MICROPROPAGATION & MOLECULAR CHARACTERIZATION OF *PICRORHIZA KURROA* CHEMOTYPES FOR PICROSIDE-I PRODUCTION

Dissertation submitted in partial fulfillment of the requirement for the degree
of

MASTERS OF TECHNOLOGY
IN
BIOTECHNOLOGY

By

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UNDER THE GUIDANCE OF

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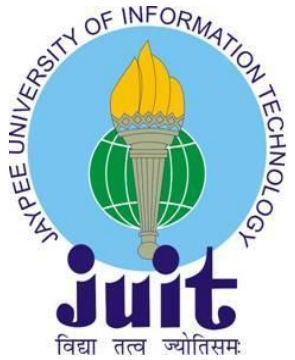

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DECLARATION

I hereby declare that the work reported in the M-Tech thesis entitled **"Micropropagation & Molecular Characterization of P*icrorhiza kurroa* Chemotypes for Picroside-I Production"** submitted at **Jaypee University of Information Technology, Waknaghat, India,** is an authentic record of my work carried out under the supervision of **Dr. Hemant Sood**. I have not submitted this work elsewhere for any other degree or diploma.

……

Sanchita Sharma

Department of Biotechnology and Bioinformatics

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Date ……

SUPERVISOR'S CERTIFICATE

This is to certify that the work reported in the M.Tech thesis entitled **"Micropropagation & Molecular Characterization of *Picrorhiza kurroa* Chemotypes for Picroside-I Production"**, submitted by **Sanchita Sharma** at **Jaypee University of Information Technology, Waknaghat**, **India,** is a bonafide record of his / her original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

…….

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This project report is part of the requirements for the degree of Masters of Technology in Biotechnology at Jaypee University of Information Technology. I would like to thank my project guide **Dr. Hemant Sood** for her guidance, supervision, inspiration and support throughout the duration of the project. She was a mentor during the development of this project and helped me visualize the entire concept. I would like to extend my thanks to Head of Department **Dr. Sudhir Sayal**, and all faculty members of Biotechnology department for their kind cooperation.

I also acknowledge the help rendered by my beloved parents, without their constant support and inspiration I would not have reached at this stage.

Sanchita Sharma

Place:

Date:

ABSTRACT

Picrorhiza kurroa is a medicinally important perennial herb found at an altitude of 3000-5000 m commonly known as "Kutki" or "Kutaki" from Scrophulariaceae plant family located in the Himalayan region. Plant is now listed as endangered species. It has many valuable compounds which offer many uses of *Picrorhiza kurroa* such as hepatoprotective, antioxidant, anti-inflammatory, antiallergic, antiasthmatic, anti-oxidant, anticancerous, anti-bacterial, anti-anaphylactic, anti-malarial, cardioprotective and immune-modulating and potential for treatment of vitiligo. The two main iridoid compounds present in *Picrorhiza kurroa are* Picroside-I and Picroside-II which are used in many herbal drugs commercially available in different combinations.

P. kurroa plant chemotypes have varied amount of P-I when grown in field conditions. Two chemotypes of high Picroside-I content PKS-1 and PKS-21 and two of low Picroside-I content PKS-4 and PKS-5 of *P. kurroa* were selected. The Micropropagation in multiple shoot induction medium showed 21.33 as highest no of shoots, 5.98 cm as highest shoot length and 35.60 g highest number of shoot biomass in PKS-4 followed by PKS-1, PKS-5 and least in PKS-21. The highest P-I content 0.1961mg/g was observed in PKS-21 followed by PKS-1, PKS-5 and least in PKS-4. The gene expression analysis showed expression levels of G10H, PAL, ISPE, HMGR, DXPS, GS, PMK in accordance with levels of P-I which was found to be highest in PKS-21 which proved the association of these genes to P-I synthesis. So the interventions in these genes can be done to increase the P-I content in *P. kurroa.*

For carrying out transformation in *P. kurroa*, concentration of various antibiotics to be used as selective agent need to optimized. In this study the concentration of kamamycin was optimized. 300 to 400 μg/mL concentration was found to be optimum which provides future avenues to explore genetic interventions in *P. Kurroa* chemotypes.

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LIST OF ABBREVIATIONS

cDNA	Complementary Deoxyribo Nucleic Acid
C	Celsius
C18	Carbon18
ISPE	4-Cytidine-5-diphospho-2-C-methylerythriol kinase
DXPS	1-Deoxy-D-xylulose-5-phosphate synthase
PCR	Polymerase Chain Reaction
HPLC	High Performance Liquid Chromatography
GPP	Geranyl Pyrophosphate
g	Gram
HMGR	3-hydroxy-3-methylglutaryl CoA
IAA	Indole-3-Acetic Acid
L	Liter
MEP	Methyl erythritol-4-phosphate
MVA	Mevalonate
µl	Microlitre
µM	Micromolar
mg	Milligram
mL	Millilitre
MEP	Non-mevalonate
mM	Milli Molar
MS	Murashgi and Skoog
OD	Optical Density
RNA	Ribo Nucleic Acid
PAL	Phenylalanine ammonia lyase
PMK	Phosphomevalonate kinase
P-I	Picroside-I
P-II	Picroside-II

TDZ	Thidiazuron

CHAPTER-1

INTRODUCTION

INTRODUCTION

1.1 *Picrorhiza kurroa*

Picrorhiza kurroa is a medicinally important perennial herb found at an altitude of 3000-5000m commonly known as “Kutki” or “Kutaki” from Scrophulariaceae plant family located in the Himalayan region. The vegetative propagation of plant occurs from stolons **(1).**

Classification

Kingdom: Plantae

Division: Angiosperms

Class: Dicotiledonea

Subclass: Asteride

Order: Scorphulariales

Family: Scorphulariaceae

Genus: *Picrorhiza*

Species: *kurroa*

1.2 Valuable Properties of *Picrorhiza kurroa*

It has hepatoprotective, antioxidant, anti-malarial, antiallergic, antiasthmatic, anti-inflammatory, anti-oxidant, anticancerous, anti-bacterial, anti-anaphylactic, potential for treatment of vitiligo, cardioprotective and immune-modulating properties (2, 3, 4, 5, 6, 7).It has commercial importance as many herbal formulation are present in market containing components of this plant. Some of these products are *Arogyavadhini vati, Tiktadi kwath,*

Katuki, livocare, livomap, livplus, Livokin, Tefrolivarogya, Picroliv, Picrolax capsules and suspension (8, 9, 10, 11). Out of many drugs in Indian medicine system from plant sources it is one of the source and among top fifteen plants traded for medicinal use (12, 3). It is an endangered vegetatively propagated plant (13, 9).

1.3 Valuable Compounds from *Picrorhiza kurroa*

Picrorhiza kurroa contains flavonoids, phenolics, cucurbitacins, iridoid-glucoside (14).It contains Picroside-I, Picroside-II and kutkoside as three main iridoid glycosides (13).

1.4 Biochemisrtry of Synthesis of Picrosides

Picrosides are monoterpenoides. Picrosides are classified as Picroside-I and Picroside-II on the basis of functional moiety present. Picroside-I has cinnamate as functional group moiety. Iridane moiety of Picrosides is geranyl pyrophosphate (GPP) derived. Isopentenyl pyrophosphate (IPP) leads to the synthesis of GPP which is synthesized from either mevalonate (MVA) pathway which operates in cytosol or tidyl 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway which operates in plastids or from both the pathways.

1.4.1 MVA Pathway

The condensation of two acetyl-CoA molecules forms acetoacetyl-CoA in the first step of mevalonate pathway. Acetoacetyl-CoA then forms 3-hydroxy-3-methylglutaryl CoA (HMG-CoA). The reduction of HMG-CoA occurs which forms MVA catalysed by HMG-CoA reductase. HMG-CoA reductase is a key regulatory enzyme of the mevalonate pathway. Then two phosphorylation steps occurs which form mevalonic-5-monophosphate (MVA-P) catalysed by mevalonate kinase. Mevalonic-5-diphosphate (MVA-PP) is formed in the successive step catalysed by the enzyme phosphomevalonate kinase. Decarboxylation of MVA-PP forms IPP which is adenosine triphosphate (ATP)-dependent. The IPP is isomerized to form DMAPP catalysed by the enzyme isomerase (IPPI) (15).

1.4.2 MEP pathway

In MEP pathway, pyruvate and glyceraldehyde-3-phosphate condensation forms 1-deoxy-D-xylulose 5-phosphate (DXP) catalysed by the enzyme 1-deoxy-D-xylulose-5-phosphate

synthase (DXS). DXP forms methyl erythritol-4-phosphate (MEP) by intramolecular rearrangement and reduction catalyzed by the enzyme 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR). Methyl erythritol-4-phosphate (MEP) is the first precursor for the synthesis of isprenoids in plastids. Methyl erythritol-4-phosphate (MEP) is then gets converted to 4-diphosphocytidyl-2-C-methylerythritol (CDP-ME) catalyzed by the CDP-ME synthase (CMS),then 4-diphosphocytidyl-2-C-methylerythritol 2-phosphate (CDP-MEP) is formed by catalysis with CDP-ME kinase (CMK) enzyme, methyl-erythritol 2, 4-cyclodiphosphate (ME-cPP) is then formed in next step by catalysis with enzyme ME-cPP synthase (MCS). Hydroxymethylbutenyl 4-diphosphate (HMBPP) is formed from Methylerythritol 2, 4-cyclodiphosphate (ME-cPP) catalysed by hydroxymethylbutenyl 4-diphosphate synthase (HDS). Then Hydroxymethylbutenyl 4-diphosphate (HMBPP) is converted by enzyme HMBPP reductase (HDR) into IPP and DMAPP. The biosynthesis of P-I requires synthesis of cinnamate from phenylalanine by phenylpropanoid pathway by the action of enzyme phenylalanine ammonia lyase (PAL) and iridoid moiety of P-I is formed by oxidation and cyclization of GPP followed by condensation of glucose moiety(15, 16).

Picroside-I synthesis in plant occurs in shoots (1, 3). Plant produces small amounts of Picrosides therefore to increase the yield of Picrosides the understanding of biology and molecular basis of their synthesis need to be understood (17). Picrosides biosynthesis transcriptional regulation is not known completely. In-vitro cultured plants of *P. kurroa* have less Picrosides than plants in their natural habitats necessitates identification of regulating factors (9).Genetic interventions for enhancing Picrosides production require knowledge of key genes involved in its production (18).

Among 26 chemotypes of *P. kurroa* named as PKS-1 to PKS-26 according to different locations from which they are obtained, two high and two low Picroside-I content containing plant chemotypes were used in this study namely PKS-1, PKS-4, PKS-5 and PKS-21.

1.5 Kanamycin as Selective Agent

Kanamycin is an aminoglycoside antibiotic. It is a potential selective agent used in plant genetic engineering. It is a trisaccharide composed of a deoxystreptamine and two

glucosamines produced by the soil actinomycete *S. kanamyceticus*. It protein synthesis is inhibited by binding to the ribosomal subunits in bacteria. In plant plastids and mitochondria protein synthesis is inhibited similarly. The corresponding resistance gene nptII encodes an aminoglycoside-3-phosphotransferase, which inactivates kanamycin by phosphorylation. The stringency of selection transgenic shoots with this antibiotic is highly dependent on various types of factors such as plant species, explant type, explant size, bacterial concentration, length of exposure, cocultivation period, and presence of other antibiotics (19).

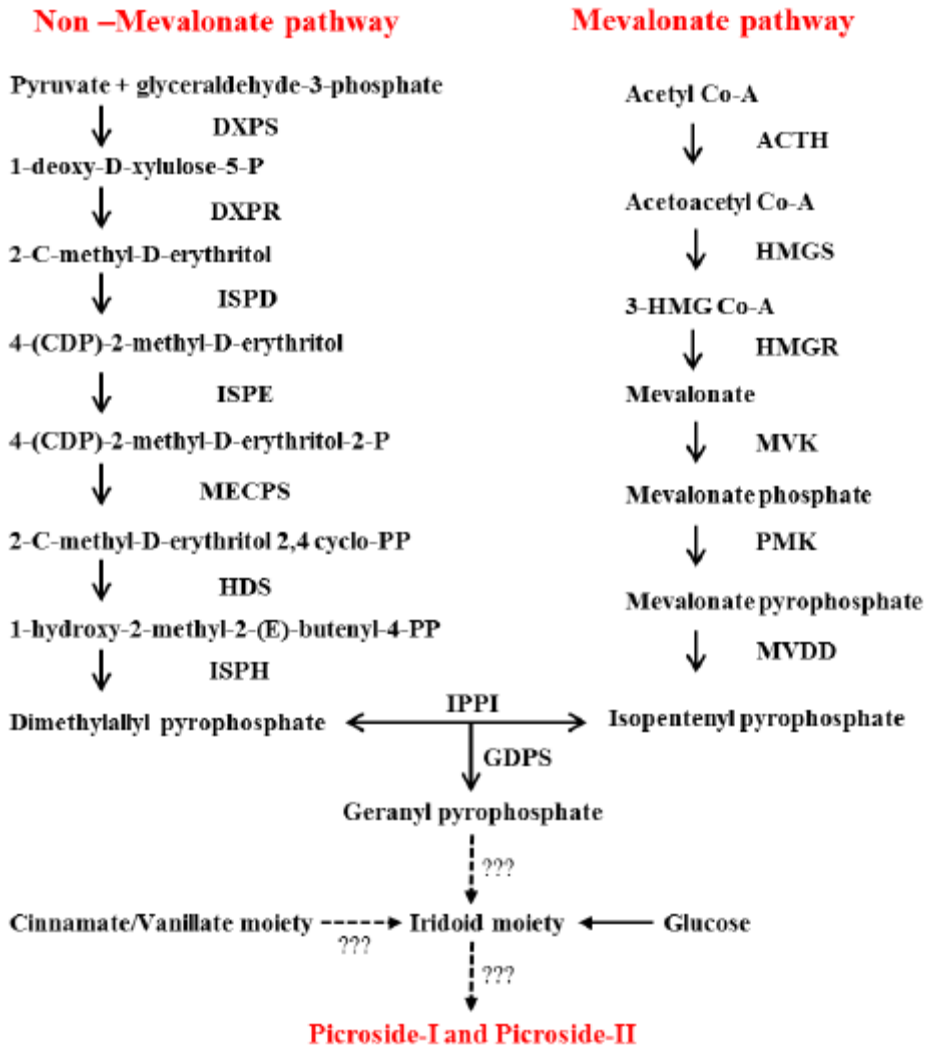


Fig 1.1: Pathway for synthesis of Picrosides in *P. kurroa* (17**)**

CHAPTER-2

REVIEW OF LITERATURE

REVIEW OF LITERATURE

2.1 *P. kurroa*

P. kurroa leaf tissues have higher Picroside-I content than roots and rhizomes. The biosynthesis and accumulation Picrosides is temperature and light dependent. It was found to be more at 15°C than 25°C temperature (16, 20).The genes *pkhmgr* and *pkdxs* plays important role in level of picrosides production. These genes are regulated by light and temperature conditions (15). Shoots contain Picroside-I whereas roots have picroside-II conferred from P-I and P-II estimation in different tissues (17).

2.2 Clonal Propagation of *Picrorhiza kurroa*

Picrorhiza kurroa clonal propagation was done from shoot tip in Murashige and Skoog medium (1962) with kinetin at concentration from 3.0 to 5.0 mg/L leads to rapid multiple shoots generation. Addition indole-3-acetic acid at a concentration of 1.0 mg/L showed improved growth of the regenerated shoots whereas in the presence of IAA there is no alteration in the growth of regenerated shoots. By transferring shoots to MS medium containing 1.0 mg/L α-naphthalene acetic acid roots were readily obtained (21). Shoot apices of *Picrorhiza kurroa* were cultured on MS medium supplemented with growth hormones at a concentration of 2 mg/l indole-3-butyric acid [IBA] and 3 mg/l kinetin [KN] (22).

2.3 Hardening of *Picrorhiza kurroa* Plants

Plants were hardened onto the pots with sand, soil and manure in ratio 1:1:1 in growth chamber and glasshouse under controlled environmental conditions (21).

2.4 Quantification of Componds of *Picrorhiza kurroa*

For quantification of Picroside-I, Picroside-II and apocynin from plant extracts of *P. kurroa* gradient RP-HPLC method using acetonitrile (ACN) and water as solvent systems was used with 1.00 mL/minute flow rate (23). Extraction of P-I and P-II from tissue cultured *Picrorhiza kurroa* was done from callus dried extract dissolved in methanol at

different time intervals by using the thin layer chromatography (TLC) and HPLC analysis. Water:Acetonitrile (75:25) were used as mobile phase in isocratic HPLC elution with 1 mL/minute flow rate and detection was done at wavelength of 270nm (4). Another High Performance Liquid Chromatography method was developed for estimation of Picroside I, Plumbagin, and Z-guggulsterone in Tablet Formulation. The Reverse Phase analysis was performed on a C18 column using the mobile phase consisting of acetonitrile as solvent A and 0.1% orthophosphoric acid in water as solvent B with the following gradient: 0-12 min, 25% A; 12-17 min, 25-80% A; 17-32 min, 80% A; and 32-37 min, 80-25% A at a flow rate of 1 ml/min. UV detection was done at 255 nm (24).

Another High Performance Liquid Chromatography method was developed for detection of sugars and Picrosides from rhizomes of *Picrorhiza kurroa* and *P. scrophulariiflora*. The extraction was done using ultrasonic extraction with evaporative light scattering detection. Ethanol:Water (1:1) were used for simultaneous extraction of sugars, Picroside-I and II. The analysis was carried out on a Zorbax amino column (250 mm x 4.6 mm i.d., 5 microm) with isocratic elution of acetonitrile:water (78:22, v/v) for the simultaneous determination of sugars (xylose, xylitol, mannitol, glucose and sucrose) and Picrosides (Picroside-I and Picroside-II) (25).

Another High Performance Liquid Chromatographic method was developed for the determination of Picroside-I in plasma. Separation was achieved using a C_{18} reversed phase column coupled with a photodiode array detector and acetonitrile 0.1 M and acetic acid (25:75) as mobile phase (26).

In another method chromatographic separations were performed on C18 column for P-I and P-II using mobile phase acetonitrile:10 mM ammonium acetate buffer of pH 3.5 in the ratio 90:10 v/v (27).

2.5 Biosynthesis of Picrosides

The catalpol and aucubin along with three other metabolites, boschnaloside, bartsioside and mussaenosidic acid leads to the formation of Picrosides in *P. kurroa*. The glucosylation of iridotrial gives boschnaloside, which undergoes hydroxylation and oxidation to form mussaenosidic acid. Further decarboxylation and dehydration forms bartsioside. Bartsioside form aucubin by hydroxylation and aucubin further gets converted

to catalpol by epoxidation. Catalpol gets converted to Picrosides and other related compounds by esterification of different free hydroxyl groups. This study has reported biosynthetic pathway for Picrosides and related compounds in *Picrorhiza kurroa* (28).

The study was undertaken to explore the status of metabolites accumulation and biosynthesis in the field grown plants of *P. kurroa*. In this study it was found that environmental parameters along with different morphogenetic stages of its developmental cycles, different age groups and different parts of plantlets have an effect on Picrosides biosynthesis and accumulation (31).

The effect of biosynthetic precursors cinnamic acid and catalpol on Picroside-I synthesis in *P. kurroa* plants shoots was studied when supplied individually and in combination. It was found that cinnamic acid and combination of cinnamic acid and catalpol stimulate P-I production. It was concluded that precursors of iridoid and shikimate/phenylpropanoid pathways are a flux limitation for P-I production in shoot cultures (32).

The effect of different temperatures were studied on biomass of shoots and P-I accumulation. The different temperatures used were 10°C, 15°C, 25°C and 30°C. MS medium was used with 3mg/L Kinetin and 2mg/L Indole-3-buyteric acid. Both highest biomass and highest P-I concentration was obtained from the shoots at 15°C (33).

2.6 Molecular Analysis in *P. kurroa*

Expression analysis of genes of non-mevalonate (MEP) and mevalonate (MVA) pathways in different tissues of *Picrorhiza kurroa* showed DXPS, ISPD, ISPE, MECPS of MEP pathway and PMK of MVA pathway showed increased expression levels in accordance with the P-I contents (17). Full-length cDNA sequences of eight genes were cloned namely 1-deoxy-D-xylulose-5-phosphate synthase, 1-deoxy-D-xylulose-5-phosphate reductoisomerase, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase, acetyl-CoA acetyltransferase, 3-hydroxy-3-methylglutaryl coenzyme A reductase, isopentenyl pyrophosphate isomerase and geranyl diphosphate synthase. The picrosides accumulation was found to be maximum in leaf tissue then in the rhizome and followed by roots and expression pattern for genes showed same pattern as well (15).

A temperature of 15°C favored picrosides accumulation as compared to 25°C and this was in agreement with the expression of 1-deoxy-D-xylulose-5-phosphate synthase *(*DXS*)* and 3-hydroxy-3-methylglutaryl-coenzyme A reductase *(*HMGR), the genes associated with picrosides biosynthesis (16).

Only GDPS gene showed high transcript level with 9 fold in roots and 20 fold in shoots (17).

Expression analysis of genes DXPS, HMGR, ISPD, PMK, ISPE, HK, IS, DAHPS, PK, GS, PAL, G10H, 10-HGO, MDH, ICDH and G6PDH in different stages of plant development was done involved in primary and secondary metabolism of plants. Development of shoots at low temperature up-regulated the expression of genes involved in secondary metabolism leading to enhanced P-I accumulation in *P. kurroa*. Quantitative expression analysis of secondary metabolism genes revealed that 7 genes HMGR, PMK, DXPS, GS, G10H, DAHPS and PAL showed high transcript abundance in fully developed plant leaf and root segments at 15°C compared to 25°C in *P. kurroa* (3).

NGS Transcriptomes of *Picrorhiza kurroa* and use of enzyme inhibitors mevinolin, fosmidomycin, glyphosate AOA, and actinomycin D unravel that geranyl pyrophosphate (GPP) moiety is mainly contributed by the non-mevalonate (MEP) route, which is further modified to P-I and P-II through phenylpropanoid and iridoid pathways, in total consisting of 41 and 35 enzymatic steps, respectively. The higher expression of the majority of the MEP pathway genes (ISPD, DXPS and ISPE) coupled with higher inhibition of DXPR enzyme by fosmidomycin, suggested that the MEP route contributed to the biosynthesis of P-I in *P. kurroa*. The higher expression of the majority of the MEP pathway genes (ISPD, DXPS and ISPE), coupled with higher inhibition of DXPR enzyme by fosmidomycin, suggested that the MEP route contributed to the biosynthesis of P-I in *P. kurroa* (10).

13 genes which codes for rate limiting enzymes hexokinase and pyruvate kinase of glycolysis, isocitrate dehydrogenase and malate dehydrogenase of Citric acid pathway, glucose-6-phosphate dehydrogenase of pentose phosphate pathway, 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase and phenylalanine ammonia lyase from shikimate pathway, hydroxymethylglutaryl CoA reductase from mevalonate pathway, 1-deoxy-D-xylulose-5-phosphate synthase from non mevalonate pathway, 4-

diphosphocytidyl-2C-methyl-D-erythritol synthase and phosphomevalonate kinase were analysed by qRT-PCR in *Picrorhiza kurroa* shoots at different time intervals i.e. at 0,10,20,30,40 days. Out of these five genes HK, DXPS, ISPD, HMGR and PMK were found to be essential for Picroside-I synthesis upto 20 days where as DAHPS and G-10-H were found to be assisting Picroside-I synthesis between 20-30 days growth period of *P. kurroa* (30).

Highthroughput de novo transcriptome sequencing was done to know the molecular changes which occurs due to the temperature differences which leads to the different Picroside accumulation in *Picrorhiza kurroa*. Large scale expression profiling through Reads per exon kilobase per million (RPKM) showed major transcriptome changes in response to temperature reflecting alterations in major biological processes and metabolic pathways. The average GC content was found in the range of 40-49% and 20 most abundant Transcription families were found in *P. kurrooa*. Putative cytochrome P450 (CYPs) and uridine diphosphate glycosyltransferases (UGTs) were identified which can be used to deduce the unknown genes responcible for the synthesis of Picrosides (16).

The two genes HMGR and DXPS were cloned by using degenerate primers and by cDNA end amplification followed by cloning of the upstream sequences that showed the presence of core sequences for light and temperature responsiveness. Protein binding to these motifs is confirmed by the elecrophoretic mobility shift assay. It was deduced that there was up regulation of HMGR and DXPS at 15°C temperature and under light conditions as compared to 25°C temperature and under dark conditions. Also there was up regulation of the genes with higher P-I content in the light conditions of the in vitro grown plants (20).

2.7 Use of Kanamycin in Transformation Protocols

A transformation protocol for Maritime Pine based on kanamycin selection was developed for Agrobacterium mediated transformation of maritime pine embryonal masses as the binary vector pBINUbiGUSint, containing neomycin phosphotransferase-II (nptII) as a selectable marker gene. Different factors, such as embryogenic line, bacterial strain, bacterial concentration, and coculture duration, were examined and optimized. For selection of transformants, 15 mg/L kanamycin was used (29).

2.8 AIM

Plant produces small amounts of Picrosides because of various factors such as plant maturity, geographical origin, environmental and agronomic conditions, genotype and chemotype. So the present study is conducted to identify the elite chemotype for the micropropagation, production of high P-I containing chemotype of *P. kurroa* and exploring the molecular basis for the synthesis of Picrosides under *in vitro* conditions. As genetic interventions for enhancing P-I production require complete knowledge of the genes and their role involved in the pathway for the production.

The objective for the current study is as follows:

2.9 OBJECTIVE

1. Micropropgation of high and low Picroside-I content chemotypes of *Picrorhiza kurroa*.
2. To estimate the Picroside-I content through HPLC analysis.
3. To check the expression of key genes for increased Picroside-I content.
4. Cytotoxic effect of kanamycin for leaf explants.

CHAPTER-3

MATERIALS AND METHODS

3.1 Plant Material

Plant material was obtained from HFRI, Shimla and grown in the glasshouse in Department of Biotechnology at Jaypee University of Information Technology, Waknaghat. Four chemotypes were selected namely PKS-1, PKS-4, PKS-5 and PKS-21 (named according to the different geographical conditions from which plant was obtained).

Table 3.1: Details of *Picrorhiza kurroa* chemotypes

Accession	**Location**	**District**	**Altitude**	**Latitude**	**Longitude**
PKS-1	Hudan	Chamba	3620	33006'27.1"N	76029'171.1"E
PKS-4	Teita	Chamba	3590	32°31′28″N	76°31′01″E
PKS-5	Moral Danda	Shimla	3354	31018'23.6"N	77045'02.1"E
PKS-21	Pattal (Tissa)	Chamba	3245	32057.321″N	76018.417"E

3.2 Media Preparation and Culture Conditions

3.2.1 Multiple Shoot Induction

MS medium (Murashige and Skoog 1962) supplemented with 3 mL/L indole-3-butyric acid (IBA), 1 mL/L kinetin (KN), 30 g/L sucrose and 9 g/L agar was used as a multiple shoot induction medium. Shoot apices were used as explants and cultured in media after surface sterilization of explants for all the chemotypes that are PKS-1, PKS-4, PKS-5 and PKS-21. Two temperatures 25 ± 2°C and 15 ± 2°C were used for maintaining plant cultures with relative humidity of 70 %, photoperiod of 16 h at 40 lmol/m2/s

photosynthetic photon flux density provided by cool white fluorescent tubes (Philips, India).

3.2.2 *In vitro* Root Induction

The *in vitro* grown shoots *P. kurroa* chemotypes PKS-1, PKS-4, PKS-5, and PKS-21 were subcultured for *in vitro* root induction. The same MS medium was used with higher concentration of IBA. The shoots were incubated for 30 days to form roots before hardening.

3.3 Hardening and Acclimatization of *in vitro* grown plants

In vitro grown plants having well established roots are taken out from the culture jars, washed with water to remove the culture media and transferred to the pots containing 1:1:1 ratio of perlite, vermiculite and coccopeat. The plants are covered with inverted jars to maintain the moisture. After 15 days to acclimatize the plants jars were removed every morning and evening for one hour and placed back. After 30 days jars were removed completely.

3.4 Quantification of Picroside-I by HPLC

Preparation of sample was done by grounding fresh samples in liquid nitrogen and then suspended in 80% methanol, sonicated followed by centrifugation and then filtered through 0.22 μm filter.

Picroside-I content of *in vitro* grown *Picrorhiza kurroa* chemotypes PKS-1, PKS-4, PKS-5 PKS-21 at 15 ± 2°C and 25 ± 2°C estimated using isocratic reverse phase HPLC Waters Spherisorb reverse phase C18 column (4.6 mm x 250 mm, 5 μm). 0.05% TFA in MilliQ water is used as mobile phase A and Metanol:Acetonitrile in ratio 1:1 was used as mobile phase B with a flow rate of 1 mL/minute in 70:30 of mobile phase A : Mobile phase B. The detection was done using Waters 2996 photodiode array detector at wavelength 270nm at Retention time of 14.154 for P-I.

3.5 Expression Analysis of Key Genes

3.5.1 Selection of Genes Associated with Picroside-I Pproduction

Total seven genes were selected which are associated with Picroside-I production namely HMGR, ISPE, PMK, DXPS, PAL, GS and G10H for expression analysis.

3.5.2 RNA Isolation

Total RNA was isolated from shoots of PKS-1, PKS-4, PKS-5 and PKS-21 *Picrorhiza kurroa* chemotypes from the cultures maintained in plant tissue culture chambers at 25 ± 2°C and 15 ± 2°C by using QUIGEN ® RNA Isolation Kit by following instructions as specified by the manufacturer. RNA quality and quantity was checked using NanoDrop spectrophotometer (Thermo Scientific) by measuring OD values at 260, 280 & 230 and agarose gel electrophoresis stained with 1% w/v ethidium bromide.

3.5.3 cDNA Synthesis

cDNA synthesis was done using BioRad cDNA synthesis kit according to the instructions given by the manufacturer.

3.5.4 Expression analysis using Real Time PCR

RNA isolated from shoots of *P. kurroa* chemotypes PKS-1, PKS-4, PKS-4 and PKS-21 were used for checking the expression of selected pathway genes. Equal RNA from each sample is used to prepare cDNA and then used in real time PCR. The protocol used: 95° for 3 minutes followed by 40 cycles of denaturation, annealing and extension steps at 95°C for 10s, 48-60°C for 30s and 72°C for 20s respectively. The melt curve analysis was done at 55°C to 95°C with an increment of 0.5°C per step. A Housekeeping genes 26S rRNA was used as an internal control.

Table 3.2: List of primers used in real time PCR procured from Pandit *et.al*

Genes	Forward Primer	Reverse Primer	Size of fragment (bp)	Annealing Temprature (°C)
26s	CACAATGATAGGAAGAGCCGAC	CAAGGGAACGGGCTTGGCAGAATC	500	58
HMGR	CGTTCATCTACCTTCTAGGGTTCTT	GACATAACAACTTCTTCATCGTCCT	100	60
PMK	TGGATGTTGTCGCATCAGCACCTGG	GTAATAGGCAGTCCACTCGCTTCAA	100	58
ISPE	TTCATCTAGATAAGAAGGTGCCAAC	CCTCTACCAGTACAATAAGCAGCTC	110	55
DXPS	ACATTTAAGTTCAAGTCTGGGAGTG	ATGTGCACTCTCTTCTCTTTTAGGA	110	55
PAL	GCAAGATAGATACGCTCTAA	GTTCCTTGAGACGTCAAT	136	49
G10H	TATCGAGCTTTTCAGTGGAT	GATGTGAGTCCTGTCGATTT	136	52
GS	TGGGTAGATTAGAAGCCAGA	CTGGTGATTTCTACCAGCTC	139	52

3.6 Effect of Kanamycin on leaf explants

Effect of kanamycin was evaluated on *in vitro* callusing of leaf explants. 50 mg/mL stock solution of kanamycin was prepared in autoclaved distilled water and filtered by membrane filter of 0.2 μm. The working concentrations of 0, 10, 20, 30, 40 & 50 mg/L were used. MS media supplemented with TDZ 0.5 mL/L was used. Different concentrations of kanamycin were added to the prepared autoclaved media cooled upto 50-60°C.

CHAPTER-4

RESULT & DISCUSSION

RESULT

4.1 Micropropagation

The plants were micropropagated in MS media supplemented with kinetin at a concentration of 1 ml/L and Indole 3- Butyric Acid at a concentration of 3 ml/L. Shoots were subcultured after every 30 days Cultured *in vitro* shoots of *P. kurroa* were incubated at 15 ± 2°C (Fig. 5.1, 5.2, 5.3 & 5.4) and at 25 ± 2°C (Fig. 5.5, 5.6, 5.7 & 5.8) and various growth parameters were recorded such as number of shoots, length of shoots and plant biomass. Plants incubated at 25 ± 2°C showed less growth as compared to the plants at 15 ± 2°C recorded in the form of various parameters. At 15 ± 2°C PKS-1 showed 20.67 average number of shoots with average length of 4.27 cm and plant biomass of 33.89 g, 21.33 average number of shoots with average length of 5.98 cm and plant biomass of 35.60 g in PKS-4, 4.67 average number of shoots with average length of 3.33 cm and plant biomass of 25.36 g in PKS-5 and 4.33 as average number of shoots with average length of 2.33cm and plant biomass of 15.09 g in PKS-21(Table5.1). At 25 ± 2°C PKS-1 showed 8 average number of shoots with average length of 3.63 cm and plant biomass of 27.02 g, 10 average number of shoots with average length of 4.10 cm and plant biomass of 28.84 g in PKS-4, 4 average number of shoots with average length of 2.41cm and plant biomass of 19.73 g in PKS-5 and 3.33 average number of shoots with average length of 1.66 cm and plant biomass of 14.36 g in PKS-21(Table5.2).

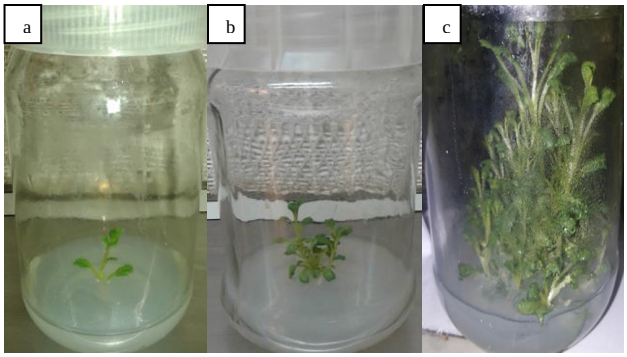


Fig 4.1: PKS-1 grown at 15°C. (a) At day 1 (b) At week 2 (c) At week 4

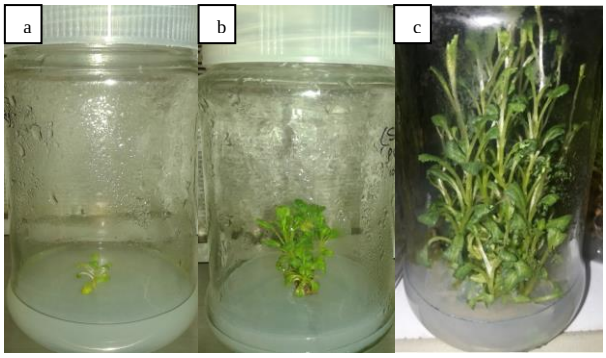


Fig 4.2: PKS-4 grown at 15°C. (a)At day 1 (b) At week 2 (c) At week 4

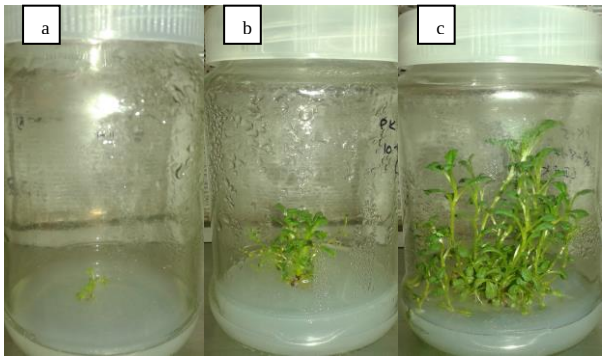


Fig 4.3: PKS-5 grown at15°C. (a) At day 1 (b) At week 2 (c) At week 4

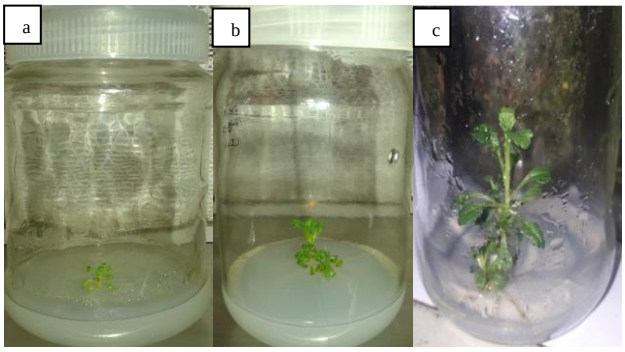


Fig4.4: PKS-21 grown at 15°C. (a) At day 1 (b) At week 2 (c) At week 4

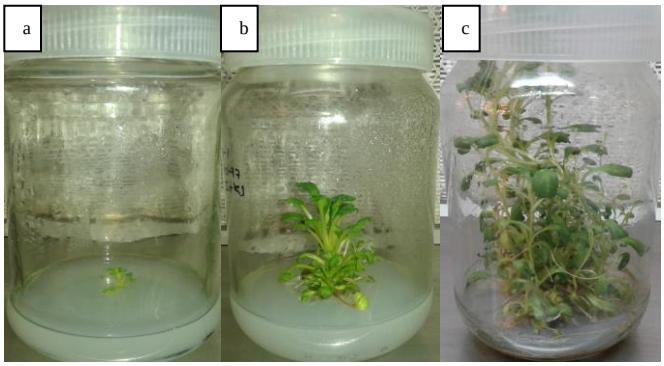


Fig 4.5: PKS-1 grown at 25°C. (a) At day 1 (b) At week 2 (c) At week 4

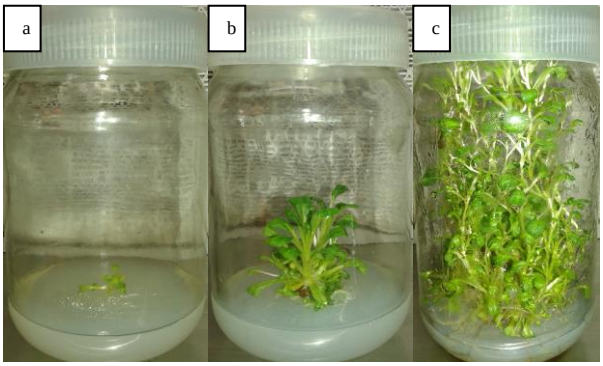


Fig 4.6: PKS-4 grown at 25°C. (a) At day 1 (b) At week 2 (c) At week 4

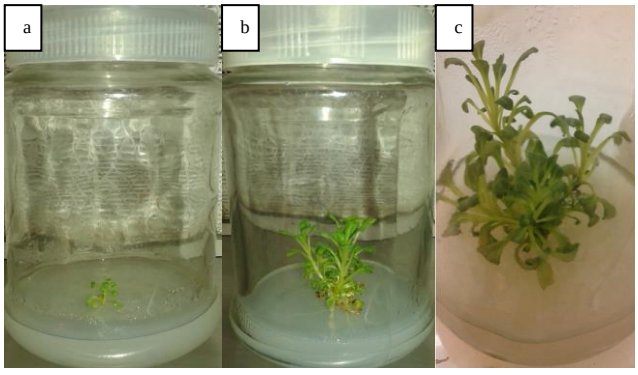


Fig 4.7: PKS-5 grown at 25°C. (a) At day 1 (b) At week 2 (c) At week 4

Fig 4.8: PKS-21 grown at 25°C. (a) At day 1 (b) At week 2 (c) At week 4

Table 4.1: Growth and Developmental Parameters of plant chemotypes PKS-1, PKS-4, PKS-5 and PKS-21 of *P. kurroa* at 15°C

Growth Parameters	**PKS-1**	**PKS-4**	**PKS-5**	**PKS-21**
No of shoots	20.67±2.52	21.33±2.52	4.67±0.58	4.33±1.53
Length of Shoot	4.27	5.98	3.62	3.31
Plant Biomass	33.89	35.60	25.36	15.09

Table 4.2: Growth and Developmental Parameters of plant chemotypes PKS-1, PKS-4, PKS-5 and PKS-21 of *P. kurroa* at 25°C

Growth Parameters	**PKS-1**	**PKS-4**	**PKS-5**	**PKS-21**
No of shoots	8±2.00	10±2.00	4±1.00	3.33±0.58
Length of Shoot	3.62	4.10	2.41	1.66
Plant Biomass	27.02	28.84	19.73	14.36

4.1.2 *In vitro* Root Induction

The *in vitro* grown shoots were subcultured in rooting medium for root induction. All chemotypes PKS-1, PKS-4, PKS-5 and PKS-21 of *P. kurroa* have shown 5 to 6 roots per shoot.

4.2 Hardening and Acclimatization of *in vitro* grown plants

In vitro grown plant chemotypes PKS-1, PKS-4, PKS-5 and PKS-21 were acclimatized for environment conditions and percentage survival rates were found to be near 50% except in PKS-5 which showed much less survival rates in glasshouse conditions. I*n vitro* grown

plant chemotypes PKS-1, PKS-4 and PKS-21of *P. kurroa* showed percentage survival rate of 41.67%.

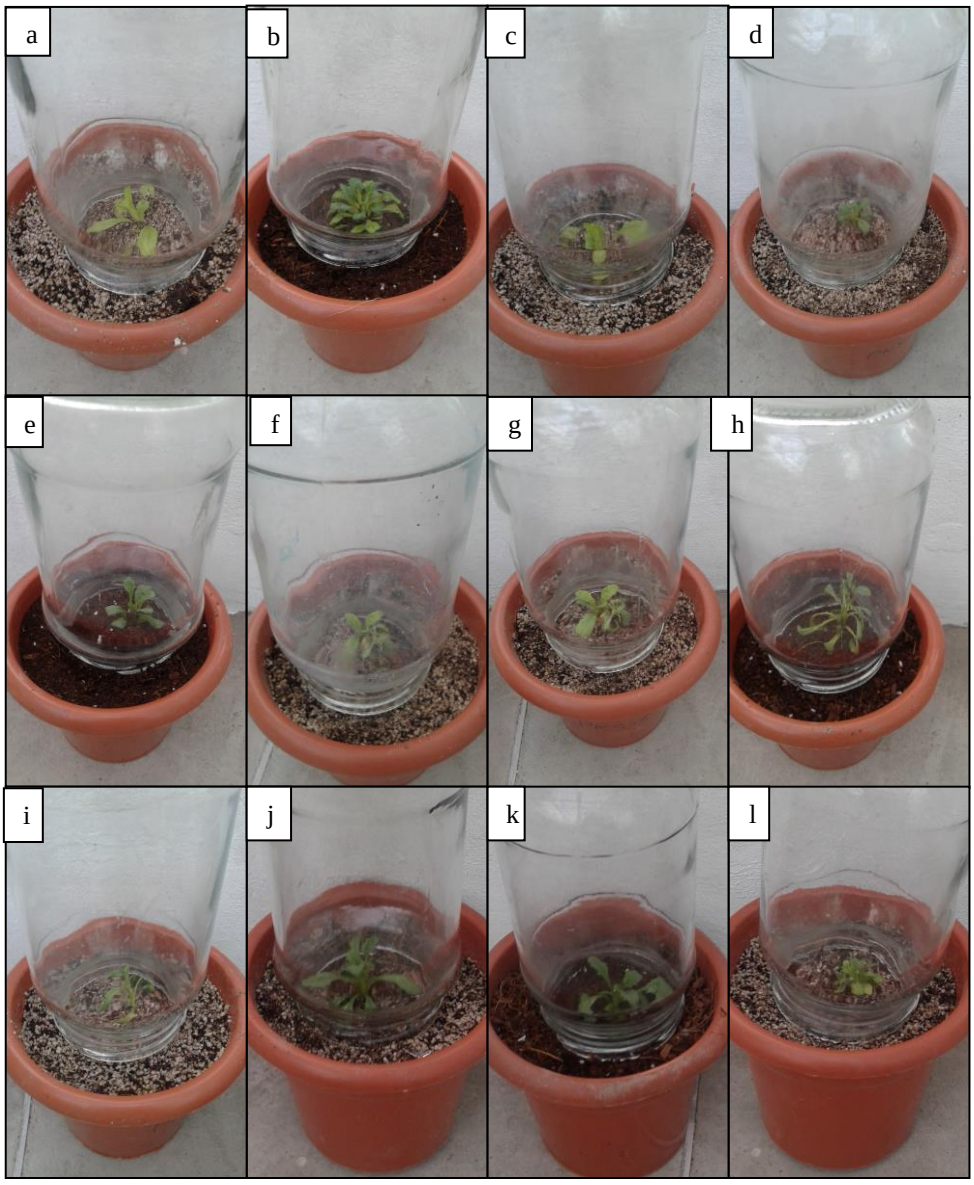


Fig 4.9: Hardening of *in vitro* grown plants of *P. kurroa* a,b,c:PKS-1d,e,f: PKS-4 g,h,i:PKS-5 j,k,l:PKS-21

4.3 HPLC Analysis for estimation of amount of Picroside-I

The Picroside-I amount for the PKS-1, PKS-4, PKS-5 and PKS-21 shoots comes out to be 0.0901 mg/g, 0.0881 mg/g, 0.102 mg/g and 0.1961 mg/g respectively. Maximum Picroside-I concentration was found in PKS-21 followed by PKS-5, then in PKS-1 and least in PKS-4 chemotypes of *P. kurroa*.

HPLC analysis of plant samples prepared showed the following chromatograms:

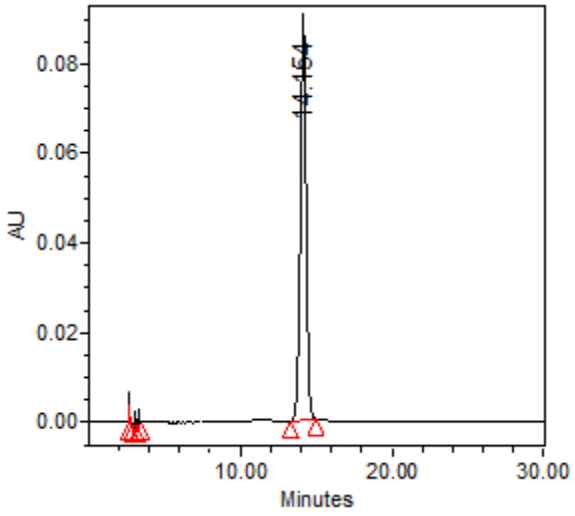


Fig 4.10: Chromatogram showing Standard peak for P-I

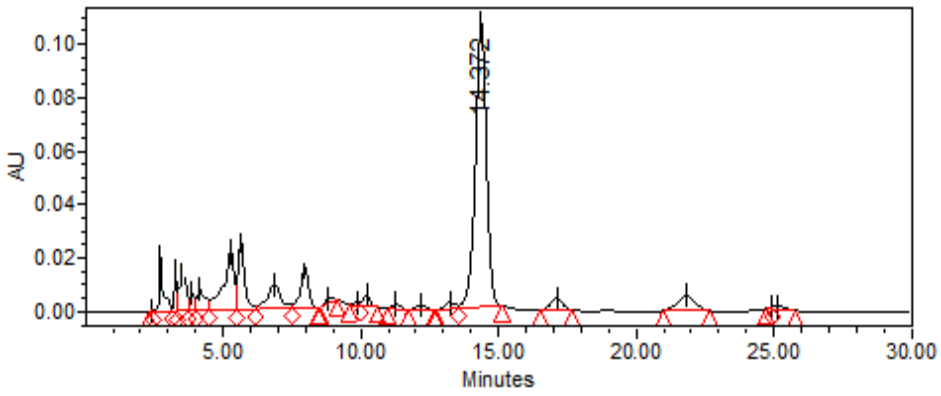


Fig 4.11: Chromatogram showing peak for P-I content of PKS-1

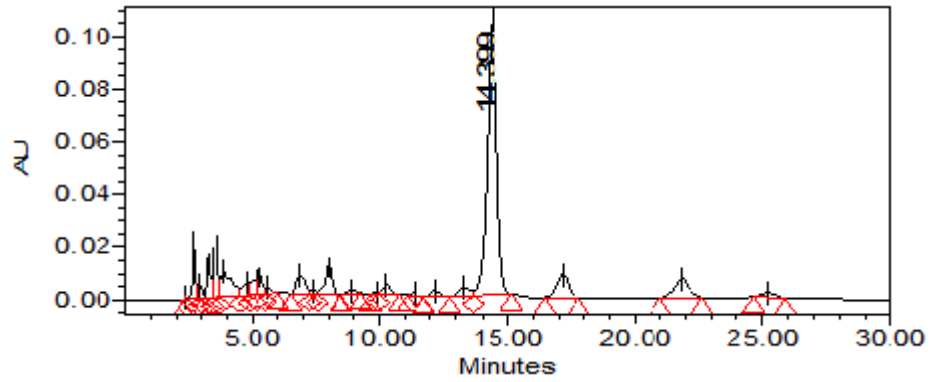


Fig 4.12: Chromatogram showing peak for P-I content in PKS-4

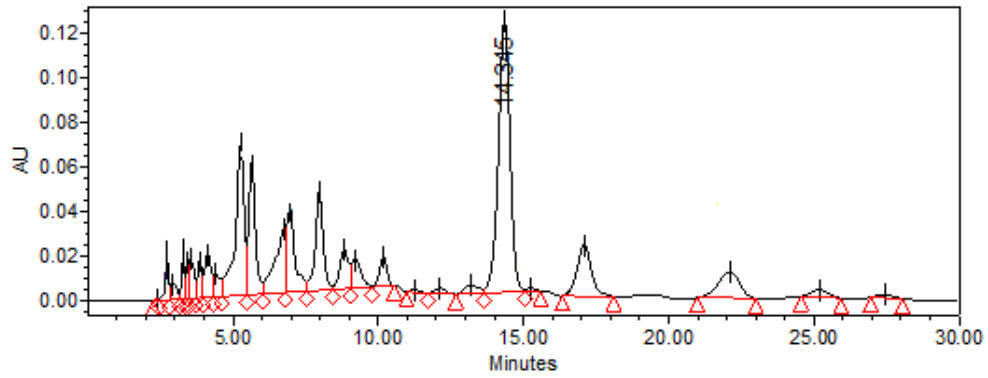


Fig 4.13: Chromatogram showing peak for P-I content of PKS-5

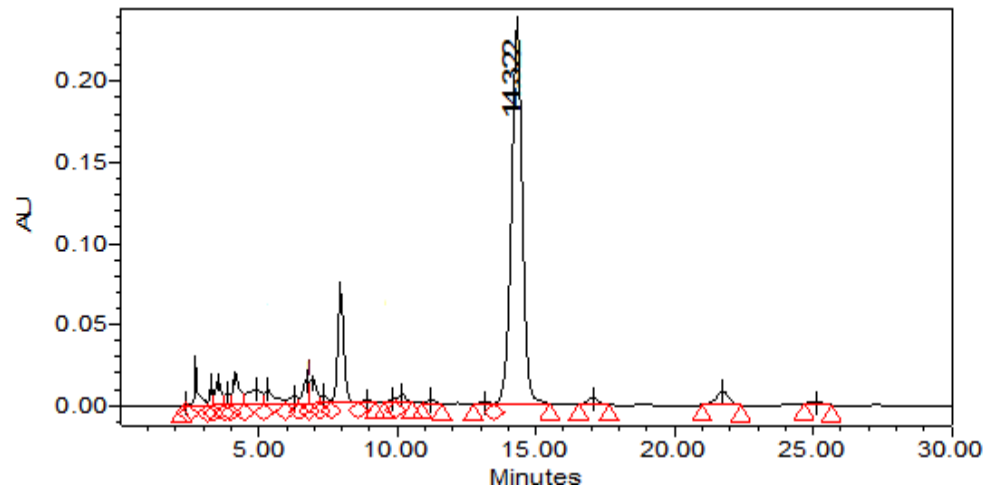


Fig 4.14: Chromatogram showing peak for P-I content of PKS-21

Table 4.3: P-I content in shoots of PKS-1, PKS-4, PKS-5 and PKS-21 chemotypes of *Picrorhiza kurroa*

Sample Name	Retention Time	Area	% Area	Amount(mg/g)
PKS-1	14.372	2968925	47.75	0.0901
PKS-4	14.399	2904608	52.22	0.0881
PKS-5	14.345	3372304	27.67	0.1023
PKS-21	14.322	6462611	62.34	0.1961

4.4 Real Time Expression Analysis

The expression analysis for seven key genes HMGR, ISPE, PMK, DXPS, PAL, GS and G10H was done. The gene expression of PKS-1 at15°C for G10H was 0.38 fold, for PAL it was 0.72 fold, for HMGR it was 0.13, ISPE it was 0.60 fold, for DXPS it was 0.77 fold, for GS it was 0.60 fold, for PMK it was 0.80 fold. The gene expression of PKS-4 at 15 °C for G10H was 0.33 fold, for PAL it was 0.60 fold, for HMGR it was 0.08, ISPE it was 0.57 fold, for DXPS it was 0.66 fold, for GS it was 0.72 fold, for PMK it was 0.16 fold. The gene expression of PKS-5 at15 °C for G10H was 0.43 fold, for PAL it was 1.13 fold, for HMGR it was 0.18, ISPE it was 1.10 fold, for DXPS it was 0.76 fold, for GS it was 0.57 fold, for PMK it was 0.31fold. The gene expression of PKS-21 at15 °C for G10H was 0.80 fold, for PAL it was 1.41 fold, for HMGR it was 0.45, ISPE it was 2.62 fold, for DXPS it was 1.74 fold, for GS it was 1.56fold, for PMK it was 1.50 fold.(Table 15.2). The gene expression of PKS-1 at 25 °C for G10H was 0.17 fold, for PAL it was 0.63 fold, for HMGR it was 0.50, ISPE it was 0.39 fold, for DXPS it was 0.27 fold, for GS it was 0.27 fold, for PMK it was 0.42 fold. The gene expression of PKS-4 at15 °C for G10H was 0.32 fold, for PAL it was 0.27 fold, for HMGR it was 0.98, ISPE it was 0.57 fold, for DXPS it was 0.65 fold, for GS it was 0.10 fold, for PMK it was 0.08 fold. The gene expression of PKS-5 at15 °C for G10H was 0.21 fold, for PAL it was 0.31 fold, for HMGR it was 0.13, ISPE it was 0.20 fold, for DXPS it was 0.10 fold, for GS it was 0.22 fold, for PMK it was

0.19 fold. The gene expression of PKS-21 at25 °C for G10H was 0.81 fold, for PAL it was 0.92 fold, for HMGR it was 0.39, ISPE it was 1.85 fold, for DXPS it was 0.18 fold, for GS it was 0.19 fold, for PMK it was 0.18 fold.(Table 15.3).

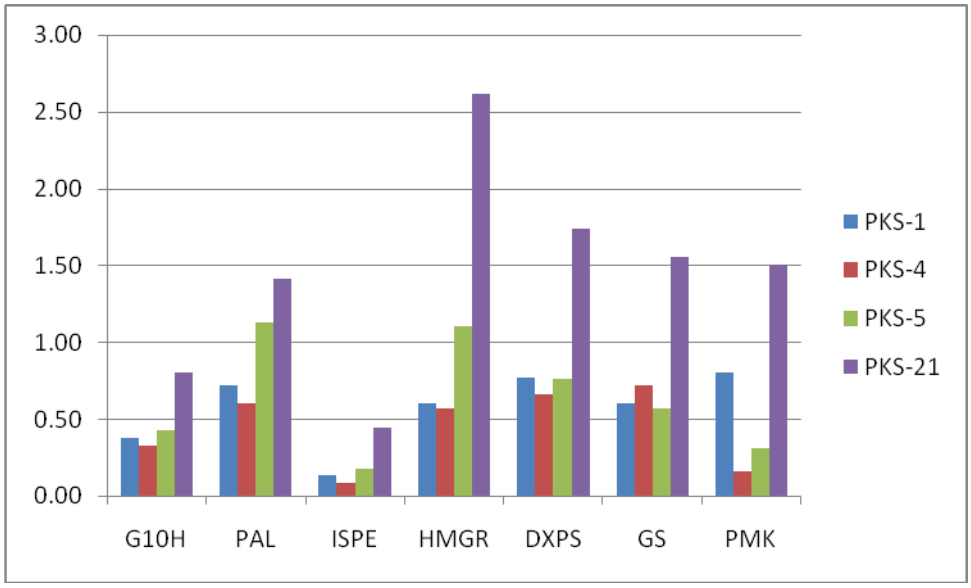


Fig 4.15: Expression of Key Gene in *P. kurroa* shoots incubated at15 °C

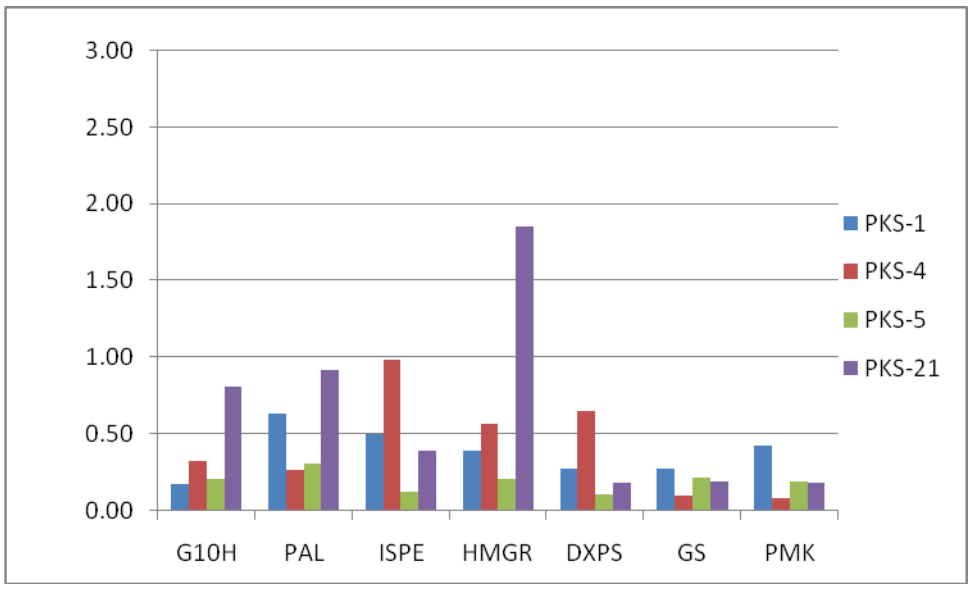
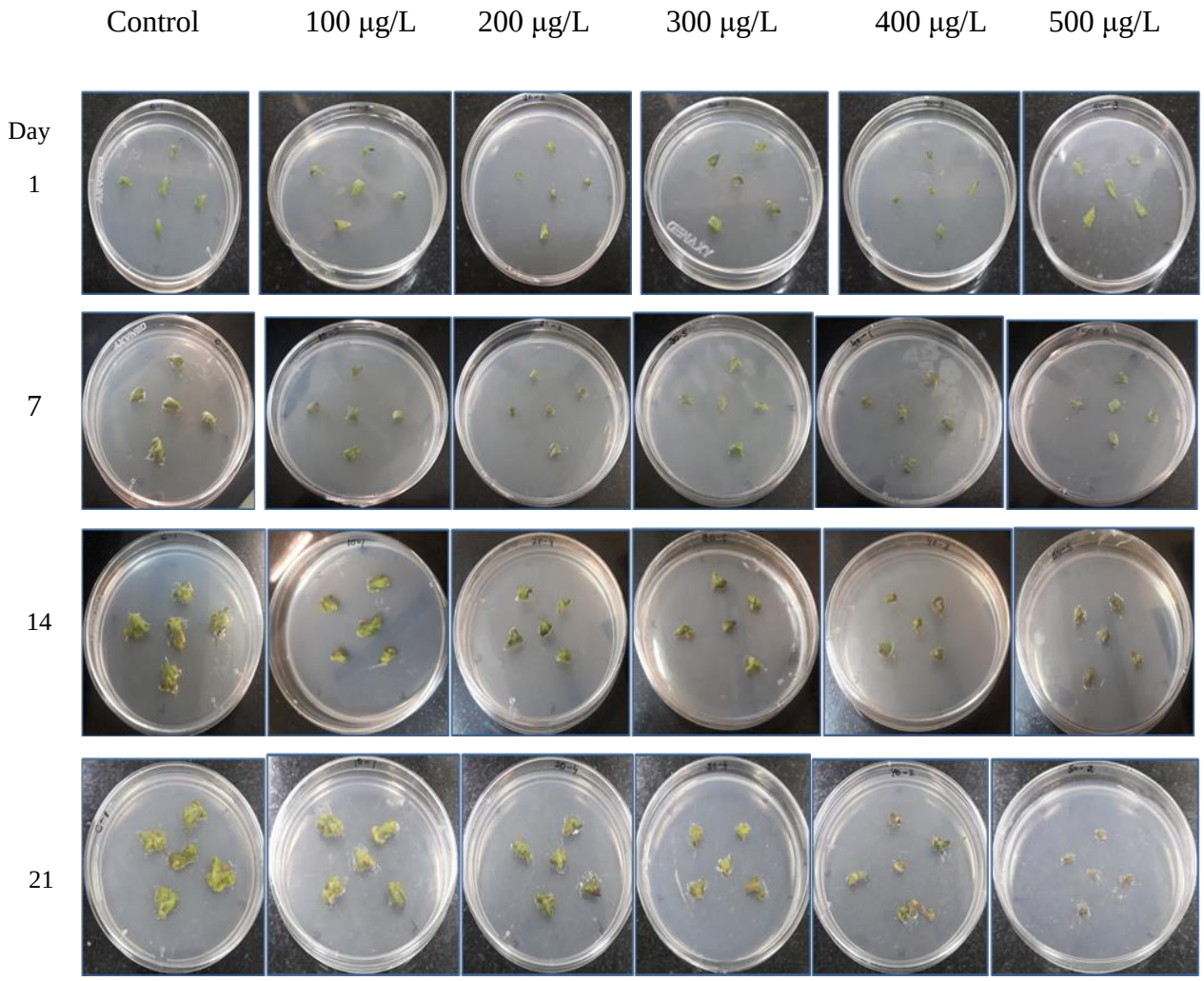


Fig 4.16: Expression of Key Gene in *P. kurroa* shoots incubated at 25°C

4.5 Effect of Kanamycin on Leaf Explants

Effect of kanamycin on leaf explants was assessed by adding different concentrations of kanamycin to the MS media supplemented with TDZ (0.5 mL/L). The concentrations were used in range of 0 to 500 µg/mL. In control cultures having no kanamycin used the callusing occurs within 3 to 4 days. The callusing was partially inhibited at 10 and 20 concentration. The concentration of 300µg/mL to 400µg/mL showed about 50 percent inhibition of callus. There was complete inhibition of callusing at 50 concentrations. So this range of concentration can be used for transformation from leaf explants along with other antibiotics.



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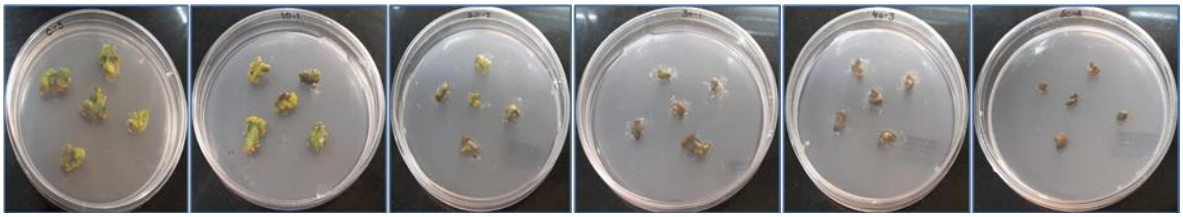

Fig 4.17: Effect of kanamycin on callusing from leaf explants of *P. kurroa*

Table 4.4: Effect of kanamycin on callusing from leaf explants of *P. kurroa* recorded as weight in grams

	Control	**10 µg/mL Conc.**	**200µg/mL Conc.**	**300µg/mL Conc.**	**400µg/mL Conc.**	**500µg/mL Conc.**
Day 0	7.82±0.53	7.9±0.75	7.78±0.55	7.72±0.61	8.22±1.4	7.9±0.76
Day 7	37.36±2.70	23.5±0.67	17.16±0.55	14.58±2.87	12.56±1.42	11.14±1.91
Day 14	78.82±6.9	54.4±2.8	46.28±1.76	42.5±2.5	38.48±4.2	16.26±4.94
Day 21	168.88±27.50	114.1±12.22	71.26±4.97	66.60±7.82	44.58±6.83	17.64±6.17
Day 28	219.58±23.45	170.78±12.61	127.92±13.90	87.54±9.27	47.3±3.70	20.26±0.98

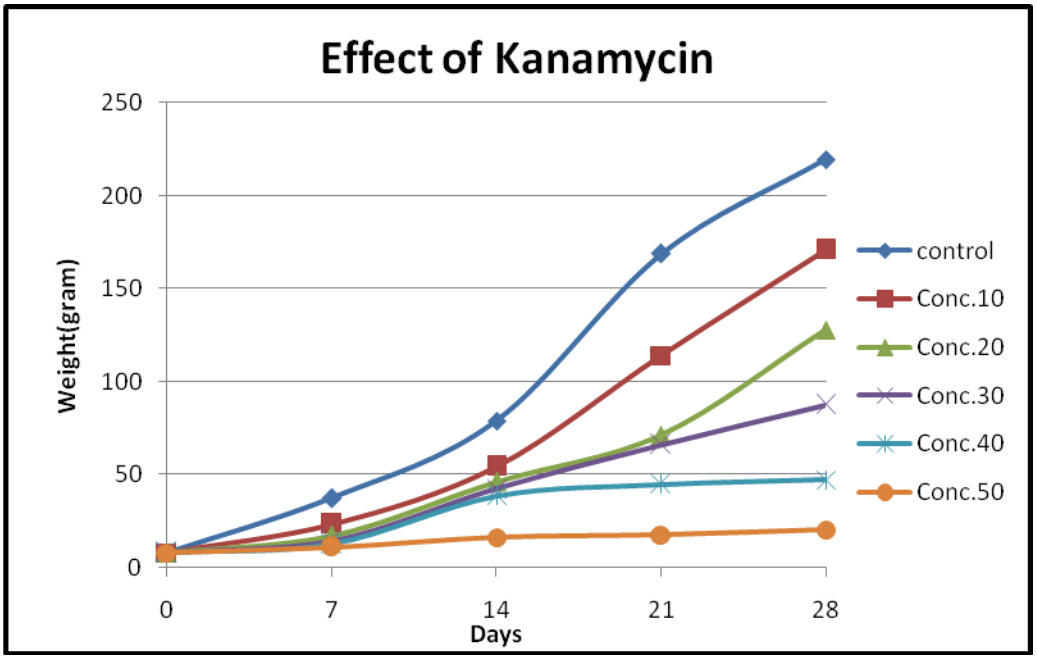

Fig 4.18: Effect of different concentrations of Kanamycin on leaf explants of *P. kurroa*

4.6 DISCUSSION

Micropropagation of the four chemotypes in multiple shoots induction medium showed highest shoots, shoot length and highest biomass in PKS-4, followed by PKS-1, PKS-5 and least in PKS-21 among the four chemotypes of *P. kurroa* at 15°C and same pattern of growth was followed at 25°C as well. With respect to temperatures the growth of four chemotypes PKS-1, PKS-4, PKS-5 and PKS-21 was more at 15°C as compared to 25°C this was in accordance as reported by Sood & Chauhan for *P. kurroa*.

The survival rate for PKS-1, PKS-4, and PKS-21 was found to be almost same of about 50% whereas survival rate for PKS-5 was very less in greenhouse conditions. The survival rate for *P. kurroa* chemotypes was less as compared to the *P. kurroa* reported by Neha et.al which was about 70%.

The P-I content in PKS-1 was 0.0901 mg/g, in *in vitro* grown shoots which was less as reported by Kirti et.al as compared to the field grown shoots and green house grown shoots. The P-I content in PKS-4 was 0.0881 mg/g, in *in vitro* grown shoots which was less as reported by Kirti et.al as compared to the field grown shoots and green house grown shoots. Also the P-I content in PKS-5 and PKS-21was less when compared to the field grown shoots and green house grown shoots as reported by Kirti et.al.

The gene expression of seven genes namely HMGR, ISPE, PMK, DXPS, PAL, GS and G10H for the PKS-21 showed highest expression for all the genes among four chemotypes PKS-1, PKS-4, PKS-5 and PKS-21. Followed by expression of genes in PKS-5 of all the genes namely HMGR, ISPE, PMK, DXPS, PAL, GS and G10H and then by PKS-1 and least expression of genes in PKS-5 which was in accordance with the P-I content in the chemotypes which was found to be highest in PKS-21 followed by PKS-5, PKS-1 and PKS-4.So this indicates that these all seven genes HMGR, ISPE, PMK, DXPS, PAL, GS and G10H increased expression is associated with increased production of Picroside-I.

In control medium of kanamycin antibiotic assay for callus induction from leaf explants of *P. kurroa* was observed to be started after 3 to 4 days. In MS medium containing kanamycin concentration of 300 µg/mL to 400 µg/mL fifty percent inhibition of the callus was observed so this concentration can be used for transformation procedures for *P.*

kurroa. Partial inhibition of callus was observed in medium containg 100 µg/mL to 200 µg/mL concentration of kanamycin whereas complete inhibition of callus occurs in medium containing concentration of 500 µg/mL of kanamycin from leaf explants of *P. kurroa*.

CHAPTER-5

CONCLUSION

CONCLUSION

The study carried out on identifying high content chemotype of *P. kurroa* is providing complete information for growing and propagating this plant for its pharmaceutical and herbal use. We have carried out optimization of culture medium for multiplication of all the chemotypes PKS-1, PKS-4, PKS-5 and PKS-21 of *P. kurroa* and collected data for their morphological developments. MS medium supplemented with IBA 3mL/L and Kinetin 1mL/L give best results in *in vitro* multiplication of different chemotypes of *P. kurroa*. Highest content of P-I estimated by HPLC 0.1961 mg/g was observed in PKS-21 and expression analysis carried out for key genes HMGR, ISPE, PMK, DXPS, PAL, GS and G10H in all chemotypes resulted in higest fold expression in PKS-21 which was in accordance with P-I concentration. The optimization of kanamycin concentration was done to be used as aselective agent which was found to be 300 μg/mL to 400 μg/mL. So this study provides prerequisite for genetic interventions and molecular exploration for other desirable characteristics required for developing commercial aspect of *P. kurroa*.

CHAPTER-6
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ANNEXURE

Composition of Murashige and Skoog basal medium (MS MEDIUM)

STOCKS	CHEMICALS	ORIGINAL STRENGTH (mg/l)	STOCK (g/l)	FINAL VOLUME (ml/l)
A-10 X	KNO_3 $MgSO_4.7HO$ KH_2PO_4	1900 370 170	19 3.7 1.70	100
B-20 X	NH_4NO_3	1650	33	50
C-100 X	$CaCl_2.2H2O$	440	44	10
D-100 X	Na_2EDTA $FeSO_4.7H2O$	37.26 27.85	3.72 2.28	10
E-100 X	KI	0.83	0.083	10
F-100 X	H_3BO_3 $CoCl_2.6H_2O$ $ZnSo_4.7H_2O$ $CuSo_4.5H_2O$ $MnSo_4.4H2O$ $Na_2MbO_4.2H_2O$	6.2 0.025 8.6 0.025 22.3 0.25	0.62 0.0025 0.86 0.0025 2.23 0.0025	10
G-100 X	m-INOSITOL GLYCINE	100 2	10 0.2	10
H-100 X	PYRIDOXINE-HCL NICOTINE ACID THIAMINE	0.5 0.5 0.1	0.05 0.05 0.01	10

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