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Indirect Regeneration of *Picrorhiza kurroa* Royle ex Benth: An Endangered Medicinal Plant for *Ex Situ* Conservation

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ABSTRACT

An efficient rapid plant regeneration protocol has been optimized for Picrorhiza kurroa Royle ex Benth, propagation which is an endangered and venerable medicinal plant. Leaf explants were established in culture vessel containing Murashige and Skoog (MS) basal medium supplemented with different concentrations of phytoregulators namely Indole Butyric Acid (IBA) and Thiodizuron (TDZ) in order to induce callus which was observed at 0.75 mg/l IBA and 0.75mg/l TDZ with an average of 84.33±13 % frequency for callus induction. The highest value for shoot generation frequency 90±8.660254 % was observed with an average shoot number 8.33±1.0 on the medium supplemented with Benzyl Amino Purine (BAP) 0.5mg/l and kinetin 0.75 mg/l. These micro shoots were sub cultured on the root generation medium for complete regeneration of plantlets on MS medium supplemented with concentrations of 2,4-Dichlorophenoxyacetic acid (2, 4-D) ranging from 0.0mg/l - 1.0 mg/l and Naphthalene Acetic Acid (NAA) ranging from 0.0mg/l - 1.0 mg/l. Maximum root generation frequency was recorded at 2, 4-D (0.5mg/l) and NAA (0.4 mg/l) with an average percentage of 86.33±9.53 with an average number of roots 8.0±0.0.

Key words: *Indirect regeneration, Medicinal plants, Picrorhiza kurroa Royle ex Benth.*

INTRODUCTION

Ex situ conservation for sustaining biodiversity diminishing bio-resources are important to be kept without further loss in order to maintain harmony in conjunction with nature and welfare of humanity. Plant resources have immense implications on human beings in terms of providing a range of goods and services such as food, medicines, animal feed, fibres and fuel. *Picrorhiza kurroa* Royle ex Benth is widely recognized medicinal herb, endemic to alpine Himalaya[1], at an altitude of 3,000 to 4,500 m which is usually harvested before seed setting as per the requirement for subsequent trading of raw material[2,3]. The plant is listed as vulnerable species in Red Data Book[4] which subsequently is a threatened plant species and has been recognized under the 'endangered' species category, as per International Union for Conservation of Nature (IUCN) criteria due to lack of organized cultivation and overexploitation of this important plant resource which has been depleted by now considerably.

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Traditionally, the mature plants are uprooted, and their different preparations are under use for treatment of various disorders of liver, upper respiratory tract, dyspepsia , chronic diarrhea and scorpion sting[5,6]. This plant has also identified as immunomodulatory cardiovascular, antihepatotoxic, choleretic, antiinflammatory, hypoglycemic, hypolipidemic, antispasmodic, antitumor, antiviral, purgative, antioxidant, anti-phosphodiesterase, neuritogenic, molluscicidal, and leishmanicidal like biological activities[7,8,9]. The conventional propagation methods requires conjunction with *in vitro* techniques for rapid and efficient mass propagation which offers possibilities for sustainability of this endangered plant species, thus reducing the risk of extinction[10].

Presently *in vitro* propagation techniques have been extensively used for the commercial proliferation of a number of economically important species[11]. This technique is useful not only for rapid and mass multiplication of existing stock of plant species but also offer advantages for the conservation of important and endangered taxa. In view of the importance of this species, a holistic approach for the propagation of *Picrorhiza kurrooa*, using *in vitro* method and strategy has been employed for its conservation for effective utilization for human kind.

MATERIAL AND METHOD

Plant Material

The plants of *Picrorhiza kurroa* Royle ex Benth were collected from Chamoli, North West Himalayan Region of Uttarakhand in the month/year of July/2015. These plants were established in Plant Germplasm Evaluation Facility (25^0C, 65.0% RH) at College of Basic Sciences and Humanities, G.B.P.U.A. &T., Pantnagar in the same duration which was then subsequently established *in vitro.*

Callus induction:

Callus induction was attempted with leaf explants. Explants were pre cleaned with double distilled water containing 1% Tween 20 for 4-6 min. then surface sterilized with 0.1% $HgCl_2$ for 35 sec., wash with autoclaved water 3-4 times. After that explants were treated with 70% ethanol for 30 sec. rinse thoroughly 3-4 times in sterile distilled water, cut into small pieces (~ 0.4cm) and inoculated MS basal medium have 3% sucrose and 0.6% agar powder, pH of the medium was adjusted ~ 5.8.To avoid browning due to phenolics, medium was supplemented with 0.05% (Poly Vinyl Pyrolidine) PVP soluble polymer. The medium was supplemented with different concentration of IBA and TDZ medium for callus induction. The explants for callus induction were incubated in photoperiod of 16/8 hours with 27 – 33.75 µ mol sec^{-1} light intensity at 25^0C. After three weeks of culture, calluses were induced from the leaf explants and these were sub cultured to the fresh medium at the intervals of 2-weeks.

Shoot generation:

After callus induction, fully grown friable callus were cut into 2-3cm pieces and transferred to the MS medium having different combinations of BAP and kinetin. Within 2-3 weeks shoots regeneration was observed.

Root generation:

Root generation was attempted from generated micro shoots (2-4 cm length) up on transferring them in to MS basal medium supplemented with different concentrations of 2, 4- D and NAA. Root generation could be possible within 2-3 weeks.

Culture Conditions:

All cultures were at 25± 2ºC temperature under 16 hr photoperiod with 27 – 33.75 µ mol sec-1 of light intensity.

Calculation of induction frequency:

$$\text{Frequency of Callus induction} = \frac{\text{No. of leaf explants producing callus} \times 100}{\text{Total No. of leaf explants subjected on callus induction media}}$$

$$\text{Frequency of Shoot generation} = \frac{\text{No. of callus producing shoots} \times 100}{\text{Total No. of calluses subjected on shoot generation media}}$$

$$\text{Frequency of Root generation} = \frac{\text{No. of shoots producing roots} \times 100}{\text{Total No. of shoots subjected on root generation media}}$$

Sub culturing of Plantlets:

The fully regenerated plantlets were sub cultured into MS medium supplemented with GA_3(0.5 mg/l), kinetin (1.5 mg/l) and IAA (1.00 mg/l) after an interval of two weeks.

Statistical analysis:

All measurements were performed in triplicates for each combination of hormone and expressed as mean ±SE. Mean values of various treatments were subjected to one way Analysis of Variance (ANNOVA) at the 5% probability level ($p \leq 0.05$).

RESULTS AND DISCUSSION

Plantlet regeneration:

Effect of different concentrations of IBA and TDZ on Callus induction:

The explants (leaf) were cultured on MS medium supplemented with different combinations and concentrations of IBA and an analogue of cytokinin (s) TDZ to induce callus. Ample growth of callus occurred within 21 days of culture in which MS supplemented with 0.75 mg/l IBA and 0.75 mg/l TDZ with 84.33±13 % callus induction frequency, (Table-1). After obtaining callus, sub culturing was carried out every 2 weeks interval for increment of the callus growth and minimizing the phenolics[12]. Reported that MS medium supplemented with IBA (0.5mg/l) + TDZ (0.5 mg/l) were more effective for callus induction (100%) in *P. kurroa* in case of leaf derived callus[13]. Established callus cultures from different explants of *P. kurrooa* such as leaf discs, nodal and root segments of *P. kurrooa*. Callus induction percentage was recorded around 56.3 % in case of leaf on MS medium supplemented with 2,4-D (2 mg/l) and IBA (0.5 mg/l)[14] reported that MS medium with 0.25 mg/l 2,4-D and 0.25 mg/l BAP stimulated callus induction rate and direct shoot regeneration rate.) Leaf explants of *P. kurrooa* shows best results at 4.0 mg/l NAA and 1.0 mg/l Kn[15].

Effect of various concentrations of BAP and Kinetin on *in vitro* shoot multiplication:

Table-2 summarizes the effect of various PGRs (plant growth regulators) on shooting. Shoot buds were sub cultured at every 21 days. Among various treatments, MS medium supplemented with BAP (0.5mg/l) and kn (0.75 mg/l) resulted in maximum rooting (90±8.660254 %) and highest shoot number (8.333333±1.0)[12]. Reported maximum shooting percentage (100%) at BAP (1.0 mg/l) + kn (0.75mg/l). Shoot organogenesis was achieved by its sequential transfer at different media, i.e. induction of bud primordial on MS + 0.25 mg/l BAP and alteration of bud primordia into shoots on MS + 0.12 mg/l BAP with half strength nitrogen supply[15].

Effect of different levels of 2, 4-D and NAA on rooting of microshoots:

The individual micro shoots (3.0–5.0 cm length) were transfer to MS medium supplemented with various concentrations of 2, 4-D (0.0mg/l - 1.0 mg/l) and NAA (0.0mg/l - 0.4 mg/l. The best rooting response was recorded at 0.4 mg/l NAA and 0.5 mg/l 2, 4 D with 86.33333±9.53939 rooting percentage of and 8.0±0.0 root number (Table-3). Same medium was found suitable for more number of roots[12]. Successful rooting of microshoots in *P.kurrooa* can be achieved by incorporation of a variety of auxins (NAA, IBA and IAA) in the culture medium[16,17]. Although, [16] did not mention the rooting percentage but[17] achieved 89 % rooting in micro shoots with 1.0 l µM NAA in *Picrorhiza kurrooa*. 100 % in vitro rooting of micro shoots with 1.0 or 2.5 µM IBA was reported by Chandra *et al*[18].

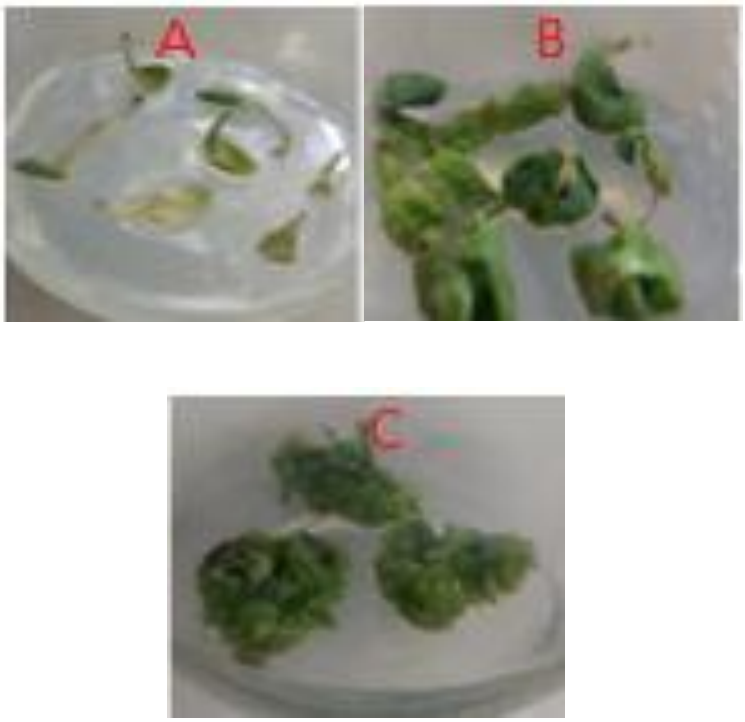

Fig. 1: Callus induction from leaf explants of Picrorhiza kurroa Royal ex Benth. in MS medium supplemented with TDZ (0.75 mg L-1) and IBA (0.75 mg L-1) (a) Leaf explants (b) After 3 weeks (c) After 4 weeks of culture

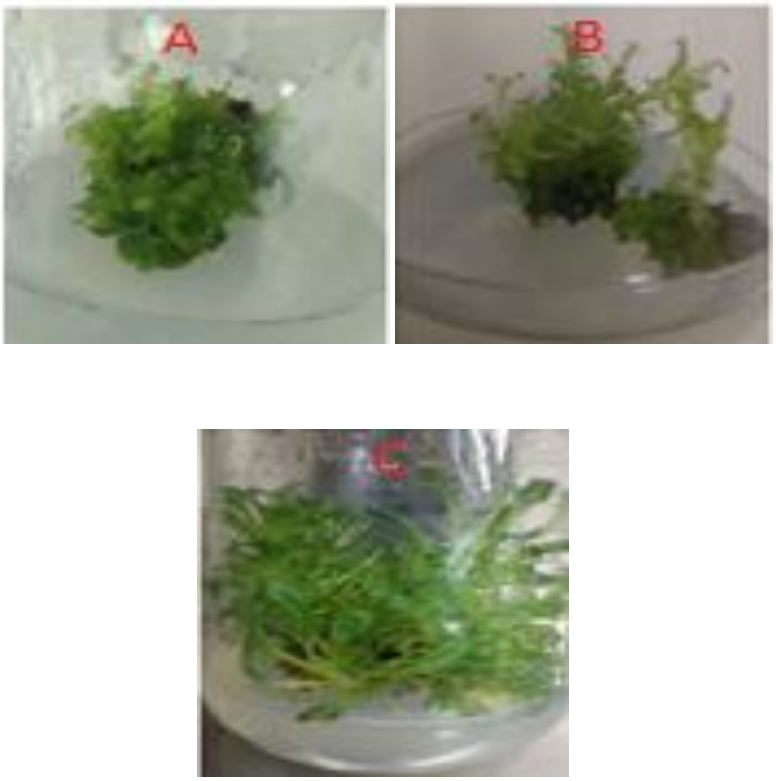

Fig. 2: Multiple shoot generation from leaf derived callus in MS medium supplemented with BAP (0.50 mg/l) and Kinetin (0.75 mg/l) (a) After 3 weeks (b) After 4 weeks (c) After 5 weeks of culture

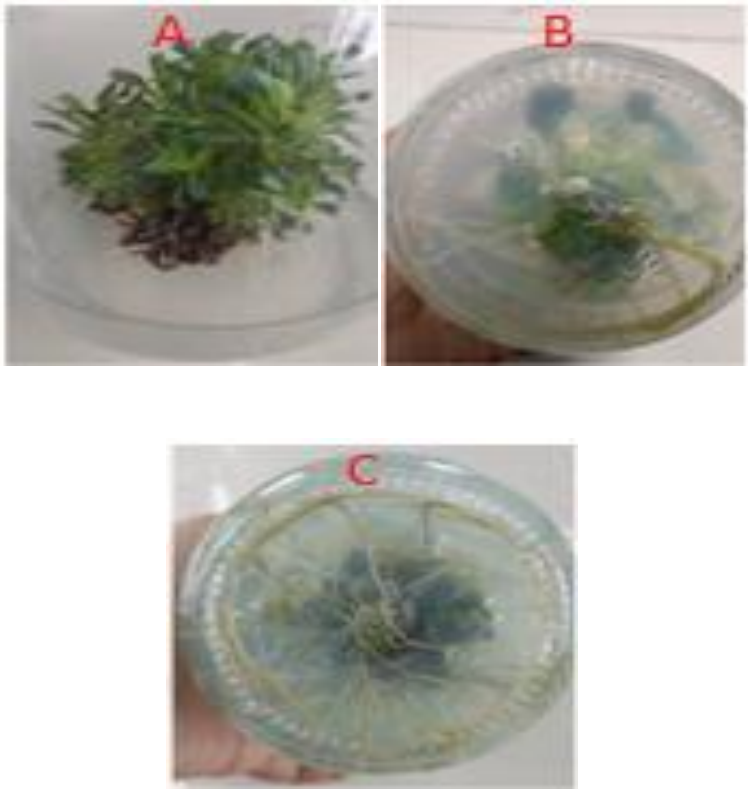

Fig. 3: Rooting of micro shoots in MS medium supplemented with 2,4- D (0.5 mg/l) and NAA (0.4 mg/l) (a) Root initiation after 3 weeks (b) After 4 weeks (c) After 6 weeks of culture

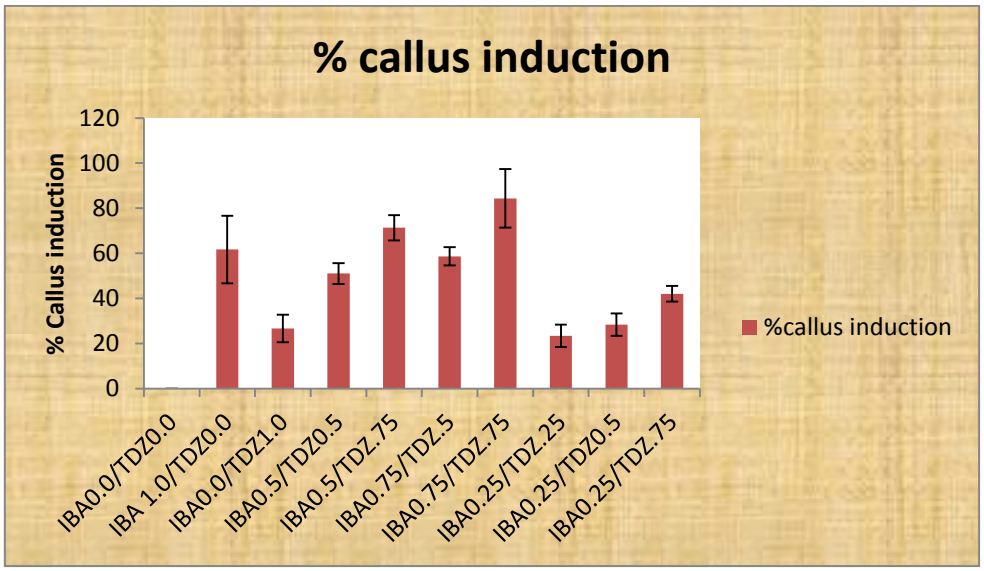

Fig. 4: % callus induction from leaf explants

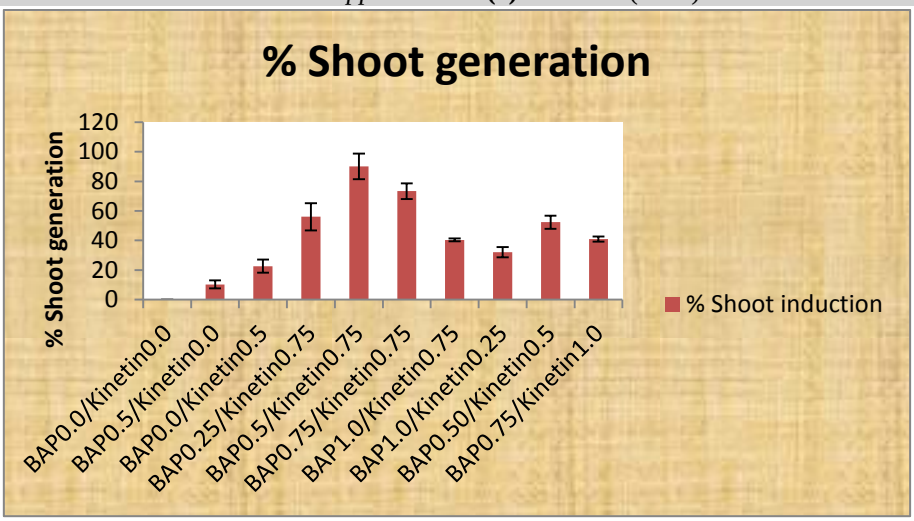

Fig. 5: % Shoot generation from leaf derived callus

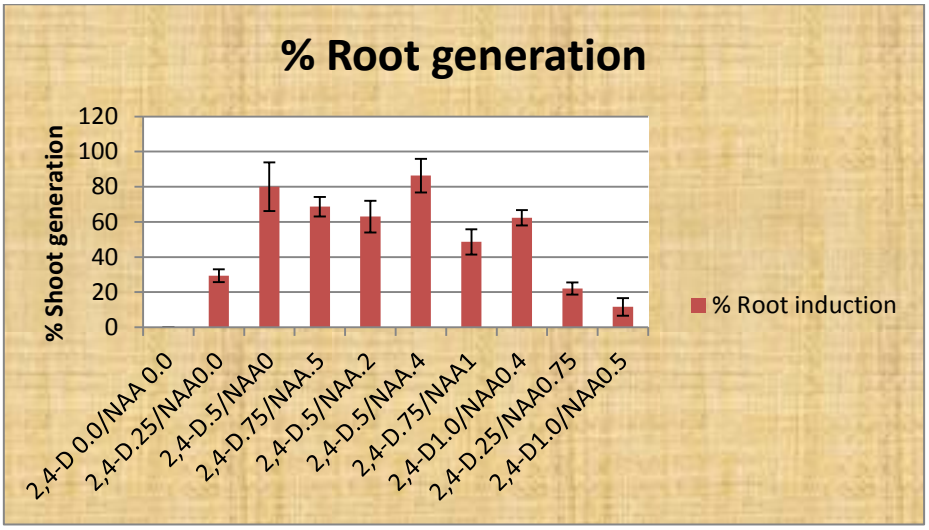

Fig. 2: % Root generation

Table 1: Effect of different concentrations of phytoregulators on callus induction from leaf explants. All values are Mean ± SE (n=3). Statistical analysis by one-way ANOVA ($p<0.05$) was found significant

S.No.	Concentrations of PGRs (mg/l)	Callus frequency [%]	Time to emergence (Days)	Type of callus
1	control	-	-	-
2	IBA 1.0/TDZ0.0	61.66±14.93	18-22	Green, compact
3	IBA0.0/TDZ1.0	26.66±6.08	20-25	Green, Friable
4	IBA0.5/TDZ0.5	51.0±4.58	18-24	Green, Friable
5	IBA0.5/TDZ.75	71.33±5.56	16-21	Green, compact
6	IBA0.75/TDZ.5	58.66±4.0	17-22	Light green, compact
7	IBA0.75/TDZ.75	84.33±13.0	14-21	Green, compact
8	IBA0.25/TDZ.25	23.33±5.0	20-22	Light green, compact
9	IBA0.25/TDZ0.5	28.33±5	20-24	Green, compact
10	IBA0.25/TDZ.75	423.46	18-20	Green, Friable

Table 2: Effect of different concentrations of phytoregulators on shoot generation from leaf derived callus. All values are Mean ± SE (n=3). Statistical analysis by one-way ANOVA ($p<0.05$) was found significant

S.No.	Concentrations of PGR's(mg/l)	Shoot generation frequency [%]	Shoot number [explants^{-1}]	Shoot length	Time to emergence(Days)
1	BAP0.0/Kinetin0.0	-	-	-	-
2	BAP0.5/Kinetin0.0	10.33±2.64	2.0±1.73	1.56±0.52	16-21
3	BAP0.0/Kinetin0.5	22.66±4.35	2.33±1.0	2.1±0.62	18-20
4	BAP0.25/Kinetin0.75	56.0±9.16	6±1.73	3.03±0.26	12-18
5	BAP0.5/Kinetin0.75	90.0±8.66	8.33±1.0	4.63±1.32	15-18
6	BAP0.75/Kinetin0.75	73.33±5.291	7±1.73	3.5±0.0	14-19
7	BAP1.0/Kinetin0.75	40.33±1.0	5±1.73	2.76±0.43	14-20
8	BAP1.0/Kinetin0.25	32.0±3.46	3±1.73	2.16±0.5	17-20
9	BAP0.5/Kinetin0.5	52.33±4.35	5.33±1.0	3.7±0.5	13-17
10	BAP0.75/Kinetin1.0	41±1.73	4.0±0.0	5.3±1.75	15-22

Table 3: Effect of different concentrations of phytoregulators on in vitro root generation of micro shoots of Picrorhiza kurroa Royle ex Benth. All values are Mean ± SE (n=3). Statistical analysis by one-way ANOVA ($p<0.05$) was found significant

S.No.	Concentrations of PGR's (mg/l)	Root generation frequency [%]	Root number [explants^{-1}]	Time to emergence
1	Control	-	-	-
2	2,4-D0.25/NAA0.0	29.33±3.60	2.66±0.0	14-18
3	2,4-D0.5/NAA0.0	80.0±13.85	7.33±1.0	12-16
4	2,4-D0.75/NAA0.5	68.66±5.56	6.0±0.0	10-14
5	2,4-D0.5/NAA0.2	63.0±9.0	5.66±1.0	12-14
6	2,4-D0.5/NAA0.4	86.33±9.53	8.0±0.0	14-20
7	2,4-D0.75/NAA1.0	48.66±7.21	3.66±1.0	15-21
8	2,4-D1.0/NAA0.4	62.33±4.35	4.0±1.73	15-18
9	2,4-D0.25/NAA0.75	22.0±3.46	1.66±1.0	13-17
10	2,4-D1.0/NAA0.5	11.66±5.0	1.3±1.0	10-15

CONCLUSION

Majority of plants comprising medicinally active ingredients are in the heavy demand by the pharmaceuticals producing pharmacies for various preparations toward their utilization in human health at present are being harvested directly from their natural habitats. Our present investigation is an attempt toward micropropagation steps for *Picrorhiza kurroa* Royle ex Benth propagation reliable method for the rapid multiplication along with its conservation in Himalayan environment. The callus induced from leaf explants could be further utilized to develop cell suspension cultures to meet out the demand of active ingredient in précised conditions and also in creating somaclonal variations for selecting desired germplasm and transformation studies as well if required.

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