

***Picrorhizakurroa* Royle ex. Benth: an ethnic resource, sustainable strategies for better conservation for community health care**

Abstract:

Picrorhiza kurroa, locally known as kutki, had been emerged now as the most promising plant. Thus, propagation and its conservation have been realized since long back owing to its demand in the local and global market. During rearing kutki plants from growers fields at biotech product testing facilities including green house facilities as well as under laboratory practices for its establishment in pot and *in vitro*, powdery mildew stress was found. Thus, pot containing soil: sand: compost in the 1:1:1 ratio where field grown plants were nurtured and nourished. However, this stress could be managed by foliar application of 0.2% (w/v) sulfur in water and 0.2% (v/v) mild (Tween-20) aqueous detergent solutions. Imidacloprid (0.2% v/v) was also required to spray to avoid sucking pests infestations. Murashige and Skoog medium while supplemented with 0.5mg/L Thidiazuron (TDZ) and 0.5mg/L Indole butyric acid (IBA) based on our previous studies and checker board for identifying suitable concentrations of growth regulators, showed good callus induction rate. Callus growth was maintained through subsequent sub-culturing based on callus growth conditions. It is worth mentioning that the use of soluble Polyvinylpyrrolidone (PVP) 0.03-0.05% w/v significantly removed phenolics exudates and sufficient for maintaining callus growth and viability. Beside, orientation of molecules is important to assess their promising biological activities. In view of this particular fact Fourier Transform Infrared (FTIR) analysis offering valuable insights for the molecules extracted from the induced callus for various functional groups. This will provide some understanding of bioactivities from this important ethnic plant resource too.

Key message:

Comparative study among *in vitro* established callus growth in cultures vessels of *Picrorhiza kurroa* and Comparative analysis of established callus cultures during propagation for terpenes were undertaken under precise growth environment.

Keywords:

Ethnic; Endangered; Iridoids; Pharmaceutical: *Picrorhizakurroa*; Polyvinylpyrrolidone

Abbreviations:

IAA Indole-3-acetic acid

IBA Indole-3-butyric acid

NAA 1-Naphthalene acetic acid

2, 4-D 2,4-Dichlorophenoxyacetic acid

PVP Polyvinyl pyrrolidone

TDZ Thidiazuron

Rf Retardation factor

HPLC High performance liquid chromatography

msl mean sea level

IPM Integrated pest management

PDI percent disease index

MS Murashige and Skoog

TLC Thin Layer Chromatography

FTIR Fourier transform infrared

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Introduction:

The International Union for Conservation of Nature and Natural Resources [IUCN] has classified *Picrorhiza kurroa* as an endangered medicinal plant species (Nayar and Sastri 1990). *Picrorhizakurroa* is known by several traditional names, mostly known as "Kutki". This plant is also found as in natural habitats in Uttarakhand Himalayan range at an altitude between 3000m to 5000m from mean sea level (msl) (Kaul et al. 1996). This plant resource is naturally distributed from sub alpine to alpine regions in the states of Jammu and Kashmir, Himachal

Pradesh(Uniyal et al. 2011), Uttarakhand, Sikkim and Arunachal Pradesh(Bhardwaj et al.2021). This plant also found in green pastures, near springs and under the shrub canopies. Kutkosides (terpene glycosides nitrogenous derivatives) from kutki plant rhizome, extracts reported from the rhizome of *P. kurroa*referred asIridoid glycosides which are further characterized such as picroside I, picroside II, picroside III, picroside IV etc. Cucurbitacins like compounds as well have also been reported from this particular plant. Isolations and identifications were performed using high performance liquid chromatography (HPLC) coupled with detectors(Sultan et al. 2016).Veronicoside, pikuroside, 4-hydroxy-3-methoxy acetophenone, and other phytochemicals had also been identified in distinct *P. kurroa* extracts preparations(Salmaet al. 2017). Carbohydrates like D-mannitol and metabolic intermediates of amino acids like vanillic acid, cinnamic acid are found prominently beside the other significant chemical components that are mainly derived from this specific plant(Navya et al. 2018; Krupashree et al. 2014)

—In view of above owing to the fact of important derivatives inherent capacity, this plant is already well recognized for potential usages in our traditional medicine system. The studies confirm the potential of modulating human immune system(Bhandari et al.2008). *Picrorhiza kurroa*, health care properties are attributed to pharmacological potential including the protection of the liver ailment(Ansari et al. 1988)by virtue of anti-oxidative properties(Chander et al. 1992)and anti-cancerous activity including modulation of the immune system(Gupta et al. 2006).Local growers are traditionally maintaining this germplasm by propagating *Picrorhizakurroa*in order to utilize this plant resource to overcome ailments including high fever and stomach aches locally with success upto their satisfactory level(Masood et al. 2015). Local value of this plant resource *P. kurroa* reveals a plethora of biological active compounds probably with wide range of therapeutic management. Large range of liver, digestive and immune disorders including respiratory ailments and scorpion stings operations, while this plant has been able to address diverse health concerns (Visen et al.1998;Verma et al. 2009).The choleric, anti-inflammatory attributes, supporting liver functions and alleviating upper respiratory tract discomforts are managed well using this plant extracts. Moreover, it's hypoglycemic, cardiovascular, and anti-viral properties hold promise for modern medicines as well(Basu et al. 1984; Hussain et al. 1984; Joy et al. 2000; Ansari et al. 1988). Such a versatile pharmacological profile of *Picrorhiza kurroa* compels continuing to be a source for herbal remedies as a potential source for various health, lifestyle and wellness issues. Application of *in vitro* culture methods optimization which would suits at working laboratory and bioproduct testing facilities including greenhouse facility was performed. This in turn also would be

beneficial to develop micro propagation strategies which shall help the growers community to provide them back quality planting material which might help in mitigating the declining population of *Picrorhiza kurroa*. Preserving the production of its specific derived metabolites in its natural habitats had also been attempted (Patial et al. 2012; Rawat et al. 2013; Sood and Chauhan 2010). Traditional propagation methods are also important which involve rhizomes and stolons. Though such methods are time-consuming and demanding a significant quantity of plant material, however the growers' community maintained such plants losing their profits compared to seed-based propagation, which in such plants is a real constraint due to their low seed germination rates (Rawat et al. 2013).

In the said context the present experimental plan was undertaken to further optimize protocols precisely for *in vitro* establishment of *Picrorhiza kurroa* Royle ex. Benth post corona pandemic collecting field grown germplasm from mid mountains of Uttarakhand. Suitable explants selected under various light intensities, sterilization optimization protocols of explants including explant orientation effects during inoculation for callus induction. Comparative study among *in vitro* established callus growth in culture vessels of *Picrorhiza kurroa* and comparative analysis of established callus cultures during propagation for terpenes were undertaken to pave the way and maintaining the continuity for caring this important plant resource.

Material and methods

Experimental site:

The experimental site was College of Basic Science and Humanities rear building and Molecular Biology and Genetic Engineering laboratories. The experimental materials were obtained for this investigation comprised of the plants of *Picrorhiza kurroa* were collected from Jhuni, Bageshwar Northwest Himalayan region of Uttarakhand in the month of November, 2022 from the growers' field in plastic bags along with the soil. These plants were under growth and development at the height of 520m (1706.04ft) and coordinates 29.94°N 79.90°E, propagating them from rhizome. The plants carrying 5-6 mature leaves and approximately (8-10cm height) without much volume of rhizome. These collected plants were further planted at the experimental site in the same month (November, 2022) in the pots of (1.5L) for their establishment as a readily available plant germplasm. The germplasm

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evaluation facilities maintaining 25⁰C- 28⁰C and 70-80% relative humidity for the survival of these plants. Care was also required through shifting of maintained pots consisting of single plant per pot. The leaves, stem, root and stem nodes were attempted as an explants of *Picrorhiza kurroa* Royal ex. Benth. All these explants were inoculated for the study of their *in vitro* response of callus induction.



Fig 1: *Picrorhiza kurroa* under growth in pots containing soil (collected from Dr NE Borlaug Crop Research Centre, Pantnagar-263145): sand: compost (obtained from cow dung collected from Dairy Research Farm) in the ratio of 1:1:1 w/w/w with required moisture contents.

Sterilization of explants:

The leaves, approximately 5-6 cm in size, were excised and thoroughly washed under running tap water. Subsequently, the explant(s) were washed with distilled water and treated 30 min in an appropriately diluted aqueous solution of a systemic fungicide 0.1% (w/v) Bavistin. Afterward, the explant(s) were washed with distilled water to remove any residual traces of Bavistin. The surface sterilization of the explant(s) involved a freshly prepared aqueous solution of 0.1% (w/v) mercuric chloride (HgCl₂) for 35 seconds. The explant(s) were washed with distilled water, followed by a 30 seconds immersed in 70% ethanol. Finally, the leaf sample was washed again with autoclaved distilled water under laminar air flow cabinet.

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Inoculation of explants and callus induction

Explants (1.5 cm approximately) was carefully inoculated in MS medium containing 3% sucrose and 0.8% agar with pH= 5.8 ± 0.03 . This provides an optimal environment for growth and development of callus. To assess the impact of phenolic oxidation (browning), different concentrations of polyvinylpyrrolidone (PVP) ranging between 0 to 0.9 g/L were added to the MS basal media. To promote the desired growth pattern, medium supplemented with varying concentration and combination of growth regulators IBA (0.0 – 1.5mg/L) and TDZ (0.0- 1.0 mg/L). Periodic sub-culturing was implemented every 15 days, ensuring that the culture received fresh nutrients and growth regulators, thus facilitating their development in controlled and optimized environment.

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Culture growth parameters:

The major micro environmental factors such as light intensity, wavelength and temperature and darkness in the incubation chamber play often crucial role in influencing organ dedifferentiation in order to develop callus like biomass. However, optimal growth and development of inoculated explants during incubation for developing suitable cultures, there must be an exposure which is required upto 16-hour light and 8-hour dark for photoperiodic cycle as an essential. According to the observations with light intensity while maintained between 27 and 33.75 $\mu\text{mol sec}^{-1}$ alongwith the temperature which was kept at $25 \pm 2^\circ\text{C}$ with the humidity levels at 60-70%.

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Results and discussion:

— Powdery mildew is a widespread fungal disease affecting various plant species, including *Picrorhiza kurroa*. As it was evidenced during rearing collected plants in pots from the [growersgrower's](#) field under study. These plants were found infected by Powdery mildew alongwith sucking insects (aphids and thripes) pests as well. Further Powdery mildew infection and sucking [insectsinsects'](#) infestation were managed during pot cultivation applying an integrated pest management (IPM) practices including the recommended doses of pesticides.

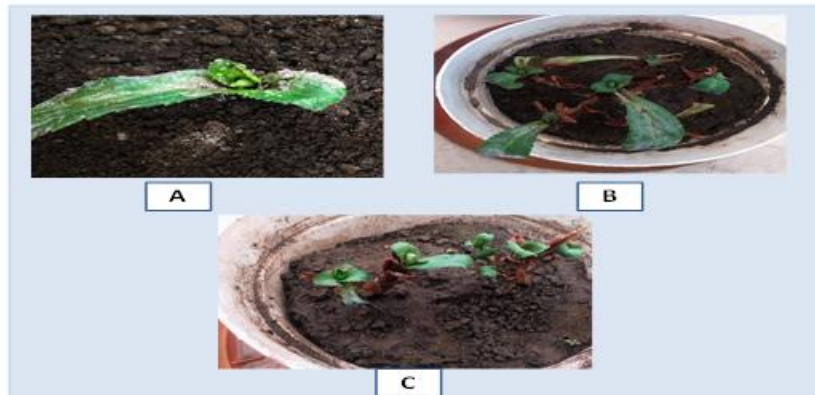


Fig 2: (A), (B) Symptomatic plants observed and identified for powdery mildew, sucking insects and (C) recovered plant after pest management.

$$\text{Percent Disease Index (PDI)} = \frac{\text{Sum of Grade}}{\text{Total no of leaves analysed} \times \text{Maximum disease}} \times 100$$

$$\text{PDI} = \frac{273}{50 \times 9} \times 100 = \frac{27300}{450} = 60.66\%$$

Experimental process while observing growth and development of potted plants, it was noticed that powdery mildew disease was found deadly. Hence, immediate attention required to prevent further infection. Thus, foliar applications of sulphur 0.2% (w/v) suspension, and mild (Tween-20) aqueous detergent 0.2% (v/v) solutions were quite effective for powdery mildew management and as a result these plants could be recovered upto 39.34% extent. The appropriate concentration of imidacloprid 0.2% (v/v) was found to be more effective while managing sucking insects pest in *Picrorhiza kurroa* in potted cultures. Since *Picrorhiza kurroa* is vulnerable in the grower field as well as while maintaining in pot cultures of collected field grown plants, therefore, conservational strategies ought to be adopted to maintain the germplasm in order to attain maximum benefits in sustainable manner. Keeping this fact we attempted callus induction which was successfully achieved in selected basal media as per MS medium

constituents. On the basis of experiments conducted previously at our laboratory, thidiazuron (TDZ) while replaced by other cytokinins was found suitable in the concentration ranging upto 1.5mg/L, present experimentation showed TDZ (0.5 mg/L), and indole butyric acid (IBA) (0.5 mg/L) were optimum for callus induction while trying various combination of growth regulators and various explants derived from potted cultures. The callus induction percentage was observed ranging 40% to 100% depending upon size of explants, nature of explants, orientation of explants, explants from different maintained pots and nature of plant growth regulators as well including cytokinins and auxins. The induced callus growth parameters(**Table 1**) exhibited green coloration with varying size in terms of growth while observing at same interval while weights of such induced calluses were observed ranging between 1545mg to 8456 mg. Response of various auxins (2, 4- D /IBA /NAA/IAA) on callus induction frequency from leaf explants on MS basal medium supplemented with 1.5% sucrose was also performed and range less than 60% (**Table 2**).

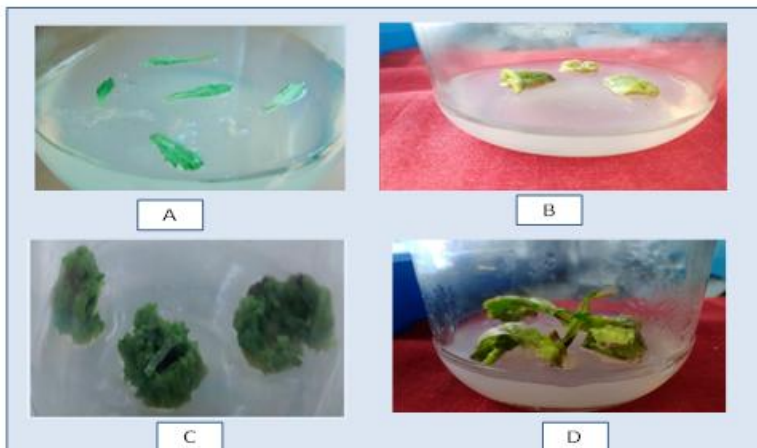


Fig 3: (A) Leaf explant 0 day (B) 14Days induced callus (C) 28 Days old callus (D) Direct organogenesis from one of the inoculated shoot explants

Table 1: Callus Induction from leaf explants of *Picrorhiza kurroa* Royle ex. Benth on MS basal Medium supplemented with various combinations of IBA and TDZ concentrations

S.no	MS +3% sucrose + Concentration of PGRs(mg/L)	Callus Frequency (%)	Time to emergence(Days)	Type of callus
1	IBA 0.0/TDZ 0.0	0.0 ±0.0	-	-
2	IBA0.50/TDZ 0.0	41.66 ± 1.04	19-24	Pale yellow fragile
3	IBA0.75/TDZ0.0	50.00 ± 1.14	19-23	Pale yellow fragile
4	IBA1.0/TDZ0.25	66.66 ± 0.10	17-21	Green compact
5	IBA 0.75/TDZ0.25	58.33 ± 1.39	15-21	Green compact
6	IBA 1.0/TDZ 0.25	75.00 ± 0.23	15-19	Green compact
7	IBA 0.5/TDZ 0.5	91.66 ± 2.09	14-18	Green compact
8	IBA 0.75/TDZ 0.5	83.33 ± 1.99	15-19	Pale yellow fragile
9	IBA 1.0/TDZ 0.5	75.00 ± 0.93	14-20	Green compact
10	IBA 0.5/TDZ 0.75	66.66 ± 0.72	17-22	Green compact
11	IBA 0.75/TDZ 0.75	83.33 ± 0.22	16-18	Green compact
	CD	2.2643		

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	SE(M)	0.7720		
	SE(D)	1.0918		

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Table 2: Response of various auxins on callus induction frequency from leaf explants on MS basal medium supplemented with 1.5% sucrose

Sr. No.	MS+ 1.5% sucrose + Auxin (mg/L)				
	2,4-D	IBA	NAA	IAA	Callus induction frequency (%)
1	0	0	0	0	0
2	0.5	0	0	0	16.66 ± 0.17
3	1	0.5	0	0	33.33 ± 0.10
4	1.5	0.5	0	0	41.67 ± 0.48
5	2	0.5	0	0	58.33 ± 0.79
6	2	1	0	0.5	50.00 ± 0.99

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7	0	1	0.5	1	33.33 ± 0.76
8	0	1	1	0	41.66 ± 0.34
9	2	0	2	0	33.33 ± 0.72
10	0	0	1.5	1	41.66 ± 0.30
11	0	0	2	1.5	58.33 ± 0.51
C.D	1.2679				
SE(M)	0.4323				
SE(D)	0.6114				

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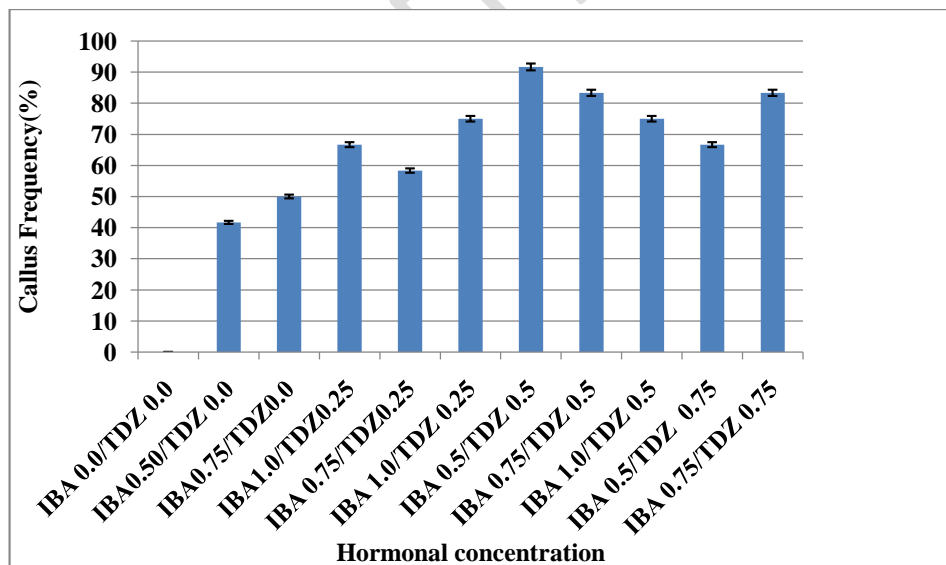
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a)



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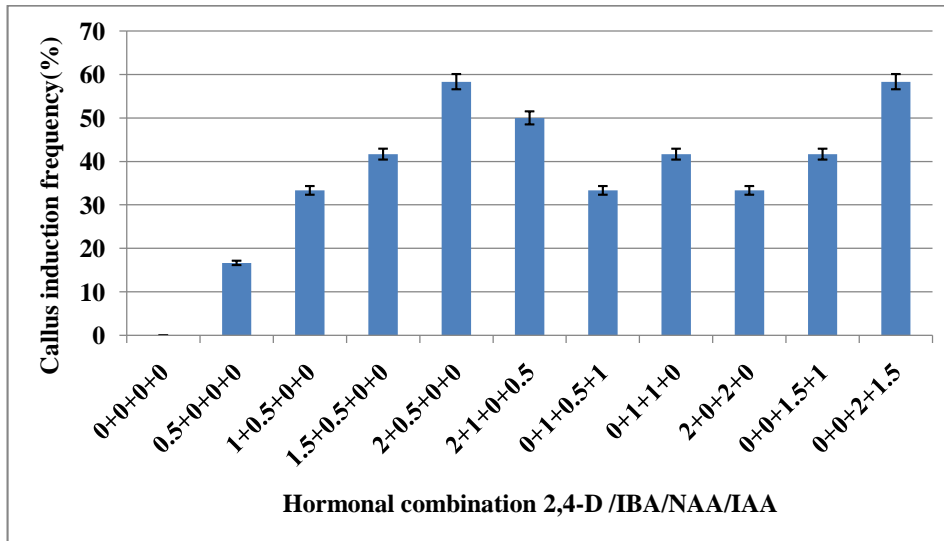


Fig 4: Callus induction frequency of leaf explants a) Response of various hormonal combinations of IBA and TDZ b) Response of various auxins combinations of 2, 4-D/ IBA/NAA/IAA

Table 3: Response of concentrations of soluble PVP to subside phenolics exudates during callus cultures of *Picrorhiza kurroa*

Culture	Concentration of PVP (g/l)	Colour of callus	Survivability of callus after sub culturing (%)
A	0.0	Brown	0%
B	0.1	Light brown	25%
C	0.3	Dark Green	80%
D	0.5	Light green	72%
E	0.7	Brown	20%
F	0.9	Black	0%

Response of soluble polyvinyl pyrrolidone (PVP):

The leaf explants were obtained from this plant germplasm maintained at our facilities which was also *in vitro* cultured aseptically after thorough different required sterilizations and washings. These explants were then inoculated into the growth medium and kept for incubation under a photoperiod of 16/8 hours (light/dark) with a light intensity ranging between 27-33.75 $\mu\text{mol sec}^{-1}$ and maintaining temperature of $25\pm 2^\circ\text{C}$ with the relative humidity 60-70%, the assessment of the impact of phenolics for their oxidations leading to browning of induced callus and other adverse effect on growth. In order to avoid different concentrations of PVP (ranging between 0 to 0.9g/L) were added to the culture vessels having suitable volume of MS basal media. After the growth period of 28 days, the survival of explants and callus related data were recorded for analysis.

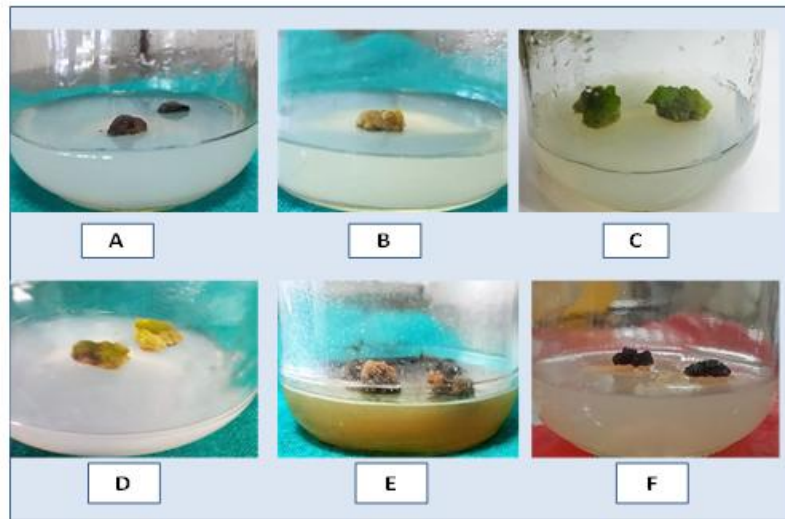


Fig 5: Effect of various concentration of soluble PVP on callus culture

(A) 0.0g/L (B) 0.1g/L (C) 0.3g/L (D) 0.5g/L (E) 0.7g/L (F) 0.9g/L

On the basis of recorded experimental data which revealed that the most suitable concentration to control the adverse effects of phenolics was found in between 0.3-0.5 g/L of soluble PVP. The presence of PVP at concentrations of 0.0 g/L, 0.7 g/L, and 0.9 g/L when supplemented in the basal growth MS medium, the browning of callus was noticed resulting into adverse effect on growth *in vitro*.

While finding the impact if any of different orientations of various explants on the establishment and subsequent induction of callus, we inoculated explants placing them in three different orientations of horizontal, vertical and tilted.

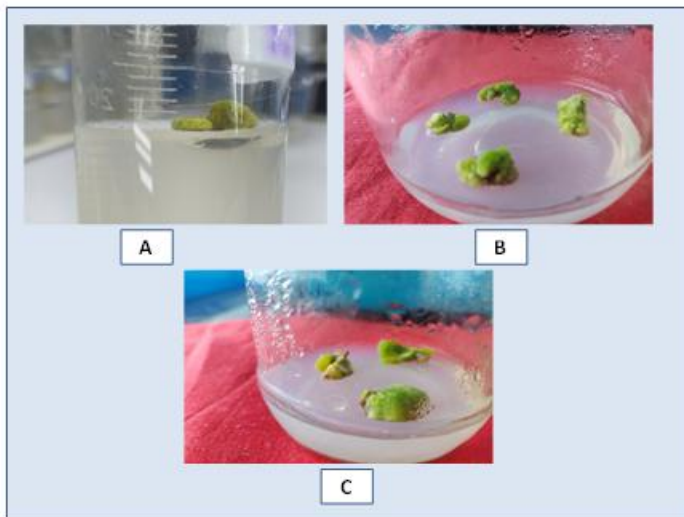


Fig 6: Orientation of explants placed on MS media supplemented with 0.5mg/l IBA+ 0.5mg/l TDZ (A) Tilted (B) Vertical (C) Horizontal

Horizontal placement of the explants resulted in a greater percentage of callus growth induction when compared to vertically and in between oriented explants. Horizontal orientation may be facilitated direct contact between the explants and the growth medium, enhancing the uptake of

nutrients and signalling of hormones, which ultimately stimulated callus formation. The results emphasize the significance of orientation in callus induction during plant tissue culture.

Determination of picroside II

Callus mass (10 mg) shades dried were finely powdered in liquid nitrogen using a pestle and mortar. Then, 1 mL of 80% HPLC-grade methanol was added to the powdered samples, and intermittent grinding was carried out for 1 minute followed by centrifugation at 10,000g for 20 minutes (Kawoosa et al. 2010). The supernatant obtained after centrifugation was utilized for picroside-II estimation.

During TLC analysis, the prepared TLC plates were marked approximately 2 cm above from bottom using a pencil. With a capillary tube or micro-syringe, the methanol extract obtained was spotted onto the marked line. In order to ensure accuracy, multiple spots were applied. Additionally, to facilitate comparison and identification, spot of pure picroside II (used as standard) was also included on the same TLC plate.

Table 4: Solvent used for Mobile Phase

Sr. No	Chemicals	Concentration for 100ml in(ml)
1	Ethyl acetate	78.125
2	Methanol	15.625
3	Glacial acetic acid	3.125
4	Formic acid	3.125

An adequate amount of a suitable solvent mixture, i.e. Ethyl acetate: Methanol: Glacial acetic acid: Formic acid in a ratio of 25:5:1:1 v/v/v/v (Gaikwad et al. 2011).

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Table 5: The Relative front (R_f), Distance travelled by isolates / total solvent run onto the plate relative to reference compound comparison were shown below:

Sample	Distance (cm)	Distance (cm)	Distance (cm)
Reference (R1)	0.0	2.8	0.0
Isolate 1	1.6	2.8	3.2
Isolate 2	1.6	2.8	3.2
Isolate 3	1.6	2.8	3.2
Isolate 4	1.6	2.8	3.2

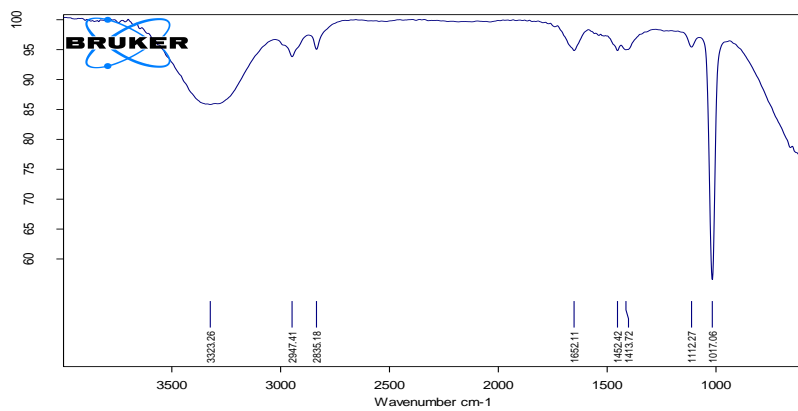
Extrapolated Relative front

$$\frac{\text{distance travelled by compound}}{\text{distance by solvent front}}$$

$$\frac{2.8}{7.5} = 0.37$$

TLC Plate visualizes under UV and R_f value calculated. The obtained R_f value of sample that is comparable to the reference compound which indicates the presence of Picroside II in extract.

FTIR Analysis of *in vitro* grown Callus Extract



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Fig 7: Fourier transforms infrared (FTIR) depicting functional groups present in the callus extracts

Table 6: Functional group and its quantified frequencies

Functional groups	Wave number (cm ⁻¹)
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H- bond, aromatic and unsaturated	3323.26
Linear aliphatic	2947.41
Methoxy(O-CH ₃), methyether, C-H stretch	2835.18
C=C Stretch alkenyl (double bond)	1652.11
Methylene C-H bend	1452.42
Vinyl C-H in plane bend	1413.72
Aromatic C-H in plane bend	1112.27
Aromatic C-H	1017.06

The observed peaks correspond to different vibrations associated with specific chemical bonds in the sample. Overall, the FTIR data indicates the presence of a diverse range of functional groups, including aromatic, aliphatic, and unsaturated compounds in the analyzed sample (Griffith 1983). These functional groups are indicative of the sample's complex chemical composition and may be associated with various biological, pharmaceutical, or industrial applications.

Discussion:

Picrorhiza kurroa presents analysis of various aspects including conservational strategies, *in vitro* tissue culture, stress control, and chemical composition. Stress control emerged as a challenge during the experiment, with Powdery mildew and sucking insects affecting the plant. Thus, foliar application of sulfur 0.2% (w/v) in water and mild (Tween-20) aqueous detergent 0.2% (v/v) were found effective for Powdery mildew stress management. Imidacloprid 0.2% (v/v) for sucking insects exhibited promising insecticide for insect pest management (Gahukar 2018). The focus of this research was to develop an *in vitro* tissue culture protocol, successfully inducing callus from leaf explants. Optimal conditions were determined by MS basal medium supplemented with 0.5 mg/L TDZ and 0.5 mg/L IBA resulting in a high callus induction frequency of 91.66% (Ahmed et al. 2012). To address phenolic exudates during callus culture, the study explored the use of Polyvinylpyrrolidone (PVP) at various concentrations. These findings suggest that 0.3-0.5 g/L of PVP were effective in controlling callus growth and viability (Amente et al. 2021). Horizontal placement allows direct contact with the growth medium,

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facilitating nutrient uptake and hormonal signaling, thereby stimulating callus induction. This highlights the crucial role of explants orientation in plant tissue culture success rate, offering practical implications for optimizing callus induction protocols (Iapichino and G 1990). TLC analysis strengthens the indication of Picroside II with extrapolated Rf value of 0.37 using standard as reference (Tiwari et al. 2012). Analysis by FTIR spectroscopy revealed a diverse range of functional groups of the molecules extracted from the callus (Griffith 1983). These findings have implications for potential pharmaceutical, biological, or industrial applications of this particular plant.

Conclusion:

Picrorhiza kurroa, by virtue of the potential demand as an international commodity for versatile applications with special reference to health care management and the fact of diminishing nature sustainable goals to protect this plant resource is inevitable. Crucial insights into this plant resource conservation requires continuous efforts in maintaining sustainability and judicious utilization adopting modern approaches are being obtained from scientific developments for this endangered more importantly recognized among Himalayan plants. Our experimentally obtained data contributes to the conservational strategies in one or another ways and provide an insight for further development experimental planning and strategies for *Picrorhiza kurroa* better management practices in terms of community goals. *In vitro* maintained cultures and developed protocols are significant insights into various parameters of growth including chemical compositions under precise growth environment. These findings hold promises for opening many avenues of utilizing the medicinal properties of *Picrorhiza kurroa* while ensuring its survival in the Western Himalayan region. Further research and development of tissue culture and related techniques could significantly be able to prove worthy conserving endangered nature of this

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plant and developing innovative pharmaceutical and other commodities to cater local and global market.

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