Available online at www.scholarsresearchlibrary.com

Scholars Research Library

Scholars Research Library

Annals of Biological Research, 2015, 6 (5):7-14
(http://scholarsresearchlibrary.com/archive.html)

Annals of Biological Research
Scholars Research Library
ISSN 0976-1233
CODEN (USA): ABRNBW

In vitro regeneration of *Picrorhiza kurroa* Royal ex benth for *ex situ* conservation and sustainable utilization

D. Shephrou Helena[1], Geetanjali Kannojia[1] and A. K. Gaur

Bioprospecting and Metabolomics Lab, Department of Molecular Biology and Genetic Engineering, College of Basic Sciences and Humanities, G. B. Pant University of Agriculture and Technology,Pantnagar,(263145) Uttarakhand, India

---

ABSTRACT

Picrorhiza kurroa Royal ex Benth, a Scrophulariaceae member, is an important and highly valued medicinal herb endemic to alpine and sub alpine belts of the Indian Himalayas. Several monoterpenoid alkaloids are present in its tuberous roots which are known as picroside I, picroside II, picroside III ,cathpol and anthocynin. The plant has been overexploited for its valuable iridoid glycosides,and due to this environmental effect the plant is now considered in endangered category. The depleting status of the herb in the wild needs implementation of conservation and sustainable utilization practices. It is essential to apply biotechnological approaches to sustain the production of picrosides ex situ using in vitro regeneration to ensure its proper utilization and constant supply of useful active ingredients for nutraceutical and pharmaceutical applications. In the present research work, an attempt has been made to propagate the plant through induction of callus from leaf and shoot explants followed by regeneration. Different combinations of phytohormones based on usual practices of plant tissue culture were examined for callus induction and subsequently regeneration. Maximum callus induction was observed in MS supplemented with 0.5 mg L^{-1} TDZ + 0.3 mg L^{-1} IBA and 0.5 mg L^{-1} TDZ + 0.5 mg L^{-1} IBA in leaf and stem explants respectively. Callus derived from leaf and stem was transferred in Shoot induction medium where maximum shoot frequency was observed in 1.0 mg L^{-1} BA + 0.75 mg L^{-1} KN in leaf derived callus and 1.0 mg L^{-1} BA + 1.0 mg L^{-1} Kinetin in stem derived callus respectively. Shoots thus obtained were transferred for induction of roots.

Keywords: *ex situ* Conservation, Himalayas, Iridoid glycosides, picroside, propagation

---

INTRODUCTION

Picrorhiza kurroa Royle ex Benth belongs to Scrophulariaceae family is a medicinal herb, mainly found in the North-Western Himalayan regions of India at altitude of 3,000 to 4,500 m **[6].**It is commonly known as kutki or karu. *P. kurroa* is a rich source of hepatoprotective picrosides; picroside-i and picroside-ii and other metabolites like picroside-iii, picroside-iv, apocynin, androsin, catechol, kutkoside, etc **[15]** . In Ayurvedic medicine, the plant is used as valuable bitter tonic for liver diseases. Rhizomes and roots of this plant are widely used for the treatment of a range of liver diseases **[1,2,4]**. The medicinal importance of *P. kurroa* is due to its various pharmacological properties like antioxidant (particularly in liver) **[3]**, hepatoprotective **[5]** antiallergic and antiasthamatic **[7]**, anticancerous activity particularly in liver **[9]** and immunomodulatory activity **[8]**. Increasing demands of *Picrorhiza kurroa* Royle ex Benth metabolites in global market poses a serious threat to its natural habitat as well as natural reserves. In the International Year of Mountains (2002), *Picrorhiza kurroa* was listed as an 'endangered' herb due to reckless collection from its natural habitat. It is therefore important to protect the species through *in vitro* techniques.

In recent years, tissue culture has emerged as a promising technique to obtain genetically pure elite populations under *in vitro* conditions. *In vitro* propagation is a viable alternative for species which are difficult to regenerate by

conventional methods; where populations have decreased due to over exploitation by destructive harvesting and can effectively to meet the growing demand for clonally uniform elite plants and in conservation of germplasm of large number of threatened plant **[10]**. In the present study, tissue culture has been used to propagate and multiply this species through i*n vitro* regeneration protocol.

MATERIALS AND METHODS

Collection of plant material and establishment of axenic culture

The plants of Picrorhiza kurroa Royle ex Benth were collected from Garhwal region of Uttarakhand in the month of July from farmer's field. These plants were established in controlled environment containment facility (25^0C, 65.0% RH) at Pantnagar.

Sterlization and inoculation of explant

The explants (leaves and stems) were surface sterilized. Explants were cut into pieces of suitable size (2-3cm), and transferred to MS medium in culture vessels (jam bottles).

Callus Induction

The sterilized explants (leaves and stems) were inoculated in MS medium supplemented with growth regulators in various combinations *viz.* TDZ, 2,4-D, IAA, IBA, NAA and TDZ+NAA, TDZ+IBA, TDZ+IAA, 2,4-D+NAA, 2,4-D+IBA, 2,4-D+IAA. The explants for callus induction were incubated in photoperiod of 16/8 hours with 27 – 33.75 µ mol sec^{-1} light intensity. After three weeks of culture, calluses proliferated from the explants and were sub cultured to the new medium at 3-weeks interval.

Shoot Proliferation

Shoot induction and proliferation was carried out upon MS medium comprising cytokinin(s) in different concentrations/combinations (0.0 mg L^{-1} – 1.0 mg L^{-1}). Three calluses were used per culture vessel in triplicate.

Rooting of Micro shoots

Root induction was attempted using micro shoots (1-4 cm length) which were transferred to MS basal medium supplemented with different auxin (s) in different combinations/concentrations (0.4 mg L^{-1} – 1.0 mg L^{-1})

Observation

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

$$\text{Shoot induction frequency} = \frac{\text{No. of callus producing shoots}}{\text{Total No. of callus on shoot induction media}} \times 100$$

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

Culture Conditions

All cultures were incubated under 16 hr photoperiod with light intensity of 27 – 33.75 µ mol sec^{-1} and temperature of 25± 2°C.

Sub culturing of regenerated Plants

During the course of present investigation, the fully differentiated plants were subculture into fresh medium supplemented with kinetin 1.5 mg L^{-1},IAA 1.00 mg L^{-1},GA_3 0.5 mg L^{-1}.

Acclimatization of rooted microplantlet

The well rooted microplantlets transferred in autoclaved vermiculite and sand 1:1 ratio and were placed in greenhouse (Biotech product testing facility).

Statistical Analysis

All the experiments were performed in replicates. Three replicates each in callus induction, shoot proliferation and root induction for each combination of hormone treatments were analyzed respectively. Mean values of various treatments were subjected to one way Analysis of Variance (ANNOVA). Experiments were performed in replicates.

RESULTS AND DISCUSSION

Increasing demands of *Picrorhiza kurroa* Royle ex Benth metabolites in global market poses a serious threat to its natural habitat as well as natural reserves. It is also a native of very high altitude (3000 - 4500 m) region; it is therefore important to protect the species through *in vitro* techniques, Since nursery at *ex situ* conditions at Pantnagar cannot be maintained, therefore *in vitro* propagated plantlets were used for developing the protocols for further caulogenesis and rhizogenesis.

In the present study an attempt has been made to initiate and to establish *in vitro* regeneration protocol of *Picrorhiza kurroa* Royal ex Benth for their maintenance and generating sufficient plant material through indirect regeneration. In the optimization of regeneration of plant, first callus induction was attempted with different sterilized explants (leaves and shoots) by supplementing different concentration of auxins and cytokinins in MS-medium along with 3% sucrose and 0.7% agar.

The explants (leaves and shoots) were cultured on MS medium supplemented with different combinations and concentrations of auxin(s) (2, 4-D, IAA, IBA, NAA) and an analogue of cytokinin(s) (TDZ) to initiate callus induction. Profuse growth of callus occurred within three weeks of culture in which MS supplemented with 0.5 mg L^{-1} TDZ + 0.5 mg L^{-1} IBA and 0.5 mg L^{-1} TDZ + 0.3 mg L^{-1} IBA were highly conducive for leaf explants, (Fig 1, Fig 2) and in case of shoot explants the best callusing was observed at 0.5 mg L^{-1} TDZ + 0.5 mg L^{-1} IBA, (Fig3, Fig 4). The explants with callus induction frequency of 100%. The callus obtained from leaf explants was large in size as compared to shoot. While **[12]** reported that IBA (0.5 mg L^{-1}) + 2,4-D (2.0 mg L^{-1}) was most effective for callus induction (56.3%) in *P. kurroa* in case of leaf derived callus and 38.3% in case of stem explants. The reason can be inherent quantity of growth regulators which can selectively influence the genes to trigger differentiation of cells in culture **[13,14].** The growth of callus from explant(s) was sufficient within 25 days. However the culture for long period resulted in release of phenolic compounds in the medium causing necrosis. Frequent subculturing at 2-week intervals could minimize the accumulation of phenolic compounds and increased the rate of proliferation. Addition of anti-phenolic substances, such as ascorbic acid (0.5 mg L^{-1}) and different concentrations of PVP (Polyvinylpyrollidone), 0.5, 1.0, 1.5, 2.0 mg L^{-1} were tried to overcome this problem. 0.5 mg L^{-1} PVP was found to be most suitable for control of phenolic compounds.

In all combinations of MS with TDZ (0.5 to 1.0 mg L^{-1}) + NAA or IAA or IBA (0.3 to 1.0 mg L^{-1}) callus induction took place with callus induction percentage ranging from 40% - 100%. Callus formed was green with average to good growth (Fig 2and Fig 4) and average mass ranged from 1.234 g to 7.115 g in leaf derived callus and 0.911 g to 5.833 g in shoot derived callus (table 1).

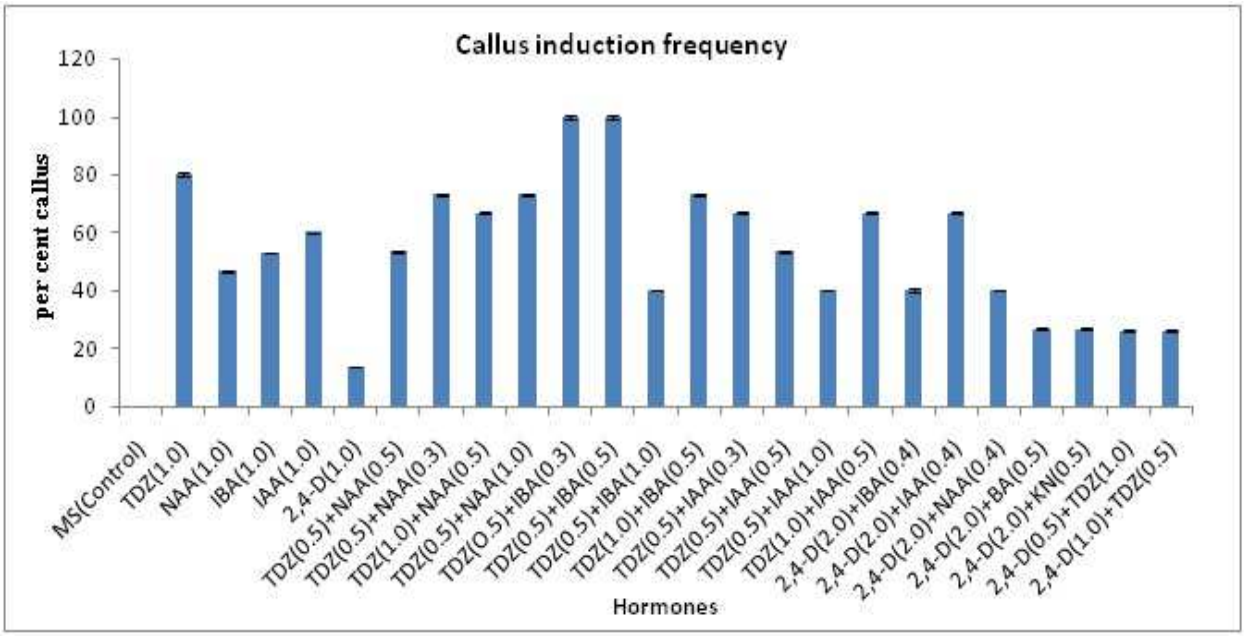

Figure 1 Leaf Callus Induction

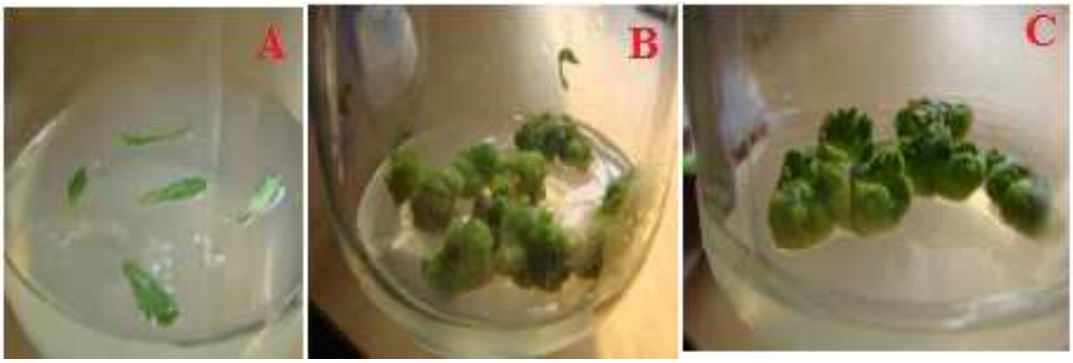

Figure 2: Callus induction from leaf explants of *Picrorhiza kurroa* Royal ex Benth. in MS medium supplemented with 0.5 TDZ mg L-1 + 0.3 IBA mg L-1 (a) Leaf explants (b) After 2 weeks of culture (c) After 3 weeks of culture

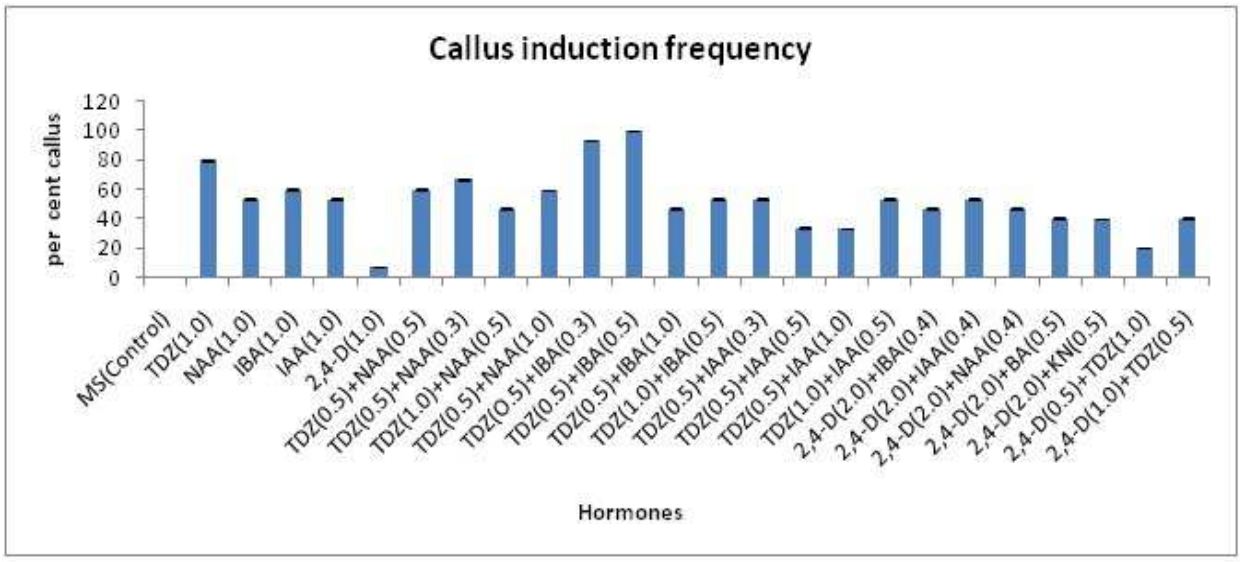

Figure 3: Shoot Callus induction

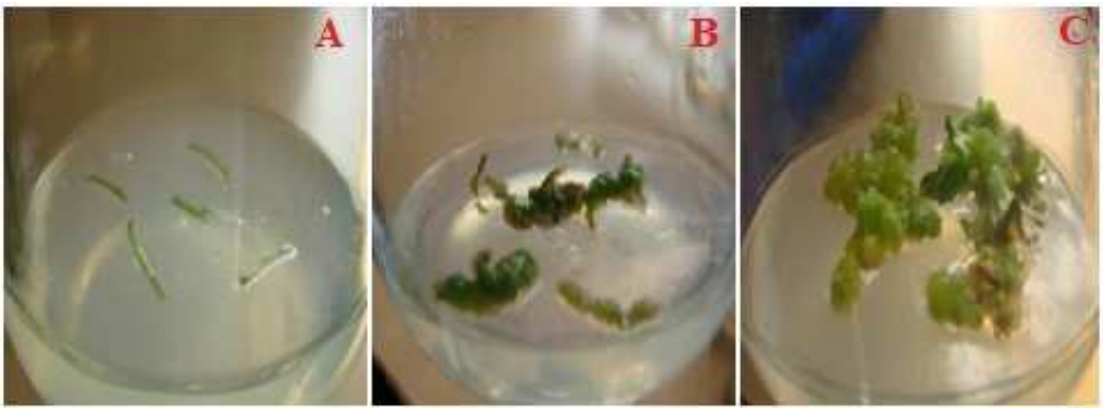

Figure 4: Callus induction from shoot explants of Picrorhiza kurroa royle ex benth. in MS medium supplemented with 0.5 TDZ mg L-1 + 0.3 IBA mg L-1 (a) shoot explants (b) After 2 weeks of culture (c) After 3 weeks of culture

Shoot proliferation

In this study regular sub culturing of callus at three week intervals prevent cessation of growth, generally caused by phenolic exudates, and promoted proliferation causing necrosis. Also, these variations in the response of *P. kurroa* could be due to ecotypic differences **[11]**. Calli were transferred to shoot induction medium comprising of MS medium + 3% sucrose and BA (0.0 mg L^{-1} – 1.0 mg L^{-1}), 0.5% PVP were employed for control of phenolics in shoot bud induction as well as shoot proliferation medium also.

Shoot buds were subcultured at every three weeks. The maximum shoot proliferation and multiplication (39) was observed on MS medium supplemented with 1.0 mg L^{-1} BA + 0.75 mg L^{-1} KN **(Fig 5)** with 88.88 % response in

shoots derived from leaf callus **(Fig 6)** and 1.0 mg L^{-1} BA + 1.0 mg L^{-1} KN **(Fig7)** with 88.88% response in shoots derived from shoot callus with a maximum of 37 shoots **(Fig 8)**, within 3 weeks of culture under 16h photoperiod. However, at higher concentrations of BA or kinetin, the rate of shoot proliferation declined per explants. Within 4 weeks of culture, the shoots derived from leaf and shoot callus proliferated and elongated to 1.5-5.5 cm **(Fig 6)** and 1.1-4.65cm **(Fig 8)** respectively in three to four weeks.

In some cases, growth was inhibited and only 8-9 shoots could elongate; some produced compact callus at the base of the explants **(Fig 8 c)**. Prolonged culture on the proliferation and multiplication media resulted in the blackening of the basal ends of the developing shoots.

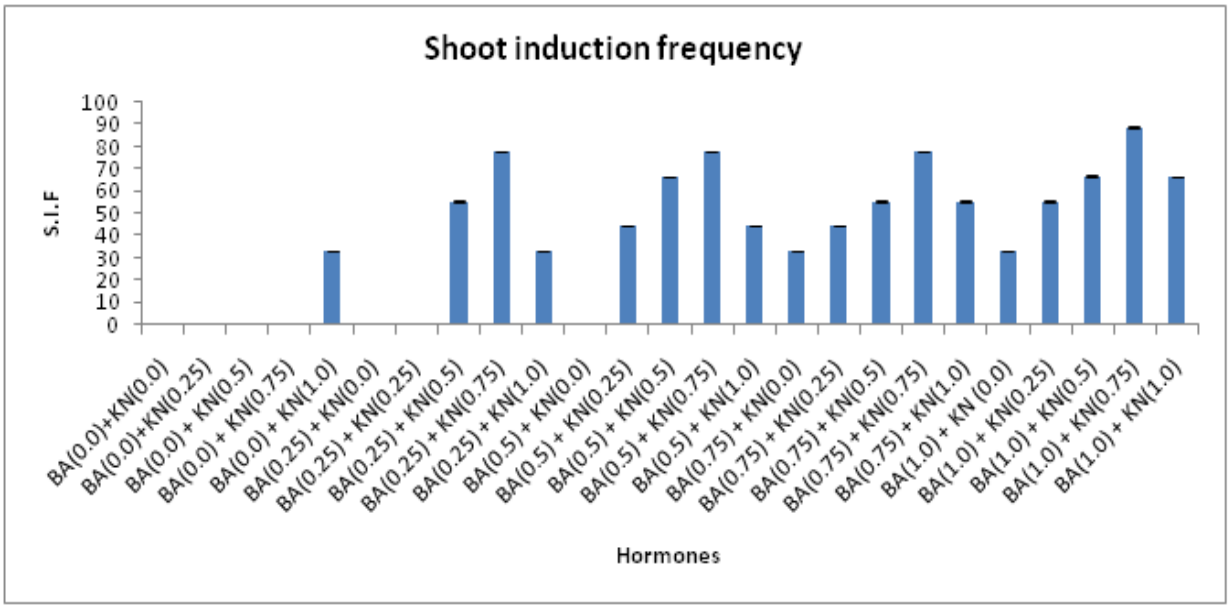

Figure 5: Shoot induction frequency from leaf explants

Figure 6: Multiple shoot induction from leaf derived callus explant in MS medium supplemented with 1.00 BA mg L-1 + 0.75 KN mg L-1 (a) After 3weeks of culture (b) After 4 weeks of culture (c) After 6 weeks of culture

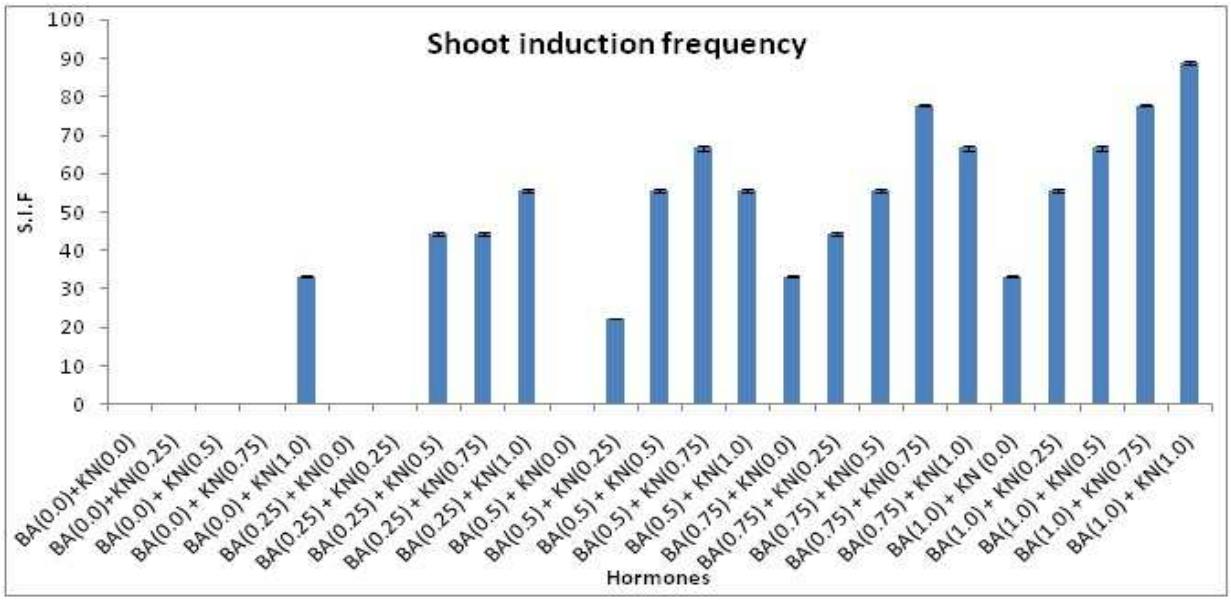

Fig.7-Shoot Induction Frequency from shoot explant

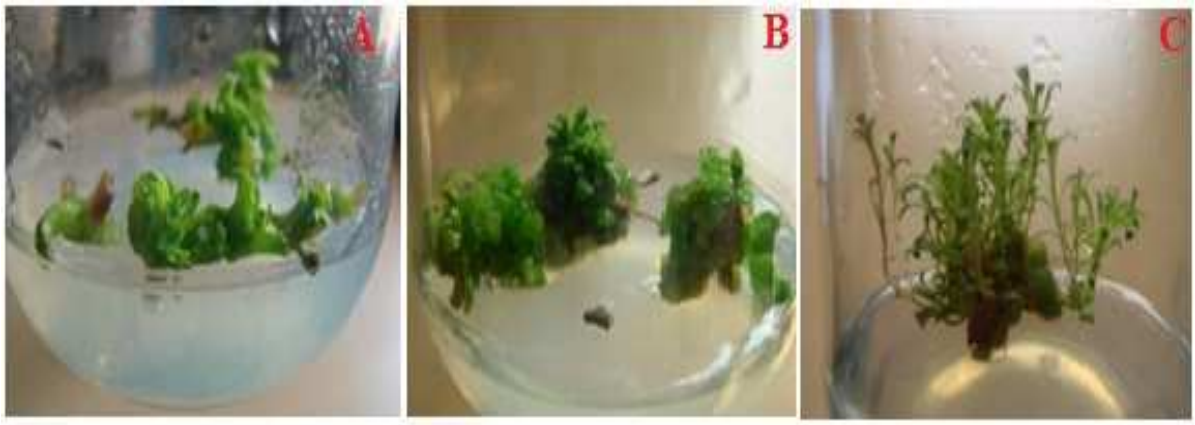

Figure 8: Multiple shoot induction from shoot derived callus explant in MS medium supplemented with 1.0 mg L-1 BA + 1.0 KN mg L-1(a) After 2 weeks of culture (b) After 3 weeks of culture (c) After 6 weeks of culture

Rooting of Micro shoots

Elongated shoots (2-5 cm long) were rooted on ½ strength MS basal medium supplemented with various concentrations of 2,4-D (0.5, 1.0 mg L^{-1}), IAA, IBA (0.4, 0.5, 0.1 mg L^{-1}) and NAA (0.3, 0.4, 0.5, 1.0 mg L^{-1}). Root initiation took place within 12-13 days. Profuse rooting occurred on medium containing NAA (0.4 mg L^{-1}) and 2-4,D (0.5 mg L^{-1})+NAA (0.4 mg L^{-1}) with rooting percentage of 88.88% with a maximum of 19 roots and 100% respectively, in roots derived from leaf explants **(Fig 9)**. 100% rooting was observed in roots derived from shoot explants on medium containing 2-4,D (0.5 mg L^{-1}) + NAA (0.4 mg L^{-1}) with maximum 16 roots **(Fig 10)**. The rooting ability was reduced with the increase in concentration of 2, 4-D, NAA, IAA or IBA in the culture medium. The percentage of shoots forming roots and days to rooting significantly varied with different concentrations of 2,4-D, NAA, IAA or IBA. Complete plantlets (5-7 cm) were recovered after 6-8 weeks on rooting media **(Fig 10C).**

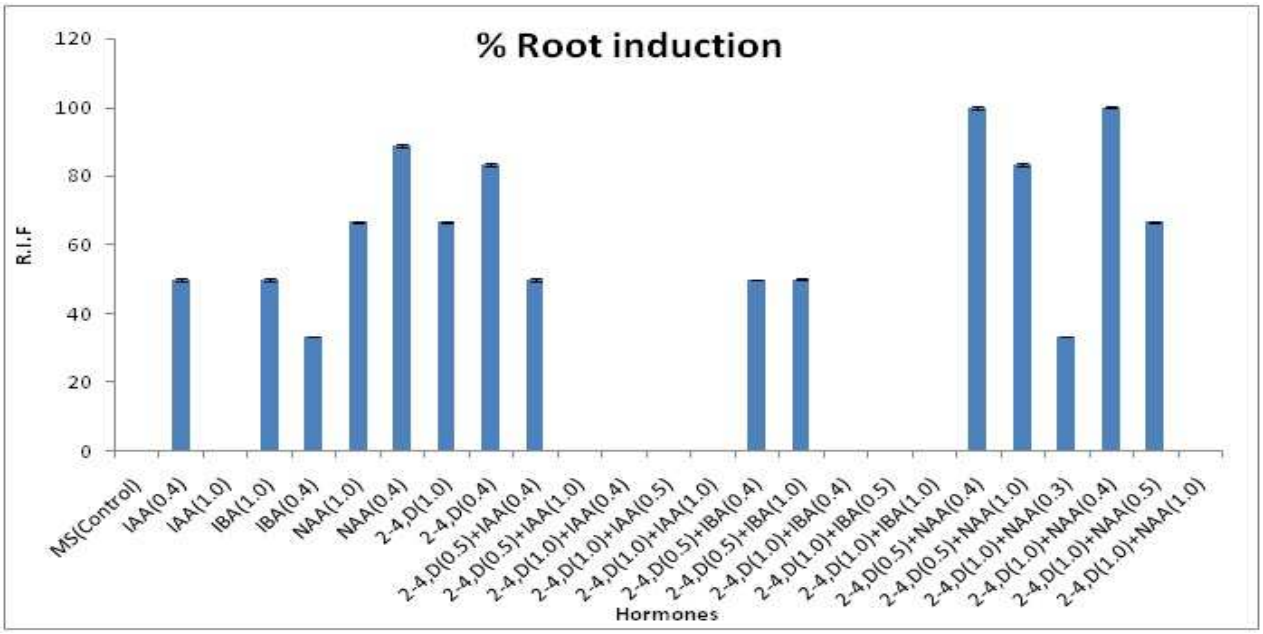

Figure 9: Root induction frequency from leaf explants

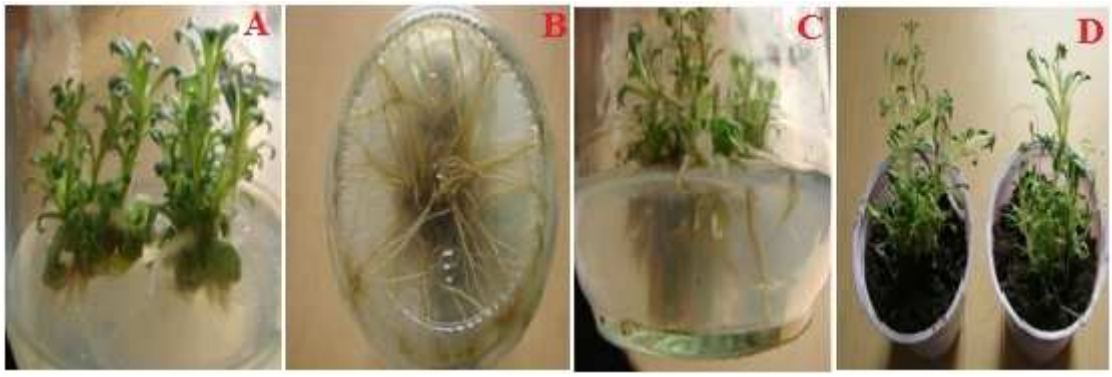

Figure 10:Rooting of adventitious roots from leaf explants in MS medium supplemented with 0.4 mg L-1 NAA (a) Root initiation (b) Root proliferation (after 20 days) (c) Root proliferation (after 30 days) (d)Complete microplantlet and Acclimatization of plant material in plastic cups

Acclimatization of plantlets

After 30 days of culture of micro shoots on rooting medium, which resulted in the sufficient rooting of shoots, the plantlets were transplanted to plastic cups containing autoclaved vermiculite and sand(1:1) ratio. These plastic cups were then kept under polyhouse having temperature 25^0C and 65.0% relative humidity **(Fig 10(D))**.

CONCLUSION

Picrorhiza kurroa Royal ex Benth, a well-known traditional herb of the *Scrophulariaceae* family is a perennial herb, growing primarily in the north-west Himalayan Mountains. Endemic to alpine Himalayas, *P. kurroa* grows wild at altitudes ranging from 3000- 5000 meters. Indiscriminate exploitation, coupled with lack of attention to the development of cultivation practices has resulted in considerable depletion of the wild stocks of this medicinal herb. In fact *P. kurroa* is now listed as one of the endangered plant species of India. Plant tissue culture offers the possibility of rapid clonal propagation and immediate conservation of invaluable germplasm. In the present study, an effort has been made to establish *P. kurroa* under *in vitro* conditions of tarai region at Pantnagar (Uttarakhand).

REFERENCES

[1] R. Anandan, M. Prabhakaran, T. Devaki, *Fitoterapia*, **1999a**, 70, 548–551.
[2] N. Anadan, R. D. Rekha, N. Saravanan, T. Devaki T.. *Fitoterapia*, **1999b**,70, 498– 501.
[3] R. A Ansari,., B.S. Aswal, and R. Chander, *Indian J. Med. Res.*, **1988**, 87, 401-404.

[4] R.A. Ansari, S.C. Tripathi, G.K. Patnaik, B.N. Dhawan, *J Ethnopharmcol*,**1991**, 34,61–68.
[5] R.Chander, N.K. Kapoor, and B.N. Dhawan, *Biochem. Pharmacol.*, **1992**,44, 180-183.
[6] N. Chettri, E.Sharma, S.D. Lama, Lyonia, **2005**, 8,89–101.
[7] W.Dorch, H. Stuppner, and H. Wagner, *Int. Arch. Allergy Appl Immunol.*, **1991**, 95,128-133.
[8] A. Gupta, A. Khajuria, J.Singh, K.A.Suri, and G.N. Quazi, *Int. Immunopharmacol*, **2006**, 6,1543-1549.
[9] K.L. Joy, N.V. Rajeshkumar, and R. Kuttan, *J. Ethanopharmacol*, **2000**,71,261-266.
[10] A. Marco, and M. D. Lledo, *In Vitro* Cell. Dev. *Biol. Plant*, **1996**,32, 42–46.
[11] S.S Mohapatra, and P.M. Greshoff, *Plant Cell Reportsm* **1982**,1, 189-192.
[12] H. Sood, and R. S. Chauhan, *African Journal of Biotechnology*, **2009**,8,1965-1972.
[13] T. A. Thorp, *Baltsville Sym. Agr. Res.*, **1983**, **7,** 285-303.
[14] T. A. Thorp, and B. Stefania,. *Adv. Cell Culture*, **1981**,1, 213-239.
[15] K. Weinges, P. Kloss, W.D. Henkels, *Justus Liebigs Ann Chem*, **1972**, 759,173-182.