OPTIMIZATION OF CULTURE CONDITIONS FOR DIFFERENT STRAINS OF *PICRORHIZA KURROA ROYLE EX BENTH.*

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CERTIFICATE

This is to certify that the work titled-"**OPTIMIZATION OF CULTURE CONDITIONS FOR SEVEN DIFFERENT STRAINS OF *PICRORHIZA KURROA***" submitted by "**DISHA KALIA AND JASLEEN KAUR AHUJA**" in partial fulfillment for the award of degree of B.Tech-M.Tech Dual degree 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.

Signature of Supervisor .....

Name of Supervisor Dr. Hemant Sood

Designation

Date 25/5/13

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Disha kalia and Jasleen Kaur Ahuja

SUMMARY

An efficient and rapid protocol for optimization of culture conditions for micropropagation of seven strains of *Picorhiza kurroa*(PKS-1, PKS-2, PKS-5, PKS-11, PKS-13, PKS-14, PKS-21) has been developed. The mother plant was obtained from different regions of Himanchal Pradesh i.e Hydanbhatori, Bhuri, Rohtang, Gue, Kungpa, Teita, Pattal. The motive of considering these seven strains from different altitude to identify and multiply the highest content strain of *P.kurroa* in *in vitro* conditions and analyse that plant giving highest Picroside-I content when grown in field condition maintained its genetic superiority or not under in vitro condition.

Mother stock that is different strains of P.kurroa was collected from field and were quantified for its Picroside content using HPLC. PKS-1,2,5 showed high content that is above 15ug/mg and PKS-11,13,14 showed intermediate content that is between 8-15ug/mg and PKS-21 content was below 8ug/mg. then explants of all the strains were cultured on MS media showing best results with KN (3mg/L) and IBA (1mg/L). Highest content strain PKS-1 gives 6-7 shoots at 15Cand 4-5 shoots at 25c.wheras PKS-5 also resulted into good growth but PKS-2 showed sluggish growth under *in vitro* conditions. Results for the growth at above mention medium was very significantly showed by intermediate and low content strains. The root induction was found good in all strains in MS supplemented with IBA(3mg/L) and Kinetin(1mg/L). Then the objective was to estimate the Picroside-I content of *in vitro* grown plant by using HPLC. We compared the results with field grown strains where one month old plant of PKS-5 grown at 15 ºC and 25 ºC was quantified for its Picroside-I content which showed higher content as compared to two year old field grown plant. Picroside-I content in field grown plant which is one month old should be 0.80ug/mg whereas when in vitro raised plants were analysed the amount of accumulation of Picroside-I was 1.77ug/mg in plant grown at 15 ºC and 0.91ug/mg in plant grown at 25 ºC. So this is reported for the first time that high content strains accumulate high content of Picroside-I even under in vitro conditions with very short duration of time.The rooted plantlets were hardened in glasshouse containing sterile soil, sand, vermiculite, cocopeaat in equal ratios of 1:1:1:1 and we successfully acclimatized and established in soil with survival rates 80%,75%, 77%, 85%, 88%, 90%, 82% for all strains respectively. So this study will help in exploring various genetic and phenotypic parameters related to metabolite production for high content strains so that their propagation can be commercialized to meet the pharmaceutical and biotech industries demand.

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LIST OF ABBREVIATIONS

Abbreviations	Full forms
KN	Kinetin
IBA	Indol-3- butyric acid
MS	Murashige and Skoog(1962)
HPLC	High Pressure Liquid Chromatography

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 rawmaterials 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 phytopharmaceutical 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). 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.

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 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.

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. As far now no such report on identification of superior strains of *P.kurroa* is available ,whereas if any strain which could have high content of metabolite will be identified so protocols for large scale multiplication and *in vitro* production of metabolite should be developed.So the present study is carried out by following objectives:

OBJECTIVES:

Optimization of culture conditions for micropropagation of seven different strains of *P.kurroa* (PKS-1,PKS-2,PKS-5,PKS-11,PKS-13,PKS-14,PKS-21).

Optimization of the culture conditions for metabolite production through shoot cultures of these seven strains.

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.

The rooting was also optimized by Arif Jan, Phalisteen S., G.T. Thomas and A. S. Shawl 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.

2.2.2.Temperature

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.

2.3.Sterilization

A protocol has been standardized for sterilization of nodal segments 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 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

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.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).

2.8.Altitude effect on Picroside content of different strains

In Katoch M et al picroside I and II content in various plant parts of *P. kurroa* collected from different altitudes.,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.

2.9.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.Differential biosynthesis and accumulation of picrosides in an endangered medicinal herb *Picrorhiza kurroa.*

Pandit et al. reported that highest amounts of P-I(8.7 %) and P-II (5.3 %) was detected in uppermost part of mature dried rhizomes compared to bottom part with 2.9 % and 2.2 % of P-I and P-II, respectively. P. kurroa grown at high altitude (Sairopa, 4,500 amsl) showed 1.75-folds increase in P-I in leaves whereas exponential increase in the P-I content was detected (0.05–1.7 %) in the leaves of different developmental stages (L1-L5) of P. kurroa grown at lower altitude(Jagatsukh, 1,900 m). Variable amounts of P-I and P-II in different growth and developmental stages of P. kurroa imply mportance of selection of plant material (rhizomes and roots).

2.11.Expression pattern of fifteen genes of non-mevalonate (MEP) and mevalonate (MVA) pathways in different tissues of endangered medicinal herb Picrorhiza kurroa with respect to picrosides content.

Pandit et al. experimented differential conditions with varying concentrations of P-I (0–27 lg/1mg) and P-II (0–4 lg/mg). They reported that four genes of 20 MEP pathway; DXPS, ISPD, ISPE, MECPS and one gene of MVA pathway PMK showed elevated levels of transcripts in shoots (57–166 folds) and stolons (5–15 folds) with P-I contents 0–27 lg/mg and 2.9–19.7 lg/mg, respectively. Further HDS and DXPR genes of MEP pathway showed higher expression *9–12 folds in roots having P-II (0-4 lg/mg). The expression of ISPH and ISPE was also high approximately ~5 in roots accumulating P-II.GDPS was the only gene with high transcript level in roots (9 folds) and shoots (20 folds). Differential biosynthesis and accumulation of picrosides would assist in regulating quality of plant material for herbal drug formulations.

2.12.Use of HPLC for Quantification of Picroside content

A new high performance thin layer chromatography (HPTLC) method for the simultaneous quantification of picroside-I and picroside-II in *P. kurroa* was described (Singh et al. 2005). Separation of picroside-I and picroside-II was achieved by mobile phase of CHCl3:MeOH (82:18, v/v) on precoated silica gel 60 F254 aluminium plate. The densitometric determination of picrosides was carried out at 290 nm, in absorption-reflection mode. The calibration curves were linear in the range of (2-5 micro g). The method is simple, specific, rapid, and reliable for simultaneous determination of Picroside-I and Picroside-II in *P. kurroa*. The proposed method was successfully applied for accurate quantification of large number of samples collected from different altitudes of western Himalayas.

CHAPTER 3:
Material and methods

The present experiments of optimization of culture conditions of different strains of Picrorhiza Kurroa Royle Ex Benth was carried out in the Department of Biotechnology, Jaypee University of Information Technology, Waknaghat, during 2012-2013.

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

3.1.Collection of Plant material

3.2.Quantification of the mother stock.

3.3. Preparation of explants

3.4.Surface Sterilization

3.5. Media Preparation

3.6. Culture conditions

3.7. Inoculation of prepared explant

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

3.9.In-vitro root induction.

3.10.Quantification of Picroside content from all different strains using HPLC.

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

3.1.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. Hydan bhatori,Monal danda,Bhuri,Rohtang ,Gue(spiti),Yungpa,Teita and Pattal were brought to the glasshouse of the laboratory of Department of Biotechnology of this University and maintained their under controlled conditions for further experimentation.

3.2.Quantification of the mother stock.

The mother stock leaves of same age were collected from the eight strains and quantified for Picroside I by HPLC.

The *in vitro* biosynthsis and accumulation status of Picroside-1 was determined in shoot cultures of *P. kurroa* by subjecting fresh samples from different experiments to chemical analysis. The shoots

including leaf material and were harvested while the leafy material was green, actively-growing, and non-senescent stage. The quantification of Picroside-1 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 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 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.

3.3.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 and nodal regions** were excised with the help of a scalpel blade and collected in water in a beaker.

3.4.Surface Sterilization

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

Bavistin - 0.5 %

Mercuric chloride - 0.1 %

The explants 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. The washed explants were then treated with Bavistin (0.5 %) for 2-3 minutes. Subsequently, the explants were washed with distilled water 1-2 times.

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.

3.5. 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 different concentrations of kinetin and IBA in the media were tried ranging from 1-3mg/ml.

3.6. 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 and 15±2°C under 16 hours photoperiod and 3000 flux light intensity.

3.7. Inoculation of prepared explants

For the establishment of the cultures, the surface sterilized explants were inoculated onto the MS Medium supplemented with various concentrations 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.

3.8.*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 IBA & KN. Subculturing for multiplication was carried out at 3 -4weeks of time interval.

Observations

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

3.9.*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.

3.10.Quantification of all different strains using HPLC

The *in vitro* biosynthesis and accumulation status of Picroside-1 will be determined in shoot cultures of *P. kurroa* by subjecting fresh samples from different experiments to chemical analysis. The quantification of Picroside-1 will be carried out 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).

3.11.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.

CHAPTER 4:
RESULTS AND DISCUSSION

4.1.Quantification of the mother stock

The field grown shoots of one and half year old plants of *P. kurroa* grown in the glasshouse of the department were collected ,washed and processed for the sample preparation for quantifying the amounts of Picroside I.We have found after quantification that out of all the seven strains selected PKS-1,PKS-2 and PKS-5 have high amount of Picroside -I whereas one strain PKS-21 had least amount and PKS-11,PKS-13 and PKS-14 showed intermediates amount of Picroside I.(Table 1)

Table 1.Quantification of Picroside-I in shoots of field grown plants of above mentioned seven strains of *P.kurroa.*

Strains	High content (above 15ug/mg)	Intermediate content (8-15ug/mg)	Low content (0-8ug/mg)
PKS-1	26.5		
PKS-2	19.2		
PKS-5	19.24		
PKS-11		13.89	
PKS-13		13.2	
PKS-14		10.93	
PKS-21			5.17

4.2. *In vitro* shoot multiplication on different combinations and concentrations of auxins and cytokinins

The surface sterilized shoot apexes and nodal regions of all the seven strains were cultured on MS media containing IBA and KN in different combinations. Out of 9 different media combinations tested for multiple shoot formation from shoot apices, MS medium containing KN (3mg/l) + IBA (1mg/l) + sucrose 3% (w/v) + agar-agar 0.8% (w/v) was found to be the best with shoot apices proliferating into multiple shoots within 8-10 days of inoculation.

The same medium was found suitable for obtaining maximum shoots per explant .We have found highest content strain PKS-1 gives 6-7 shoots on this media composition at 15 °C (Table 2) and at 25 °C the same highest content strain PKS-1 gives 4-5 shoots on this media composition (Table 3) Fig(1,2and3).

***Table 2. In vitro* shoot multiplication on different combinations and concentrations of auxins and cytokinins at 15°C**

IBA (mg/l)	KN (mg/l)	PKS-1	PKS-2	PKS-5	PKS-11	PKS-13	PKS-14	PKS-21
0	0	3	3	4	4	4	3	2
0	1	3	4	5	4	5	5	3
1	0	4	4	6	5	6	4	3
1	1	4	6	7	5	7	7	4
1	2	5	5	6	7	7	7	6
2	1	4	6	7	9	9	9	5
2	2	6	6	8	8	11	10	6
3	1	5	8	8	10	11	11	7
1	3	7	8	10	12	12	11	9

***Table 3. In vitro* shoot multiplication on different combinations and concentrations of auxins and cytokinins at 25°C**

IBA (mg/l)	KN (mg/l)	PKS-1	PKS-2	PKS-5	PKS-11	PKS-13	PKS-14	PKS-4	PKS-21
0	0	1	1	2	3	2	2	1	2
0	1	2	1	2	3	3	2	1	2
1	0	2	2	3	4	3	3	2	3
1	1	3	3	4	5	4	4	2	4
1	2	4	3	4	6	5	5	3	5
2	1	4	4	6	7	5	6	4	5
2	2	4	5	5	7	6	7	4	6
3	1	5	5	6	8	7	7	5	7
1	3	5	6	7	10	9	9	5	7

4.3. *In vitro* root induction on different combinations and concentrations of auxins and cytokinins

The Rootable shoots which were excised and put in separate culture jars containing MS Media enriched with growth hormones IBA and kinetin of different concentrations were observed after 2-3 week(s) and the best results for number of roots was seen in media containing (MS + IBA 3mg/l +kinetin 1mg/l + Sucrose 30 g/l + agar-agar 9 g/l).

This same media was suitable for obtaining maximum average number of roots per explants,PKS-1 the highest content strain at 15 °C showed 2.9 as average number of roots (Table 4) and PKS-1 at 25°C showed 2.6 as average number of roots.(Table 5)

***Table 4. In vitro* root induction on different combinations and concentrations of auxins and cytokinins at 15°C**

MS + Growth Hormones (mg/l)		PKS-1	PKS-2	PKS-5	PKS-11	PKS-13	PKS-14	PKS-21
KN	IBA							
0	0	2.4	2.5	2.7	2.8	2.6	2.4	3.2
0	1	2.7	2.6	3.2	2.9	3.2	2.8	3.6
0	3	2.8	3.1	3.5	3.3	4.3	3.9	3.8
1	3	2.9	3.8	3.9	4.8	5.5	5.3	4.1

***Table 5. In vitro* root induction on different combinations and concentrations of auxins and cytokinins at 25°C**

MS + Growth Hormones (mg/l)		PKS-1	PKS-2	PKS-5	PKS-11	PKS-13	PKS-14	PKS-21
KN	IBA							
0	0	1.6	1.7	1.5	2.1	1.4	1.8	1.7
0	1	1.5	1.5	2.2	2.4	1.9	2.2	2.1
0	3	1.9	2.1	2.3	2.8	2.7	2.9	2.8
1	3	2.6	2.7	2.9	3.5	3.3	3.2	3.1

4.4.Estimation of Picroside I content in shoots of *in-vitro* grown plants

After the plants were well established and growing well on the shoot multiplication media under the *in-vitro* conditions their shoots were analysed for picroside I content.(Table 6)

It was observed that:

- When PKS-1 was grown under *in vitro* conditions at 25 °C and 15 °C it showed lower Picroside-I content as compared to the field grown plants of PKS-1
- When PKS-2 was grown under *in vitro* conditions it showed lower Picroside-I content as compared to the field grown plants of PKS-2 at 25 °C and comparable content at 15 °C.
- When PKS-5 was grown under *in vitro* conditions at 25 °C and 15 °C it showed higher Picroside-I content as compared to the field grown plants of PKS-5.
- When PKS-11 was grown under *in vitro* conditions at 25 °C and 15 °C it showed higher Picroside-I content as compared to the field grown plants of PKS-11
- When PKS-13 was grown under *in vitro* conditions it showed higher Picroside-I content as compared to the field grown plants of PKS-13 at 15 °C and comparable content at 25 °C.
- When PKS-14 was grown under *in vitro* conditions at 25 °C and 15 °C it showed comparable Picroside-I content as to the field grown plants of PKS-14

- When PKS-21 was grown under *in vitro* conditions at 25 °C and 15 °C it showed higher Picroside-I content as compared to the field grown plants of PKS-21.

Table 6: HPLC results showing the data analysed for Picroside I content

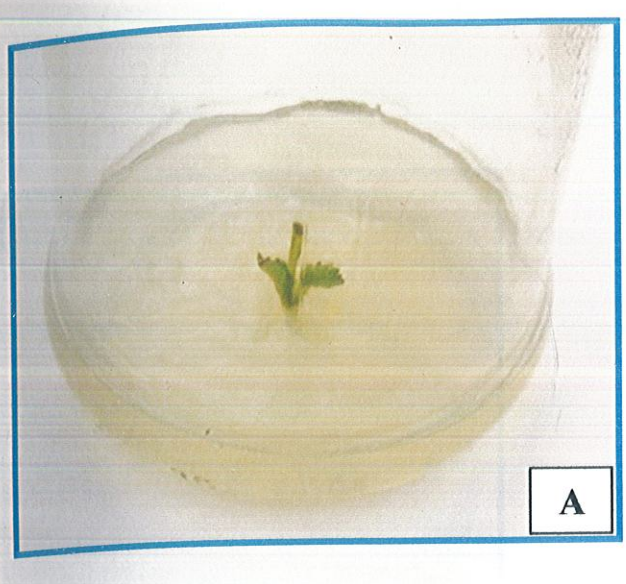
Sample	P. kurroa strains from different temp. analysed	Picroside-I µg\mg of fresh shoot weight at 15 °C	Picroside-I µg\mg of fresh shoot weight 25 °C
1.	PKS-1,(KN+IBA)	0.50	0.48
2.	PKS-2, (KN+IBA)	0.77	0.62
3.	PKS-5,(KN+IBA)	1.77	0.91
4.	PKS-11,(KN+IBA)	1.49	1.41
5.	PKS-13, (IKN+IBA)	0.62	0.55
6.	PKS-14, (KN+IBA)	0.49	0.45
7.	PKS-21,(KN+IBA)	0.64	0.55

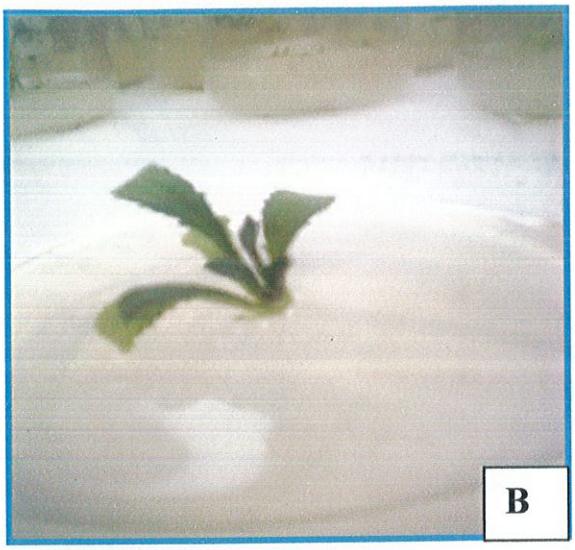
4.5.Hardening of *in vitro* rooted plantlets in glass house

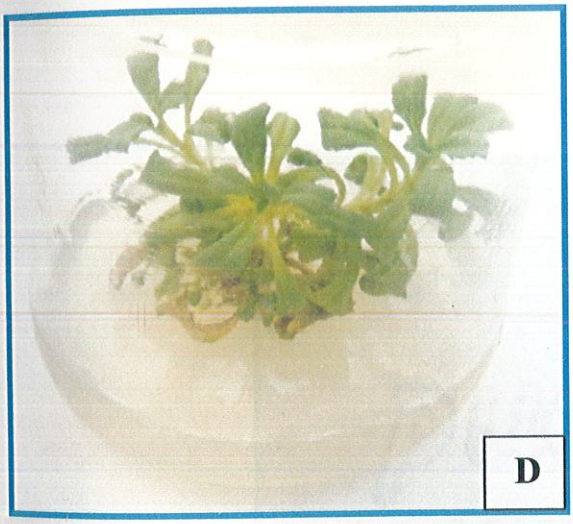
After 5 weeks of growth on rooting medium, roots were well developed and the plantlets from all rooting media was taken out from all culture jars and after the treatment already mentioned, were potted in potting plastic pots containing potting mixture perlite+vermiculite+coco-peat (Table 7)(Figure 6). 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 perlite, vermicultite, coco peat gave us the best results in terms of plant survival and growth.

Table 7.Growth and Survival of hardened plants in different potting mixtures under glass house conditions.

Strains	Percentage of survival in potting mixture **Perlite+Vermiculite +coco-peat**
PKS-1	80
PKS-2	75
PKS-5	77
PKS-11	85
PKS-13	88
PKS-14	90
PKS-21	**82**







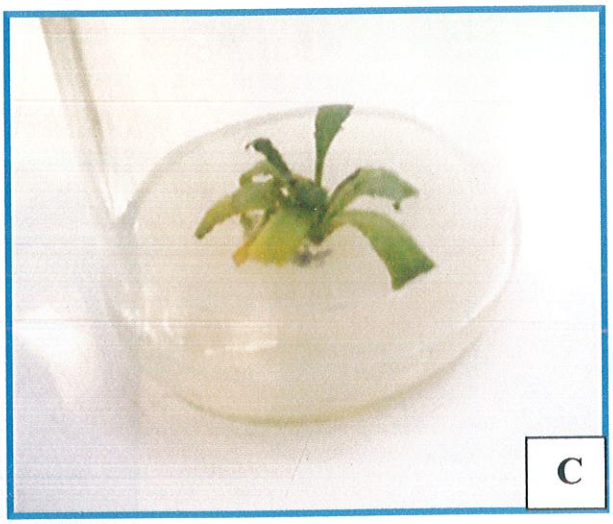
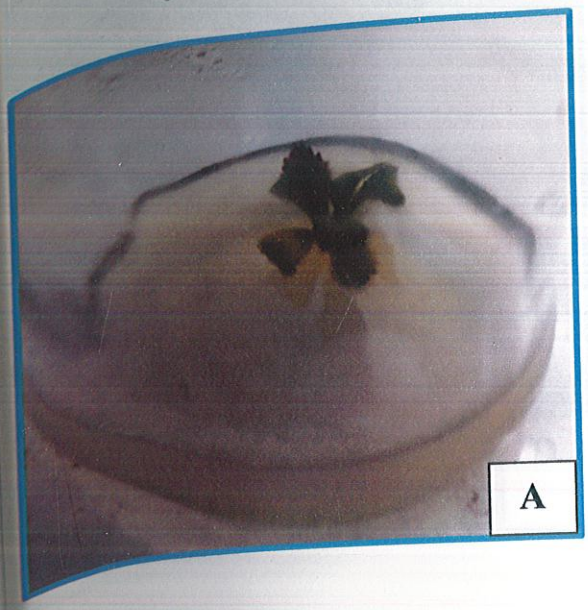


FIGURE 1: *In vitro* shoot multiplication using shot apex as a starting culture for all pks strains

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**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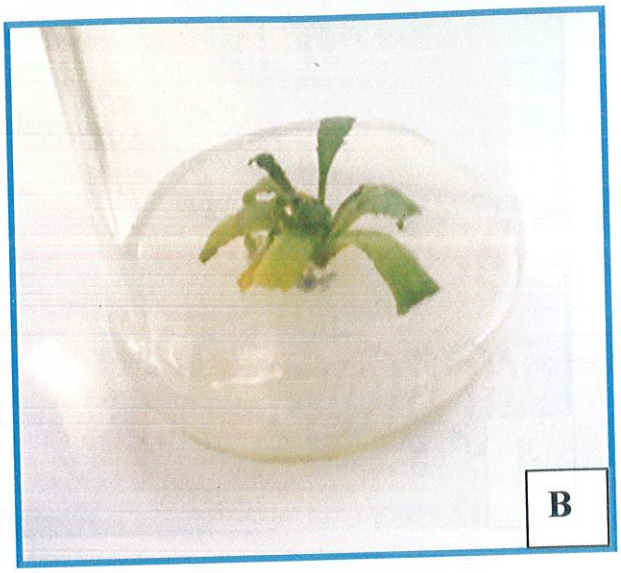
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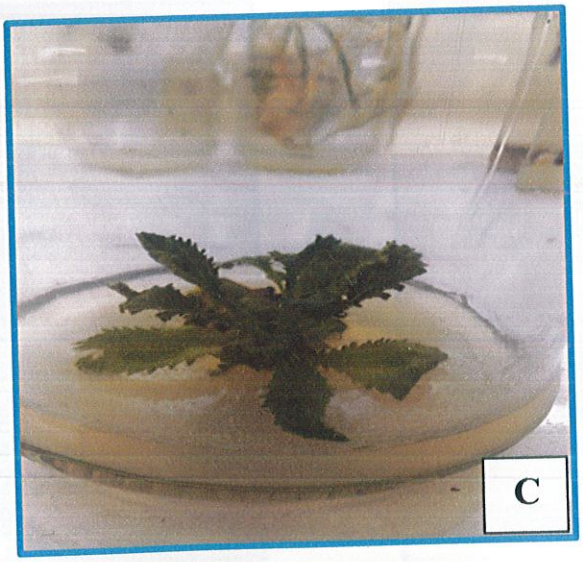
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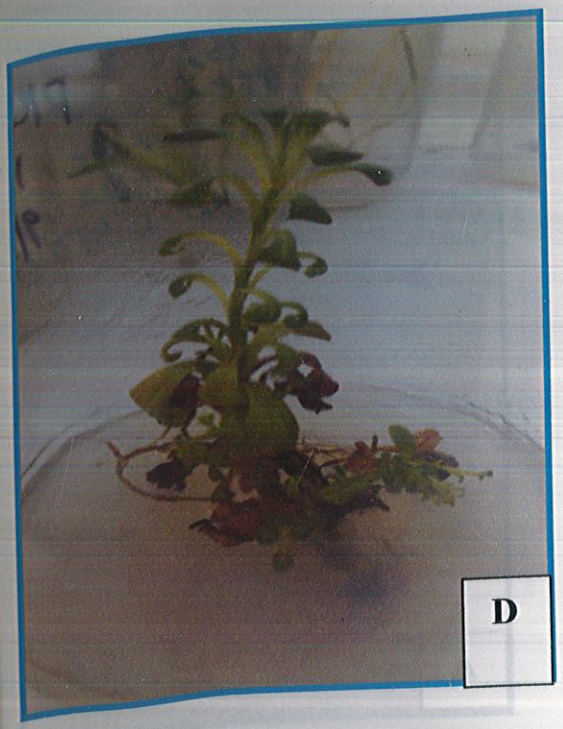
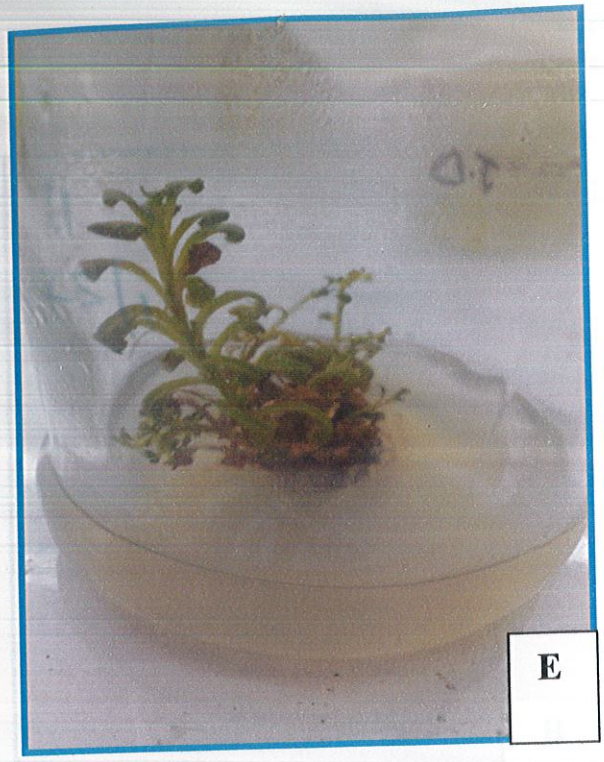
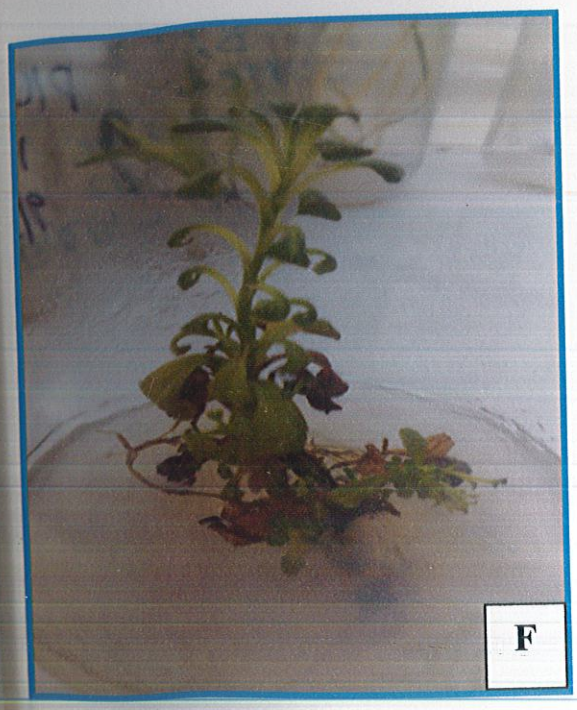
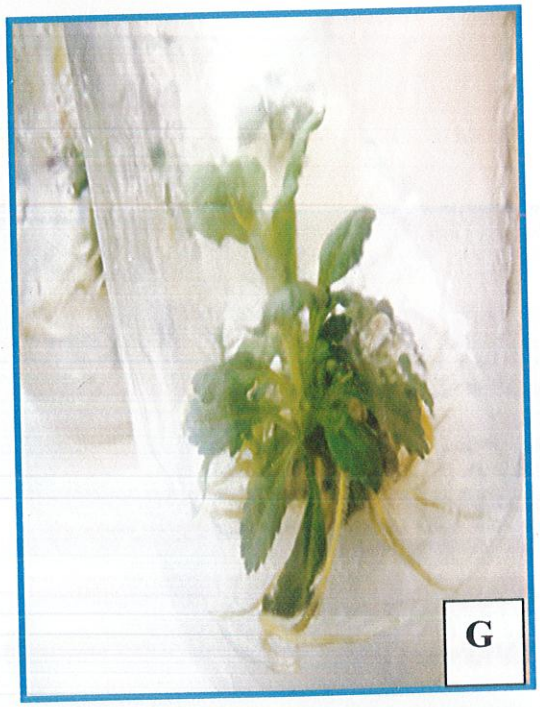


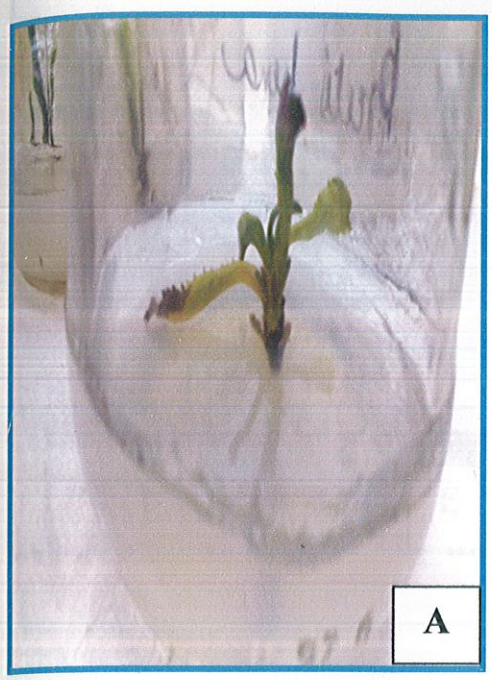


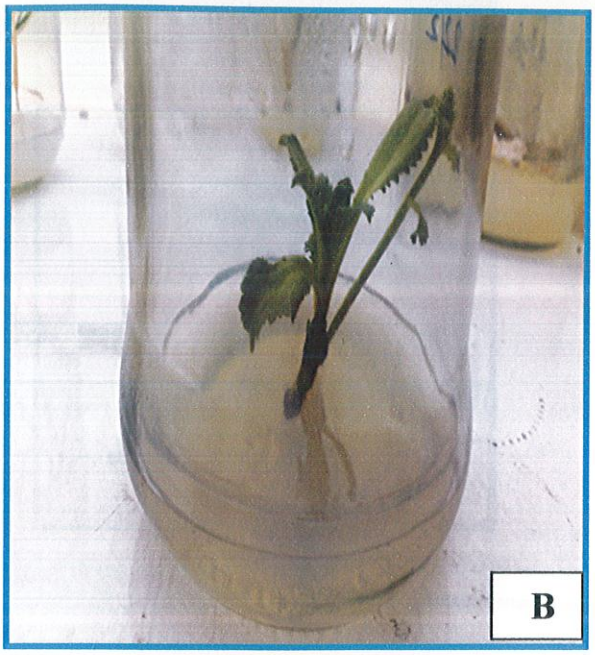


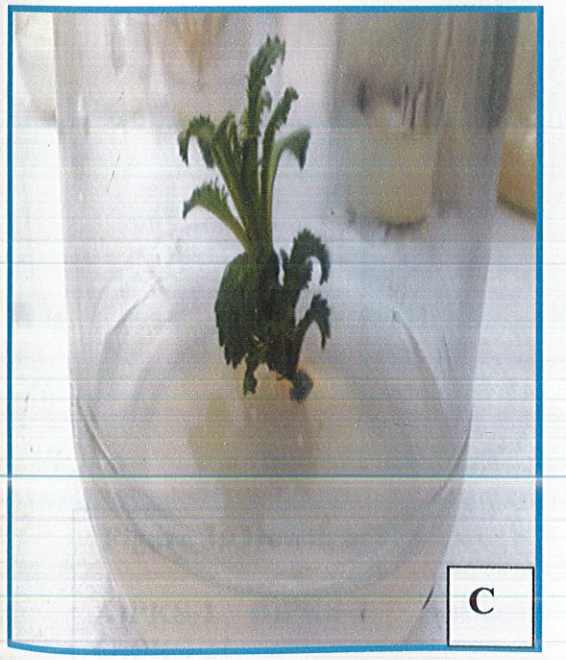


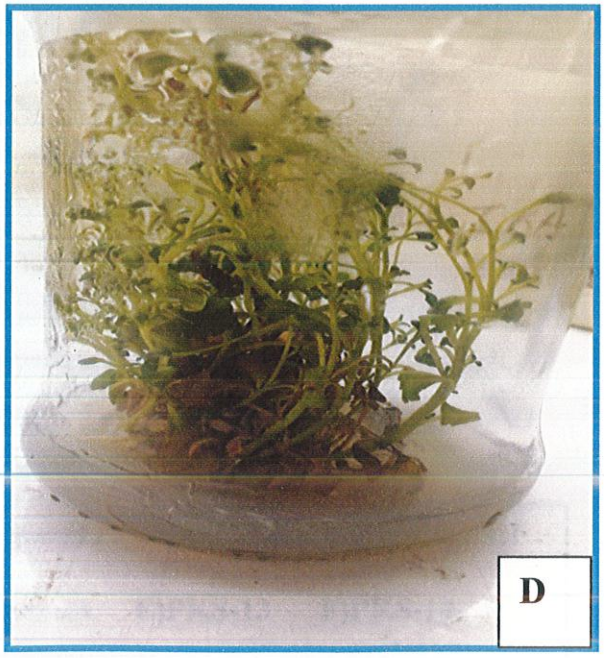
gure 2:Growth of shoot at 25 °C on medium containing KN:IBA in ratio 3:1

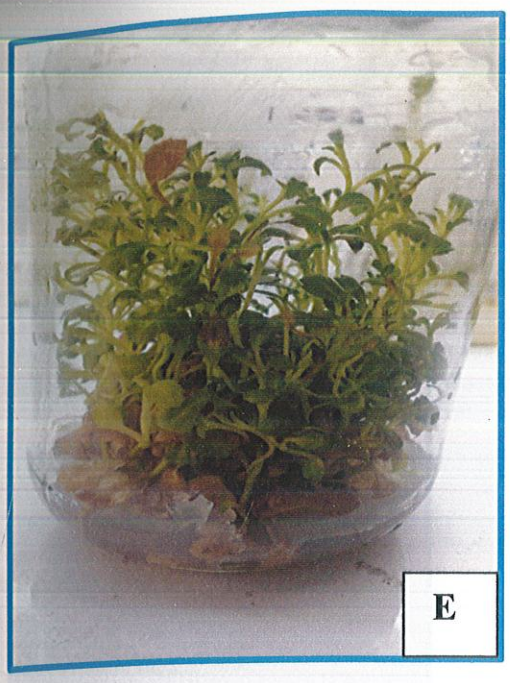
)PKS-2 B)PKS-1 C)PKS-5 D)PKS-11 E)PKS-13 F)PKS-14 G)PKS-21











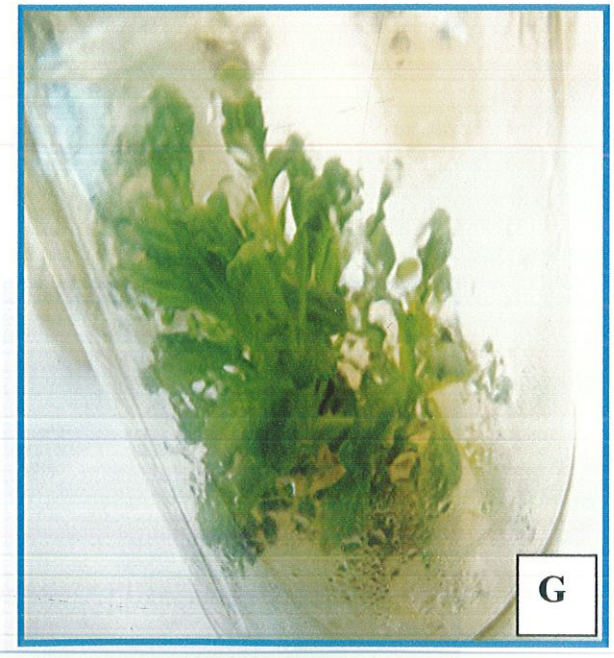
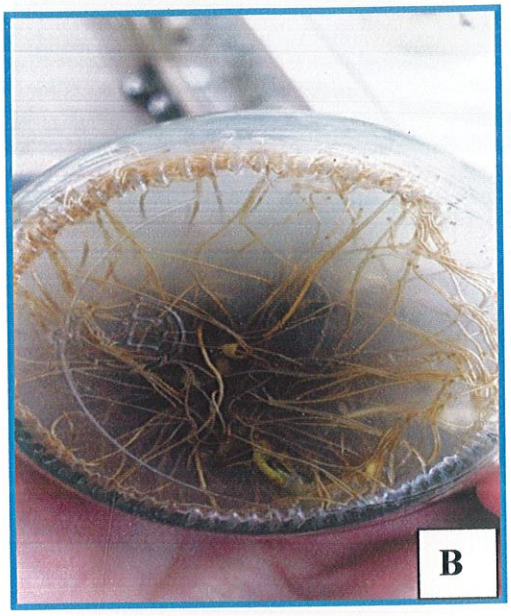


Figure 3:Growth of shoot at 15 °C on medium containing KN:IBA in ratio 3:1

A)PKS-1 B)PKS-2 C)PKS-5 D)PKS-11 E)PKS-13 F)PKS-14 G)PKS-21



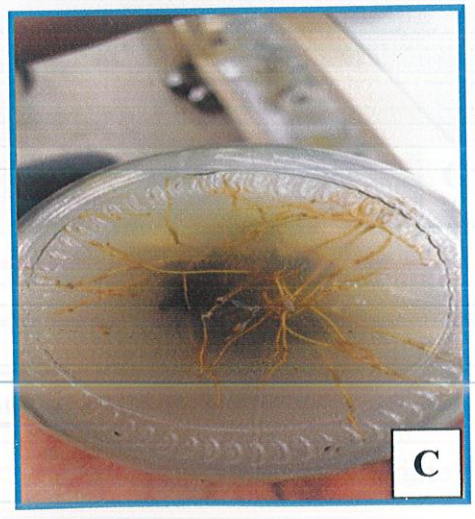
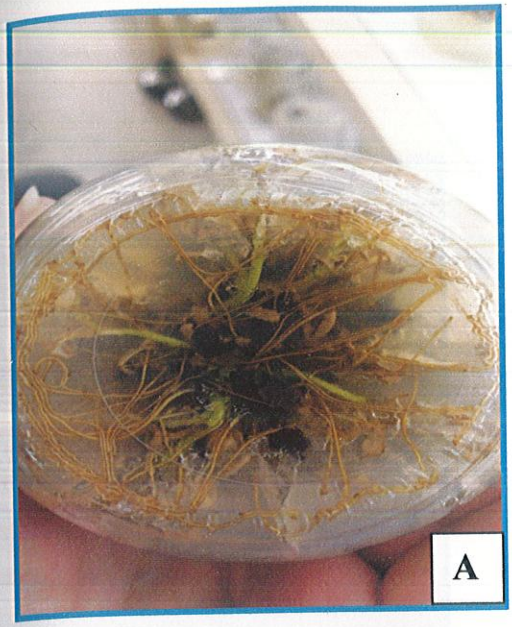
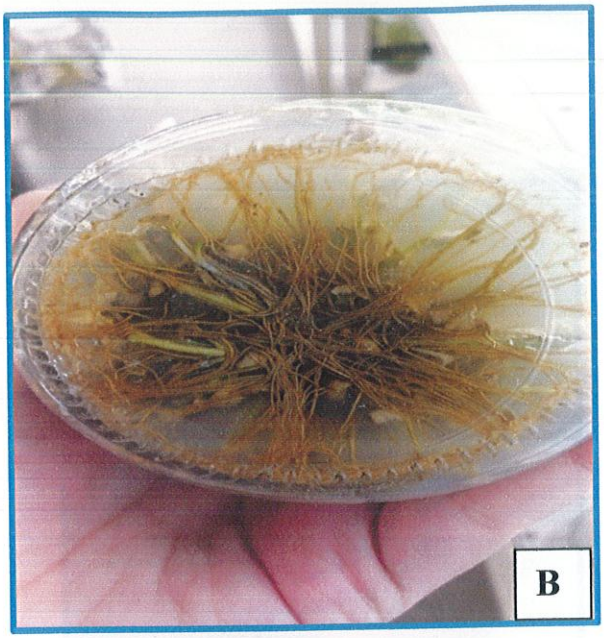


Figure 4:In vitro rooting at 25 °C on IBA:KN in the ratio 1:3

A)PKS-1(high) **B)PKS-13(intermediate)** **C)PKS-21(low)**





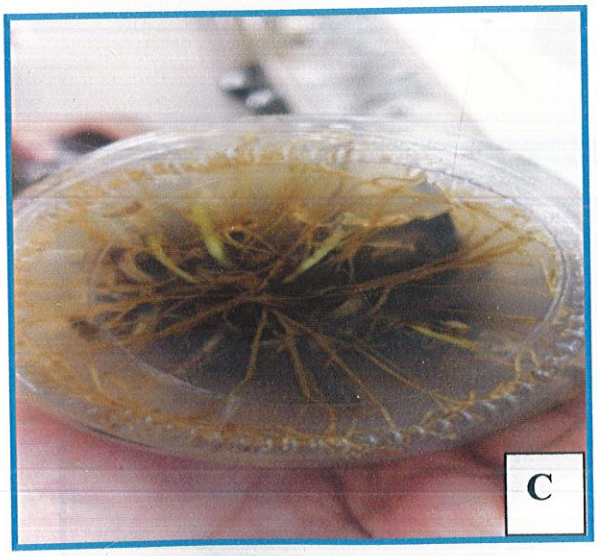
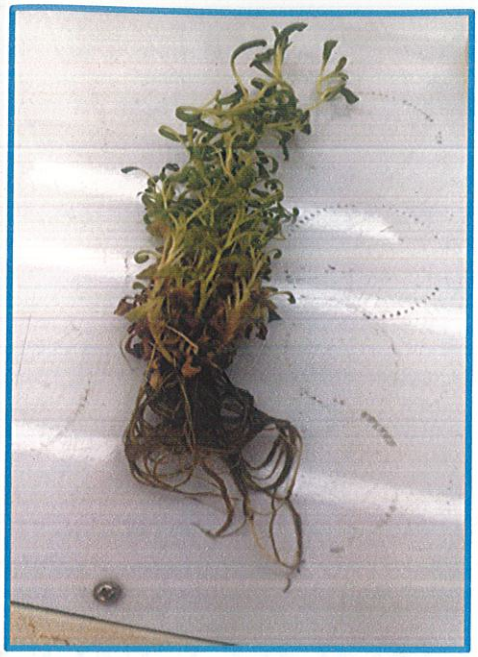


Figure 5:In vitro rooting at 15 °C on IBA:KN in the ratio 1:3

A)PKS-1(high) **B)PKS-13(intermediate)** **C)PKS-21(low)**



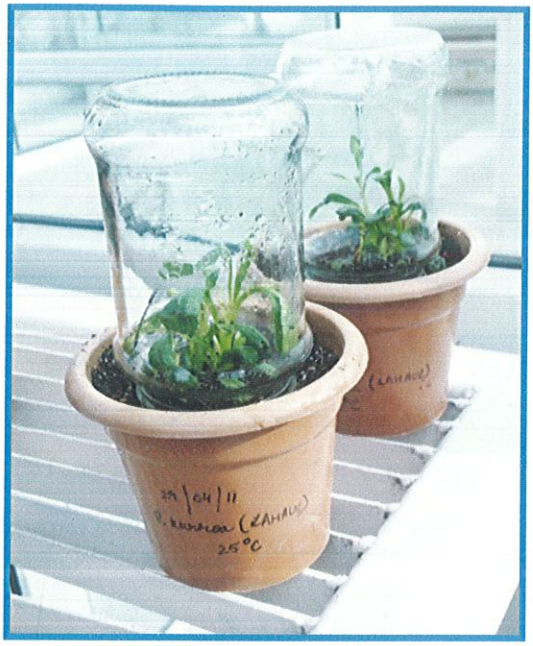


FIGURE 6: a) *In vitro* developed plantlet transferred for hardening in glass house b) Hardened plants ready for field transfer

CONCLUSION

The present study has thus optimized different culture conditions for the micropropagation of seven different strains of *Picrorhiza kurroa it was observed that* explants of all the strains when cultured on MS media showed best results with KN (3mg/L) and IBA (1mg/L). Highest content strain PKS-1 gave 6-7 shoots at 15 °C and 4-5 shoots at 25 °C,wheras PKS-5 also resulted into good growth but PKS-2 showed sluggish growth under *in vitro* conditions. Results for the growth at above mention medium was very significantly showed by intermediate and low content strains. The root induction was found good in all strains in MS supplemented with IBA(3mg/L) and Kinetin(1mg/L).

Then the objective of estimating the Picroside-I content of *in vitro* grown plant by using HPLC was accomplished.The results showed that when PKS-1 was grown under *in vitro* conditions at 25 °C and 15 °C it showed lower Picroside-I content as compared to the field grown plants of PKS-1,PKS-2 showed lower Picroside-I content as compared to the field grown plants of PKS-2 at 25 °C and comparable content at 15 °C and PKS-5 when grown under *in vitro* conditions at 25 °C and 15 °C showed higher Picroside-I content as compared to the field grown plants of PKS-5.While when PKS-11 was grown under *in vitro* conditions at 25 °C and 15 °C it showed higherPicroside-I content as compared to the field grown plants of PKS-11,PKS-13 showed higher Picroside-I content as compared to the field grown plants of PKS-13 at 15 °C and comparable content at 25 °C and PKS-14 when grown under *in vitro* conditions at 25 °C and 15 °C showed comparable Picroside-I content as to the field grown plants of PKS-14 and lastly the lowest content strain PKS-21 when grown under *in vitro* conditions at 25 °C and 15 °C showed higher Picroside-I content as compared to the field grown plants of PKS-21.

Thus the study of these strains which is reported for the first time not only helps in identification of high content strain but also optimised culture conditions can be further explored for large scale production of Picroside-I enriched shoot which would meet the industrial demand without threatening natural population.

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ANNEXURE

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 -	m-INOSITOL	100	10	10ml/l

100X				
	GLYCINE	2	0.2	
H - 100X	PYRIDOXINE-HCl	0.5	0.05	10 ml/l
	NICOTINE ACID	0.5	0.05	
	THIAMINE	0.1	0.01	

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