.0	4.5	3.3	1.3
1	**1**	**1**	**8-9**	**15-17**	**3.0 *0.5**	**1.4*0.3**	**27.8**	**8.5**	**3.9**	**1.7**
1	2	0	8-9	18-19	2.1*0.4	1.3*0.3	17.2	5.0	3.5	1.3
0	2	1	6-8	18-19	2.0*0.4	1.4*0.3	18.2	5.5	3.4	1.3
1	0	2	6-8	15-17	3.0*0.6	1.2*0.4	16.0	4.5	4.3	1.8
2	0	1	5-6	13-14	3.3*0.7	1.2*0.4	16.5	5.0	4.5	1.9
2	0	2	5-6	13-14	3.3*0.8	1.1*0.4	17.8	5.5	4.8	2.3
3	**0**	**1**	**6-7**	**11-12**	**4.1*1.1**	**1.6*0.4**	**17.8**	**7.0**	**5.7**	**2.9**

4.3 *In vitro* rooting on prepared MS media

The Rootable shoots which were excised and put in separate culture jars containing MS Media enriched with rooting growth hormones IBA of different concentrations (Table 2) were observed after 1-2 week(s) and the best results for number of roots and root length was seen in media containing (MS + IBA 4mg/l + Sucrose 30 g/l + agar-agar 9 g/l).

TABLE 3: *In vitro* root induction on different combinations and concentrations of auxins

MS + Growth Hormones (mg/l)		Days to root formation	Avg No. of Roots per explant	Avg root length per explant (cm)
KN	IBA			
0	0	18-20	2.1	0.3
0	1	14-16	2.7	0.45
0	3	12-14	3.8	0.45
1	3	12-14	4.3	1.5
0	4	8-10	5.5	2.1

4.4 Hardening of *in vitro* rooted plantlets in glass house

After 5 weeks of growth on rooting medium, roots were well developed and the plantlets from all rooting media was taken out from all culture jars and after the treatment already mentioned, were potted in potting plastic pots containing different potting mixtures (Table 5). After a week, when the plants showed initial signs of establishment in pots, with the appearance of new leaves, the pots were initially uncovered for overnight. After 2 weeks of transfer to pots, and when the plantlets were totally uncovered, we observed that the potting mixture of sand, soil with perlite,

vermicultite, coco peat in ratio 1:1:1 gave us the best results in terms of plant survival and growth.

Table 4: Growth and Survival of hardened plants in different potting mixtures under glass house conditions.

Potting Mixture (Sand+Soil)	Ratio	No. of Leaves	Percentage Survival of plants(%)
Vermiculite	--	17	25
Perlite	--	18	25
Coco-peat	--	15	50
Perlite+Vermicultie	1:1	18	50
Perlite+coco-peat	1:1	21	75
Vermiculite+coco-peat	1:1	23	50
Perlite+Vermiculite +coco-peat	**1:1:1**	**25**	**75**

Table 5: HPLC results showing the data analysed for Picroside I and Picroside II content

Sample	Plant part analysed	Picroside-I µg\mg of fresh shoot weight	Picroside-II µg\mg of fresh root weight
1.	Lahaul *in vitro* shoot,15°C(IBA+KN)	4.85	-
2.	Lahaul *in vitro* root, 15°C (IBA+KN)	-	-
3.	Lahaul *in vitro* shoot,25°C(IBA+KN)	1.02	-
4.	Lahaul *in vitro* root, 25°C (IBA+KN)	-	-
5.	Lahaul *in vitro* liquid MS media transferred to solid Ms media, 25°C (GA3+IBA+KN)	-	-
6.	Lahaul in vitro explants grown in liquid media(120rpm), 25°C (IBA+KN+GA3)	-	-
7.	*Agrobacterium rhizogenes* (strain 15384) infected shoot,15°C	3.05	-
8.	*Agrobacterium rhizogenes* (strain 15384) infected root,15°C	-	3.08
9.	*Agrobacterium rhizogenes* (strain 15384) infected shoot,25°C	-	-
10.	Lahaul *ex vitro shoot*	9.05	-
11.	Lahaul *ex vitro root*	-	2.02

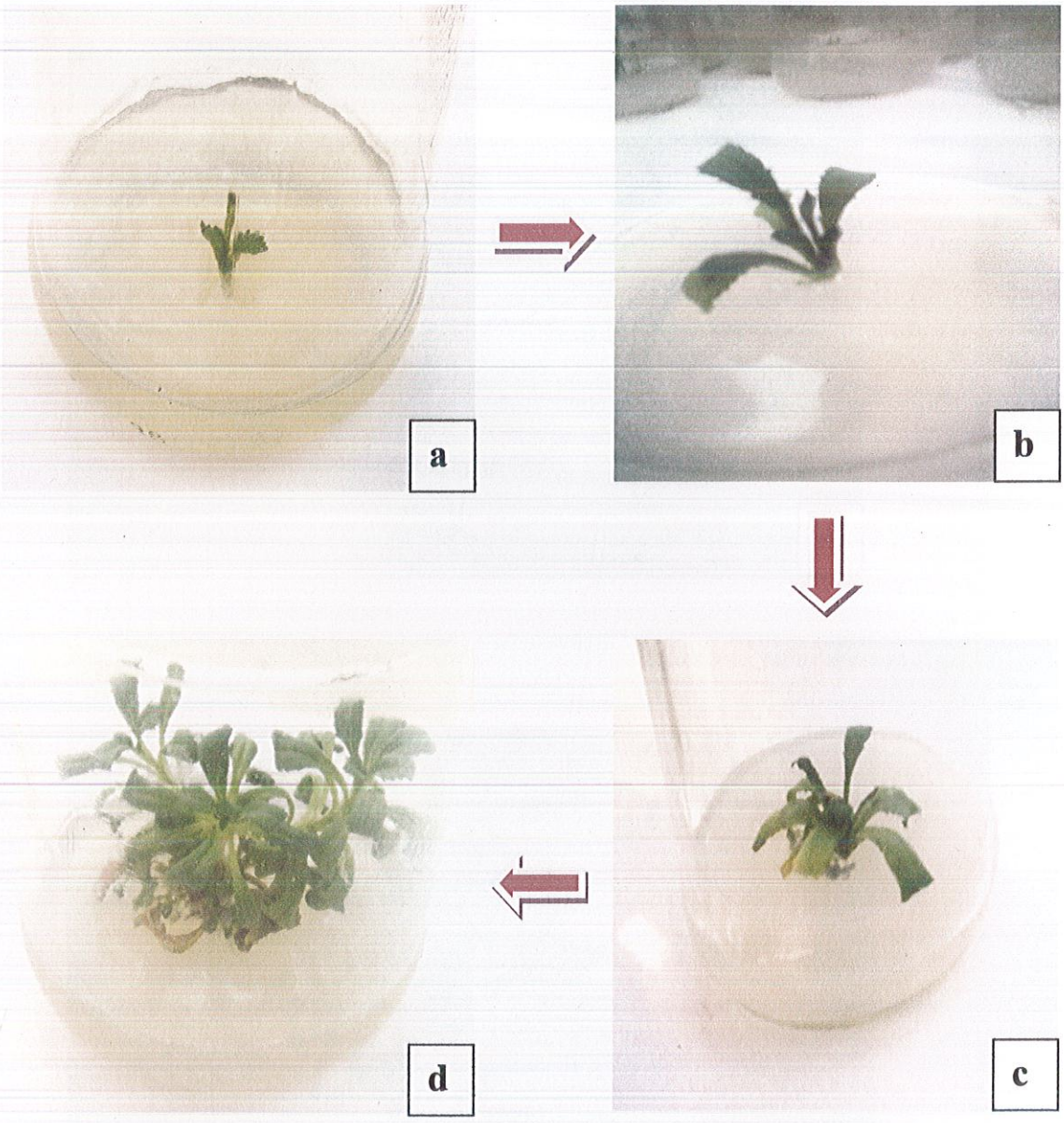


FIGURE 1: *In vitro* shoot multiplication using shot apex as a starting culture.

a) shoot apex cultured in the medium b) shoot proliferation after 2 weeks

c) shoot proliferation after 3-4 weeks d) shoot proliferation after 5-6 weeks


a


b

FIGURE 2: Subculturing done on different growth hormone combinations of MS media.

a)shoot proliferation on GA3+KN+IBA at 15°C

b)shoot proliferation on GA3+KN+IBA at 25°C

c)shoot proliferation on KN+IBA at 15°C

d)shoot proliferation on KN+IBA at 25°C

FIGURE 3: Growth of shoots in a liquid medium containing IBA+KN+GA3 at 25°C at 120rpm

FIGURE 4: *In vitro* root induction and grown plantlet after 4 -5 weeks of planting.

FIGURE 5: Plantlet ready to be transferred to field.

FIGURE 6: *In vitro* developed plantlet transferred for hardening.

a

FIGURE 7: a) Plant acclimatized to the field condition.

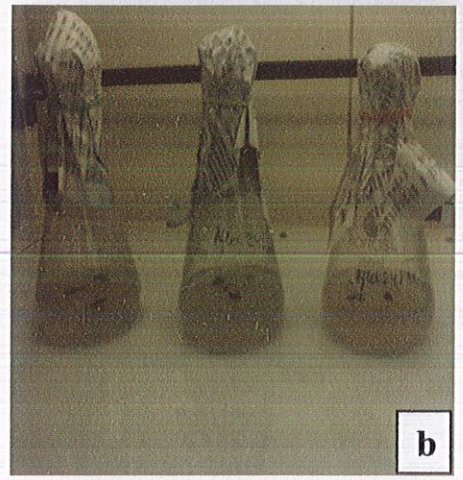


Figure 8: Hairy root cultures

a.) Co-cultivation

b.) Leaf discs infected with *A. rhizogenes* transferred to MS basal media after 24 and 48 hours.

c.) Shoot apex that was infected with *A. rhizogenes* using a syringe.

Figure 9: Growth of *A. rhizogenes* infected plants incubated at

a) 25°C and b) 15°C after being subcultured to a fresh media (Auxin free media)

CONCLUSION

In vitro requirement is an efficient means of *ex situ* conservation of plant diversity and it assists in sustainable maintenance of the present day dwindling germplasm on long term basis, especially for medicinal plant. The established the *in vitro* propagation system of *P. kurroa* and high proliferation rate was achieved with uniform and vigorous growth in the field. This method achieved a proliferation rate of approximately 10 folds (based on the nodes per explant) or 3-4 folds (based on shoots per explant) per month. This high proliferation rate could only be achieved using micro propagation rather than the traditional methods because; at present the rate of propagation in nature is far less than the rate of exploration. As we have developed a successful and rapid propagation procedure for *in vitro* propagation of different strains of *P. kurroa* and we have found that plant grown under in vitro conditions accumulate Picroside-I (4.85 µg/mg) within 6 months of their age, which would definitely be useful for saving the field grown plantlets from getting uprooted. Whereas *Agrobacterium* infected shoots also accumulate Picroside-I (2.53 µg/mg) and roots Picroside-II(4.6 µg/mg) within 2 months of their growth and development. For production of these metabolites the incubation conditions definitely played an important role as results from 15°C not only gave good growth and development but also acted as a catalyst for the production of Picrosides in all above mentioned experimented shoots. So we would like to conclude here that above mentioned procedures not only act as efficient source of *in vitro* propagation but also give good accumulation of Picroside-I but could be used as a alternate source for the large scale production of pure Picroside-I and Picroside-II .

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ANNEXURE – I

Composition of used Murashige and Skoog (1962) basal medium (MS Medium)

STOCKS	CHEMICALS	ORIGINAL STRENGTH (mg/l)	STOCK (g/l)	FINAL VOLUME
A - 10X	KNO_3	1900	19	
	$MgSO_4.7H_2O$	370	3.7	100ml/l
	KH_2PO_4	170	1.70	
B - 20X	NH_4NO_3	1650	33	50ml/l
C - 100X	$CaCl_2.2H_2O$	440	44	10 ml/l
D - 100X	Na_2EDTA	37.26	3.72	10 ml/l
	$FeSO_4.7H_2O$	27.85	2.28	
E - 100X	KI	0.83	0.083	100 ml/l
F - 100X	H_3BO_3	6.2	0.62	
	$CoCl_2.6H_2O$	0.025	0.0025	
	$ZnSO_4.7H_2O$	8.6	0.86	

	$CuSO_4 .5H_2O$	0.025	0.0025	10 ml/l
	$MnSO_4 .4H_2O$	22.3	2.23	
	$Na_2MbO_4 .2H_2O$	0.25	0.025	
G - 100X	m-INOSITOL	100	10	10ml/l
	GLYCINE	2	0.2	
H - 100X	PYRIDOXINE-HCl	0.5	0.05	
	NICOTINE ACID	0.5	0.05	10 ml/l
	THIAMINE	0.1	0.01	

BRIEF BIO-DATA OF STUDENTS

1. Ms.Sanya Bakshi

- CGPA- 7.9 (equivalent to 82%)
- Stream – Biotechnology Dual Degree
- Industrial training- Immunology Lab, school of Life Sciences, JNU, New Delhi
- Area of Interest- Plant Tissue Culture and Vaccines
- Mobile no.- 9816607195
- E-mail Id- sanya_bakshi@yahoo.co.in

2. Ms. Sneha Gulati

- CGPA-7.5
- Stream – Biotechnology Dual Degree
- Area of Interest-Plant tissue culture and Fermentation
- Industrial training- Plant tissue culture division, NBPGR (Pusa), New Delhi
- Mobile no.- 9816728860
- E-mail Id- sneha4oct@gmail.com