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Full Length Research Paper

Propagation of *Picrorhiza kurroa* Royle ex Benth: An important medicinal plant of Western Himalaya

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This study is aimed at developing propagation methods and *ex situ* conservation for *Picrorhiza kurroa,* an endangered medicinal plant of western Himalaya. Regeneration using leaves from mature plant of characterized germplasm is beneficial because the source plant is not damaged. A regeneration protocol was standardized by using leaves from aseptic shoot cultures, raised from *ex vitro* leaves. Maximum regeneration percent (94.33) and significantly higher shoot number (38.0) was evident in middle portion of leaf at 2.32 µM of kinetin (Kn). Abaxial surface that was in touch with the medium was more responsive as compared to adaxial surface. The time of exposure to thidiazuron (TDZ) was emphasized as 15 days interval, gave the best response in terms of shoot number (42.0). For shoot multiplication, Kn at 2.32 µM was optimum. Microshoots with well developed root system were obtained in MS basal medium after 4 weeks. Incubation of cultures at low temperature (15°C) for ten days enhanced the survival percent under green house conditions and could be correlated with the development of thick cuticle and well differentiated leaf tissues (palisade and spongy parenchyma). Flow cytometric analysis was performed to check the genetic stability of *in vitro* plantlets. In a parallel study, seed progenies of these germplasm were raised under *ex situ* conditions. Its reproductive cycle was also studied for successful domestication.

Key words: Regeneration, seed germination, flow cytometric analysis, *ex situ* conservation, reproductive cycle

INTRODUCTION

The western Himalaya is a rich source of plant bio-resources comprising of a large number of economically important species that are used in pharmaceutical as well as alternative system of medicines. Many of these plant species have become rare and are threatened with extinction due to over exploitation. *Picrorhiza kurroa* (family Scrophulariaceae) is a representative endemic, medicinal herb, widely distributed throughout the higher altitudes of alpine Himalayas from west to east, between 3000 to 4500 m above mean sea level (amsl).

P. kurroa is the principle source of the glycosides, picroside I and II, and kutkoside which are extracted from dried rhizomes and roots of 3 to 4 year old plants. Being application in the treatment of anemia, and is also used a hepato-protective and diuretic, *P. kurroa* finds wide as antiperiodic, stomachic, cathartic and cholagogues (Husain, 1984). Although *P. kurroa* propagates mainly through roots or suckers in nature, indiscriminate collection of its underground parts for extraction of active constituents has led to considerable depletion of natural populations. Its propagation through seeds is also poor (Chandra et al., 2006). Moreover, there is an acute lack of attention towards its cultivation practices. As a result, the plant is now listed as an endangered plant species. Therefore, it is extremely important to explore the different methods of propagation and conservation of *P. kurroa.*

Micropropagation of selected high-yielding lines has the potential to cater to the demands of the pharmaceutical industry. On the other hand, understanding the barriers of seed germination and reproductive behavior of *P. kurroa* can enable its domestication as well as germplasm conservation under *ex situ* conditions. Despite the reports

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of Lal et al. (1988), Upadhyay et al. (1988), Chandra et al. (2004, 2006), Sood and Chauhan (2009a, b) and Jan et al. (2010), a high degree of variation in the regeneration response of different accessions of high altitude plants makes it impossible to employ a single standardized method for all accessions.

The present study was undertaken with the objectives of testing the feasibility of *in vitro* propagation of selected and chemically characterized germplasm, and raising seed progenies under *ex situ* conditions. Leaf explants from mature plant is a beneficial proposition as the mother plant is not damaged. Seeds maximize the genetic diversity of local ecotypes (Fay and Muir, 1990), and have the ability to adapt and adjust genetically to changing and uncertain environments. Moreover, they are useful for generating ample stocks for evaluation, rapid plant restoration and utilization. The reproductive cycle of *P. kurroa* was also studied as the findings were expected to enable successful domestication and *ex situ* conservation.

MATERIALS AND METHODS

Plant material

Plants of *P. kurroa* were collected from its natural habitat (Rohtang pass; 4000 m amsl, 32°23′ N, 77°15′ E, India) and maintained in polyhouse in the experimental farm of CSIR-Institute of Himalayan Bioresource Technology, Palampur, HP, India (1300 m amsl; 32°06′ N, 76°33′ E).

Regeneration from *ex vitro* leaves

Leaves from selected plants, maintained under polyhouse conditions were used as explant for raising aseptic shoot cultures. Leaves were washed thoroughly with running tap water, surface disinfected with streptomycin sulfate (0.02%: w/v) and Bavistin (0.02%: w/v) and rinsed with distilled water. Surface sterilization was done with mercuric chloride (0.02%: w/v) followed by 70% (v/v) ethanol in laminar air flow cabinet. Then the explants were rinsed with autoclaved distilled water for 5 to 6 times and inoculated on Murashige and Skoog (1962) medium (MS) containing 3% sucrose and 0.8% agar for initial screening. The pH of the medium was adjusted to 5.7 with 0.1 N HCl or 0.1 N KOH prior to the inclusion of agar and autoclaved for 20 min at 15 psi pressure and 121°C.

Leaf segments were inoculated on medium containing thidiazuron (TDZ) (0.5 µM) for 15 days with their abaxial and adaxial side touching the medium and later transferred to kinetin (3.48 µM) for shoot proliferation. These plant growth regulators (PGRs) were added to the medium before autoclaving. The PGRs used were based upon protocol already standardized in our laboratory. All the cultures were kept at 25 ± 2°C under a 16/8 h (light/dark) photoperiod at photosynthetic photon flux density (PPFD) of 70 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent tubes (Phillips Trulite).

Regeneration from *in vitro* leaves

Effect of plant growth regulators

Fully expanded leaves (1.0 to 2.5 cm) were used for regeneration studies. Different PGRs used were: 2,4-dichlorophenoxy acetic acid 2,4-D (4.53, 9.06 and 11.32 µM) and/or indole-3-butyric acid (IBA) (2.45 and 4.90 µM); 1-naphthaleneacetic acid (NAA) (1.34, 2.68,4.02 and 5.37 µM) and/or 6-benzylaminopurine (BAP) (1.11, 2.22, 3.33 and 4.44 µM); 6-furfurylaminopurine (kinetin) (1.16, 2.32, 3.48 and 4.64 µM); and TDZ (0.25, 0.50, 0.75 and 1.00 µM). Sucrose (3%; w/v) and agar (0.8%; w/v) were invariably added to the medium. In all the experiments, leaves were transversely cut into three parts and inoculated on different media in petri plates (90 mm). Each petri plate contained 6 segments from 2 leaves with one as adaxial and other as abaxial side touching the medium. Leaves with callus or small shoot bud primordia initiated in TDZ containing medium were transferred to kinetin at 2.32 µM (showing maximum regeneration response) after 15, 30 and 45 days. Medium without PGRs served as control. Cultures were incubated at same culture conditions as described earlier.

Multiple shoot formation

In vitro regenerated microshoots (2.0 to 2.5 cm) were used in both liquid (static) and agar gelled medium supplemented with Kn and BAP (1.26, 2.32, 3.48 and 4.64 µM) for shoot multiplication experiments. 20 and 100 ml volume per 250 ml of Erlenmeyer flask was used in the case of liquid medium and agar gelled medium, respectively while MS medium devoid of PGRs served as control.

Rooting and hardening

Rooting of microshoots was obtained in MS basal medium without PGRs. Prior to hardening, rooted plantlets were kept at 5 and 15°C for 10 days and then transferred to greenhouse in small jars containing sand and soil in the ratio of 1:1. In another set of experiment, rooted plantlets were transferred directly from culture lab conditions i.e., 25°C. Plants were nourished by spraying Hoagland solution (505.5 mg/L KNO_3, 1180.8 mg/L $CaNO_3$, 493.0 mg/L $MgSO_4.7H_2O$, and 136.1 mg/L KH_2PO_4). Data was recorded for percent survival of plants after 8 weeks of transfer.

Histological studies

For histological studies, leaves at the time of inoculation, swollen leaves [after 15 days of inoculation in TDZ (0.5 µM)], leaves with shoot bud initiated (after 20 days in PGR free medium), and leaves from plantlets incubated at low temperature (15°C) after 10 days were taken. The samples were fixed in FAA (formaldehyde, acetic acid: 50% ethanol) at the ratio of 1:1:18 for three days, dehydrated in *t*-butyl alcohol series, infiltrated and embedded in paraffin wax (m.p. 56 to 58°C). Sections (12 µm thick) were cut using Rotary Microtome (Shandon Finsse ME, Thermo Electron Corp., UK), stained with Safranin-Fast green combination, and mounted in DPX [Distrene, 8 to 10 g (British resin product), 5 ml dibutylpthalate and 35 ml xylene, Ruzin (1999)]. These were photographed under a light microscope (Labophot, Nikon Corp., Japan), using digital camera (Nikon DXM 1200).

Scanning electron microscope studies

Leaf samples with callus and emerging shoot buds at different stages of development were frozen in liquid nitrogen for 2 to 5 min, mounted on aluminum stubs, carbon/gold coated using E-1010 sputter coating unit and observed under Scanning Electron Microscope (S-3400N Hitachi, Japan). The images were captured digitally.

Flow cytometric analysis

Flow cytometric (FCM) analysis was used to evaluate the genetic stability of *in vitro* raised plantlets of *P. kurroa* compared to the mother plant. *Raphanus sativus* (2C DNA content = 1.11 pg) was taken as internal reference standard. Nuclear DNA content was calculated according to the equation given by Dolezel et al. (1989).

$$\text{2C DNA content (pg)} = \frac{[\text{Sample G1 peak mean} \times \text{Standard 2C DNA content (=1.11pg)}]}{\text{Standard G1 Peak mean}}$$

For DNA content measurement, 50 mg young leaves of *in vitro* plants, mother plant of *P. kurroa* and *R. sativus* (internal reference standard) were taken. Nuclear suspensions were prepared by chopping leaves of sample and standard together on ice using sharp blade in 1 ml of Nuclei Isolation Buffer (Arumuganathan and Earle, 1991) in 60 mm petri plates. After chopping, the suspension was filtered through 40 µm filter membrane and centrifuged at 10000 rpm for 30 s. Supernatant was discarded and pellet resuspended in 600 µl Nuclei Isolation Buffer. Fluorescent dye propidium iodide (16 µl, 5 mg ml^{-1} stock) and RNase (4 µl, 0.5 mg ml^{-1} stock) added to the samples and incubated for 20 m at 37°C in dark and analyzed in flow cytometer (BD FACS-Calibur system (Becton Dickinson, Rutherford, New Jersy).

Raising seed progenies under *ex situ* conditions

Effect of medium and sucrose

Seeds collected during the month of June from the plants maintained in Institute Experimental Farm were treated with streptomycin sulphate and bavistin (0.25 mg ml^{-1}) for surface disinfection. These were surface sterilized with 0.01% (w/v) mercuric chloride for 5 min followed by 5 to 6 rinses with sterile water. These seeds were then inoculated on: (1) basal MS medium (MS0); (2) Hoagland medium; and (3) distilled water (DW) containing 0.8% agar and/or 3% sucrose. Maximum percent germination was recorded.

Effect of potting mix and pH

Seeds were sown in sand (potting mix I), sand:soil (1:1, potting mix II) and sand:soil:farm yard manure (1:1:1, potting mix III) at pH 5.6, 5.8 and 8.0 for germination under polyhouse conditions. In one set of experiment, pH of potting mixes was not adjusted. Observations of maximum percent germination and seedling survival were taken.

Effect of temperature pretreatment

The freshly harvested seeds were treated with water heated to 35, 40, 45, 50 and 60°C for 30 and 60 s prior to surface sterilization and inoculation on MS0 containing 3% sucrose. These were incubated at 28 ± 2°C in BOD incubator. Observations on percent germination were recorded after 8 weeks.

Effect of storage

The effect of storage time on seed germination was tested. For this, seeds were stored for 12 months at 4°C and germinated on MS0 supplemented with sucrose under culture lab conditions. Observations on percent germinations were recorded. Five replicates with 5 mg seeds (containing about 60 seeds) were taken for each experiment which was repeated thrice.

Documentation of reproductive phase under polyhouse conditions

Seedlings obtained from all experiments were transplanted to potting mix II in plastic pots (4" dia) and allowed to grow under polyhouse conditions. Complete life cycle of these plants was documented. Data with respect to flower initiation, fertilization/ anthesis, fruit and seed set, and stage of fruit/seed development were recorded.

Statistical analysis

Four replicates for each treatment were taken and experiments were repeated thrice. Experiments were designed employing complete randomized design (CRD). Data was analyzed using analysis of variance (ANOVA) to detect significant difference between means (Sokal and Rohlf, 1987). Means were compared using Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$ with STATISTICA release ver. 7.0 (Statsoft wipro). Data was expressed as mean ± standard error.

RESULTS

Regeneration from *ex vitro* leaves

Callus induction from *ex vitro* leaf explants occurred after 10 to 12 days on MS medium supplemented with TDZ (0.5 µM). When cultures were kept on the same medium, callus proliferation continued with very low frequency of shoot bud initiation. After transferring the calli to medium supplemented with Kn (3.48 µM), shoot bud regeneration (Figure 1A) occurred after 15 days and multiple shoots formed within 2 weeks of transfer. It was observed that the adaxial surface of leaf explant that was in contact with the medium was more responsive (data not shown). Leaves from these multiple shoots (Figure 1B) were used for further regeneration studies.

Regeneration from *in vitro* leaves

Direct shoot formation (Figure 1C) from *in vitro* leaves was observed in medium without PGRs (control), but shoot number was very low. However, in all the concentrations of TDZ and Kn, callus was initiated from cut ends of leaf segments after 7 to 10 and 12 to 15 days, respectively, but the callus formation in Kn was rather scanty. Emergence of shoot bud primordia was early in TDZ (after 18 to 20 days; Figure 1D) as compared to Kn (after 20 to 25 days) and the developmental pattern of shoot primordia varied in both PGRs. These shoot primordia developed further to form shoots (approximately 2.0 to 2.5 cm in length) in Kn containing medium. Shoot regeneration response to different

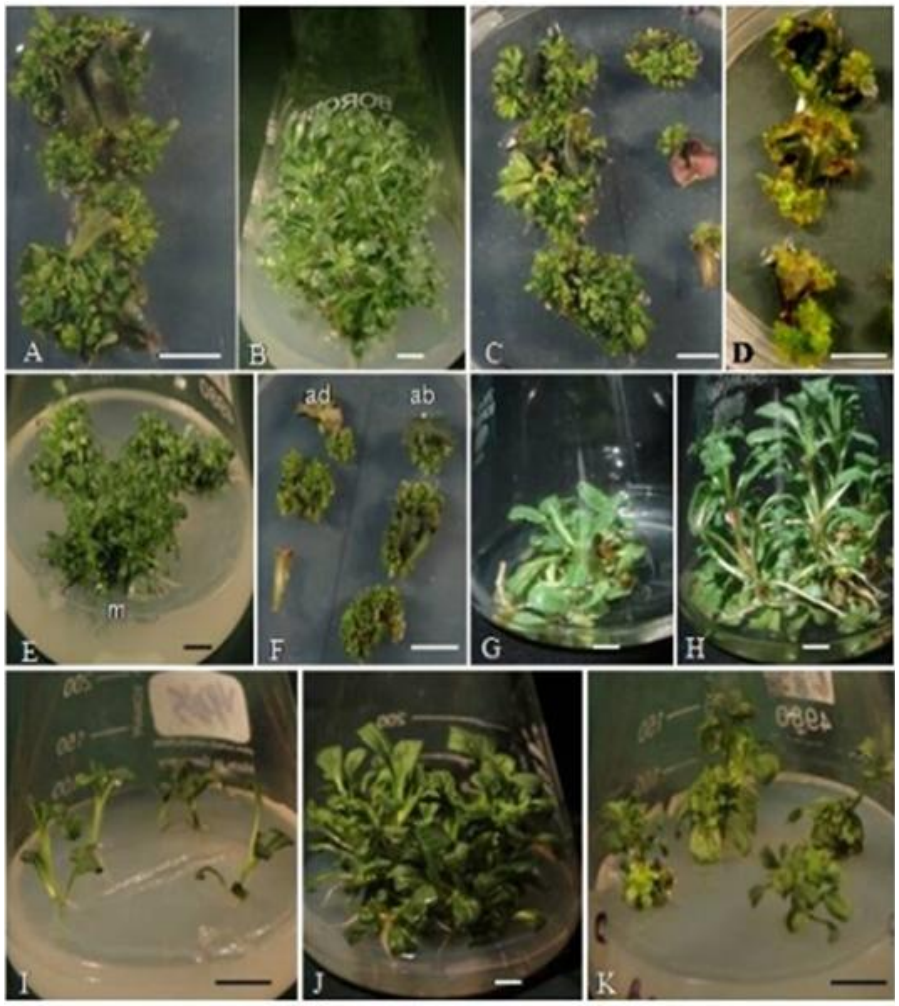

Figure 1. Regeneration and shoot multiplication: (A) Regeneration of shoot buds from *ex vitro* leaves on Kn (3.48 µM) after 15 days; (B) Multiple shoots on same medium; (C) Direct shoot formation from *in vitro* leaves on PGR free medium; (D) Shoot bud primordia from callus on TDZ (0.5 µM); (E) Maximum response from middle portion (m) of leaf; (F) Comparative response of adaxial (ad) and abaxial (ab) surface of leaf in contact with the medium; (G) Shoot culture in liquid medium at day 0; (H) Threefold increase in shoot length and leaf size; (I) Microshoots at day 0; (J) Shoot multiplication in Kn (2.32 µM); (K) Shoot multiplication in BAP (1.16 µM) (Bar line 1 cm).

concentrations of Kn is summarized in Table 1. The regeneration response was affected by concentrations of Kn and the leaf portion. Maximum regeneration percent (94.33) and significantly higher shoot number (38.0) was evident in middle portion of leaf (Figure 1E) at 2.32 µM of TDZ (0.5 µM) to Kn (2.32 µM) at 15 and 30 days interval. Average number of shoots per explant was 42.33 (transfer after 15 days; Table 2). However, the number declined to 19.66 when transferred after 30 days from TDZ containing medium (Table 3). Culturing of leaf explants beyond 30 days on all the concentrations of TDZ resulted in browning of explants. Histological studies of leaves harvested at different time intervals revealed that there was an increase in leaf thickness due to increase in the number of cell layers from 6-7 to 8-9 and cell size from 0.28 to 0.50 µm after 15 days on TDZ containing medium (Figure 2A and B). Small pockets of dividing cells or meristemoids (Figure 2C) were also observed after 15 days of culture leading to callusing or organogenesis. Histological studies of leaf segments growing on PGR free medium (control), showing organogenesis, revealed that shoot buds emerged from the leaf surface at mid rib region that was in contact with the medium (Figure 2D and E). The vascular strand was in continuity with the mother explant indicating thereby direct organogenesis. Direct emergence of shoot buds was also confirmed by scanning electron micrographs (Figure 2F). Callus induction followed by emergence of shoot bud primordia was observed in TDZ and Kn containing medium (Figure 2G and H).

Table 1. Multiple shoot formation on MS + Kn.

Kn (µM)	Response (%)	Average number of shoots/explant						Remarks
		A*			B*			
		Lower	Middle	Upper	Lower	Middle	Upper	
0	72.33	14.33 ± 2.00^{de}	15.33 ± 0.00^{de}	$7.33^{g} \pm 0.00^{h}$	0.00 ± 0.00^{e}	3.00 ± 0.00^{cde}	2.33 ± 2.00^{de}	Direct shoot bud initiation frequency low
1.16	83.33	25.33 ± 1.00^{c}	30.33 ± 0.00^{b}	12.33 ± 0.00^{def}	2.33 ± 2.00^{de}	2.66 ± 1.0^{de}	9.00 ± 0.00^{bc}	Profuse callusing, shoot bud initiation frequency a little higher
2.32	94.33	27.66 ± 1.00^{bc}	38.00 ± 0.00^{a}	12.33 ± 0.00^{c}	7.66 ± 5.00^{bcd}	17.66 ± 1.0^{a}	12.66 ± 1.00^{ab}	Scanty callus, shoot bud frequency higher
3.48	77.66	8.66 ± 2.00^{fg}	16.00 ± 0.00^{de}	11.66 ± 0.00^{ef}	0.00 ± 0.00^{e}	8.66 ± 1.00^{bcd}	3.66 ± 2.00^{cde}	Scanty callus, less shoot bud formation and very slow growth
4.64	67.00	02.00 ± 1.00^{i}	04.00 ± 0.00^{hi}	1.00 ± 1.00^{i}	0.00 ± 0.00^{e}	2.66 ± 1.00^{cde}	1.33 ± 0.00^{e}	Scanty callus, less shoot bud formation and very slow growth

A*: abaxial side of leaf in contact with medium, B*: adaxial side of leaf in contact with medium. Data are recorded after 45 days. Data represents mean of 12 replicates per treatment. Values with different superscript letters are significant at $p \leq 0.05$.

Multiple shoot formation

There was three fold increase in shoot length and leaf size in liquid medium (Figure 1G and H), but there were signs of vitrification after prolonged culturing. Such shoots reverted back to normal growth and shoot multiplication when transferred to agar gelled medium. Shoot multiplication of individual shoots (2.0 to 2.5 cm in length) was evident in all the concentrations of Kn used (Figure 1I and J).

Shoot multiplication rate increased gradually, reached its maximum (3.0) at 2.32 µM Kn and then there was decline (Table 4). Similarly, in BAP containing medium (Figure 1K), shoot multiplication rate was higher (2.0) at 1.16 µM (Table 5). This trend continued up to 45 days of experimentation on both Kn and BAP but higher multiplication was observed in 2.32 µM Kn after 45 days of culture.

Rooting of microshoots

Rooting of microshoots was evident in Kn supplemented as well as PGR free medium after developed on PGR free medium (Figure 3C), however, thin and tender roots developed on Kn containing medium. Presently, callus free rooting was observed on PGR free medium.

Hardening and acclimatization

Rooted plantlets were transferred to green house for hardening. Plants kept at 5°C turned brown after one week. However, those at 15°C showed thickened leaves (Figure 3B) as compared to those kept at 25°C (Figure 3A) due to increase in average cell size from 0.28 µm (Figure 2I) to 0.41 µm (Figure 2J) and number of cell layers from 6-7 to 7-8. The leaves in such plants also showed differentiation of leaf tissue into distinct palisade layer and thick cuticle (Figure 2J). The survival of these plants was higher (80%) under green house conditions (Figure 3D), whereas, those transferred directly from culture laboratory (25°C) showed very less survival percent (50.0).

Flow cytometric analysis (FCM)

In the present study, both direct and indirect 4 weeks of culture. Strong and stout root system organogenesis was observed. FCM analysis of

Table 2. Multiple shoot formation in *P. Kurroa**.

TDZ (µM)	Average number of shoots/explants						Remarks
	A**			B**			
	Lower	Middle	Upper	Lower	Middle	Upper	
0	3.66 ± 0.33^{ijk}	5.66 ± 0.33^{ij}	3.00 ± 0.00^{k}	3.00 ± 0.57^{i}	5.00 ± 0.00^{k}	2.66 ± 0.33^{i}	Direct organogenesis, shoot bud initiation frequency low
0.25	22.66 ± 0.66^{d}	25.66 ± 0.33^{c}	16.66 ± 0.33^{f}	12.66 ± 0.33^{ef}	19.66 ± 0.33^{b}	11.33 ± 0.66^{gh}	Profuse callusing. Shoot bud initiation frequency a little higher
0.50	30.66 ± 0.66^{b}	42.33 ± 0.66^{a}	25.66 ± 0.33^{c}	15.66 ± 0.33^{d}	22.66 ± 0.88^{a}	13.66 ± 0.33^{ef}	Scanty callus, shoot bud frequency significantly higher
0.75	19.00 ± 0.00^{e}	24.66 ± 0.33^{cd}	16.00 ± 0.00^{fg}	12.33 ± 0.33^{fg}	17.33 ± 0.33^{c}	10.3 ± 0.33^{hi}	Scanty callus, less shoot bud formation and very slow growth
1.0	9.66 ± 0.33^{h}	14.00 ± 0.57^{g}	7.66 ± 0.33^{hi}	7.00 ± 0.00^{j}	9.66 ± 0.33^{i}	4.66 ± 0.33^{k}	Scanty callus, less shoot bud formation and very slow growth

*Leaf explants transferred from TDZ (different concentrations) to Kn (2.32 µM) after 15 days. Data recorded after 45 days of transfer. **A: abaxial side of leaf in contact with medium, **B: adaxial side of leaf in contact with medium. Data represents mean of 12 replicates per treatment. Values with different superscript letters are significant at $p \leq 0.05$

source plant and *in vitro* raised plants obtained through regeneration via callusing revealed no significant difference in the 2C DNA (~2.20 pg) contents (Figure 4) indicating thereby that shoots maintained their genetic stability under tissue culture conditions.

Raising seed progenies under *ex situ* conditions

Effect of medium and sucrose

Higher germination of freshly harvested seeds in the presence of sucrose was recorded after 8 weeks on MS0 (94%) followed by Hoagland (72%) and DW (63%) under *in vitro* conditions.

However, the germination was remarkably lower in the absence of sucrose.

Effect of potting mix and pH

Freshly harvested seeds germinated within 15 days of sowing under poly house conditions when pH was not adjusted. Highest germination (47%) was recorded in potting mix II followed by potting mix III (41%) and potting mix I (34%). Irrespective of the potting mixes, germination increased considerably when pH was maintained at 5.6 to 5.8. Highest germination (95%) was recorded in potting mix II, that is, sand soil at the ratio of 1:1.

However, their survival percentage was very poor. At pH 8.0, the germination was only 30 to 35%, irrespective of potting mix.

Effect of temperature pretreatment

Seeds pretreated with water heated to 40 and 45°C for 30 and 60 s showed 98% germination after 8 weeks.

Effect of storage

Based on the earlier observations, MS0 supplemented with sucrose was used for seed

Table 3. Multiple shoot formation in *P. kurroa**.

TDZ (µM)	Average number of shoot primordia/explant						Remarks
	A**			B**			
	Lower	Middle	Upper	Lower	Middle	Upper	
0	2.00 ± 0.00^{j}	3.66 ± 0.33^{i}	1.33 ± 0.66^{j}	2.00 ± 0.00^{h}	3.00 ± 0.00^{g}	0.33 ± 0.33^{i}	Direct organogenesis, shoot bud initiation frequency low
0.25	9.66 ± 0.33^{f}	15.00 ± 0.00^{b}	10.66 ± 0.33^{e}	7.66 ± 0.33^{d}	13.66 ± 0.33^{b}	9.33 ± 0.33^{c}	Profuse callusing, shoot bud initiation frequency a little higher
0.50	11.66 ± 0.33^{d}	19.66 ± 0.33^{a}	13.66 ± 0.33^{c}	9.33 ± 0.33^{c}	17.66 ± 0.33^{a}	8.00 ± 0.00^{d}	Scanty callus, shoot bud frequency significantly higher
0.75	9.66 ± 0.33^{f}	14.66 ± 0.33^{b}	7.00 ± 0.00^{g}	5.66 ± 0.33^{e}	13.00 ± 0.57^{b}	7.33 ± 0.33^{d}	Scanty callus, less shoot bud formation and very slow growth
1.0	5.66 ± 0.33^{h}	9.66 ± 0.33^{f}	6.00 ± 0.00^{h}	3.33 ± 0.33^{fg}	7.66 ± 0.33^{d}	4.00 ± 0.00^{f}	Scanty callus, less shoot bud formation and very slow growth

*Leaf explants transferred from TDZ (different concentrations) to Kn (2.32 µM) after 30 days. Data recorded after 45 days of transfer. **A: abaxial side of leaf in contact with medium,**B: adaxial side of leaf in contact with medium. Data represents mean of 12 replicates per treatment. Values with different superscript letters are significant at $p \leq 0.05$.

germination. The germination of freshly harvested seeds was 100% on MS0 supplemented with sucrose.

However, no seed germination was evident after 9 months of storage.

Documentation of reproductive phase under polyhouse conditions

Healthy young plants were obtained from seedlings after transplantation to soil under polyhouse conditions. The *ex situ* raised plants required a total period of 20 weeks to complete their reproductive phase from flower bud initiation to seed maturity and germination (Figure 5, Table 6).

Flowering was initiated with the onset of spring that is, early February to the end of March. Fully opened, purple coloured bisexual flowers borne on a scape in an indeterminate spike developed during early to mid April. Green capsules developed in mid April and matured during summer (April end to May end). Ready to germinate, mature seeds were available during June end. Seeds germinated within 20 weeks of their development under Palampur conditions.

DISCUSSION

Micropropagation of selected elites, help in mass multiplication of high yielding lines. Present studies were undertaken in chemically characterized planting material of *P. kurroa*, enlisted as endangered plant species. Use of leaf explants from mature plants for multiple shoot formation and their subsequent utilization for regeneration and mass multiplication holds tremendous potential as the source plant is not damaged. This is an important step towards *ex situ* conservation of endangered plant species. More responsive nature of adaxial surface could be correlated to the fact that palisade parenchyma is the last tissue to cease growth and hence more responsive to nutrients as reported earlier by Welander (1988). However, higher response, when abaxial surface was in contact with the medium, in leaves from *in vitro* shoots may be explained as the leaf tissue is not differentiated under tissue culture conditions. George (1993)

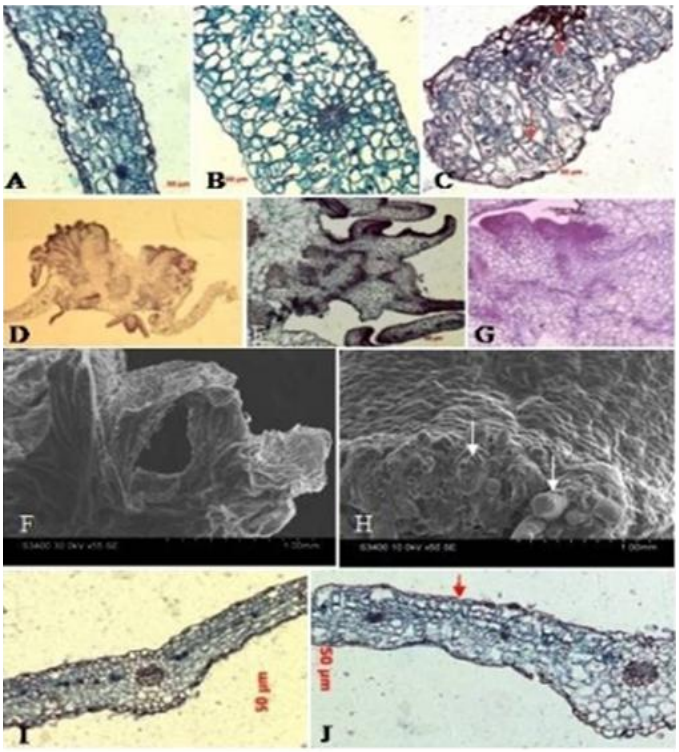

Figure 2. Light and scanning electron microscopic studies: (A) Transverse section of leaf at day 0. (B) On TDZ (0.5 µM) at day 15; Note increase in cell size and number of cell layers. (C) Meristemoids (arrow) in leaf tissue. (D to E) Shoot buds emerging from mid rib in PGR free medium. (F) Direct emergence of shoot buds from leaf. (G, H) Emergence of shoot bud primordia (arrow) from callus at cut ends. Transverse section of leaf from shoot cultures (I) at 25°C (J) at 15°C; Note increase in cell size and number of cell layers with differentiated palisade tissue.

Table 4. Shoot multiplication in MS + Kn.

Concentration (µM)	Number of shoots after days		
	15	30	45
Control	0.44 ± 0.12^{d}	1.00 ± 0.1^{bcd}	1.13 ± 0.16^{bcd}
1.16	0.63 ± 0.22^{bcd}	1.25 ± 0.37^{bcd}	1.88 ± 0.58^{b}
2.32	1.00 ± 0.2^{bcd}	1.94 ± 0.26^{b}	3.00 ± 0.44^{a}
3.48	0.75 ± 0.1^{cd}	1.63 ± 0.39^{bc}	1.88 ± 0.26^{b}

Data represents mean of 12 replicates per treatment. Values with different superscript letters are significant at $p \leq 0.05$

Table 5. Shoot multiplication in MS + BAP.

Concentration (µM)	Number of shoots after days		
	15	30	45
Control	0.13 ± 0.13^{d}	0.38 ± 0.38^{cd}	0.75 ± 0.75^{c}
1.16	0.81 ± 0.28^{bcd}	1.58 ± 0.12^{ab}	2.00 ± 0.76^{a}
2.32	0.310 ± 0.12^{cd}	0.75 ± 0.31^{bcd}	1.31 ± 0.12^{abc}
3.48	0.25 ± 0.13^{cd}	0.81 ± 0.26^{bcd}	0.94 ± 0.34^{bcd}

Data represents mean of 12 replicates per treatment. Values with different superscript letters are significant at $p \leq 0.05$.

Figure 3. Shoot cultures: at (A) 25°C and (B) 15°C. (C) Well developed root system in PGR free medium. (D) Hardened plants under greenhouse conditions (Bar line 1 cm).

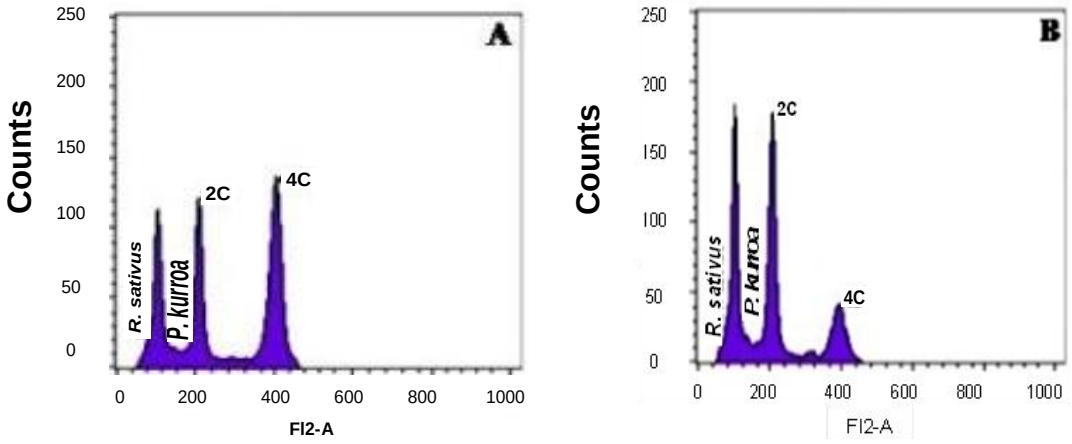

Figure 4. Histogram of relative DNA content of *P. kurroa* and *R. sativus* as internal reference standard (2C = 2.2 pg) . (A) Source plant (B) *In vitro* raised plants.

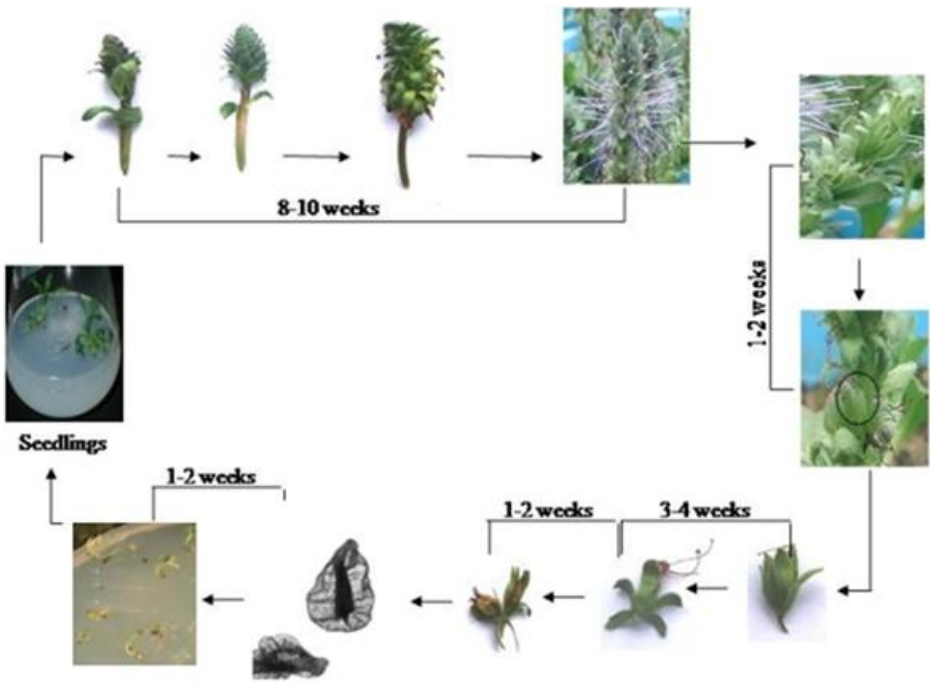

Figure 5. Reproductive phase from flower bud initiation to seed maturity and germination.

Table 6. Calendar for reproductive phase in *P. kurroa.*

Stages	Month	Time period (wk)
Inflorescence with young buds	Early February to March end	6-7 (late winter to early spring)
Inflorescence with fully opened flowers	Early to mid April	2-3 (early summer)
Immature green pods	Mid April	1-2 (summer)
Mature pods	April end to May end	3-4 (summer)
Pod ready to dehisce	Early to mid June	1 -2 (summer)
Mature seeds ready to germinate	Mid to June end	1-2 (onset of rainy season)

Seed germination under culture lab conditions occurs within 8 wks. Days from bud formation to seed set and germination takes approximately 20 weeks.

also indicated the importance of explant orientation during morphogenetic response of *in vitro* cultures. There are many reports in literature where best shoot formation and callusing was observed when abaxial surface of leaf was in contact with the medium (Sujatha et al., 2008; Bhatia et al., 2005; Mazumdar et al., 2010; Gairi and Rashid, 2005). Use of Kn at higher concentrations, that is, 3.48 µM for *ex vitro* leaves and lower concentration, that is, 2.32 µM for *in vitro* leaves, strongly indicates the relationship between PGR requirement and growth conditions of the donor plant. The results also emphasized the importance of exposure time to TDZ for high efficiency shoot proliferation. The reason may be that TDZ remains persistent in the plant tissue as is reported earlier in *Phaseolus lunatus* where both concentration and length of exposure time of TDZ play important role during organogenesis (Mok and Mok, 1985). The adverse effect of continuous use of TDZ on shoot growth and multiplication is also on record in many plant species, that is, *Cicer arietinum* (Murthy et al., 1996) *Rauvolfia tetraphylla* (Faisal et al., 2005) and *Capsicum annuum* (Ahmed et al., 2006). TDZ is known to be a strong inhibitor of cytokinin oxidase and acts by modulating the endogenous hormone level (Mok et al., 1982; Mok et al., 1987; Hutchinson et al., 1996; Murthy et al., 1998). It has been effectively used as the sole growth regulating compound for regeneration in many plant species. It evokes higher regeneration efficiency as compared to other synthetic plant growth regulators (Malik and Saxena, 1992; Gill et al., 1993; Murthy et al., 1996; Murthy and Saxena, 1998; Malik et al., 2010). Earlier, Sood and Chauhan (2009a) used 2,4-D (2 mg L^{-1}) and IBA (0.5 mg L^{-1}) for callus induction in leaf discs of *P. kurroa* and both BA and Kn collectively for regeneration. In the present study, however, leaf segments turned brown with no signs of differentiation when BAP, 2,4-D, NAA and IBA were added to the medium. Direct organogenesis at mid rib region in PGR free medium was observed as revealed through histological and scanning electron microscope studies. Earlier, Leshem et al. (1982) also observed most frequent shoot regeneration on the side of bulb-scale of *Lilium longiflorum* in which the vascular bundles are sited indicating thereby the role of vascular bundles in providing stimulus for cell division. However, on TDZ and Kn containing medium organogenesis occurred via callus phase. None of the earlier reports are supported by histological and scanning electron microscopic studies. FCM analysis of source plant and *in vitro* raised plants obtained through regeneration via callusing revealed no significant difference in the nuclear DNA contents, thereby indicating that shoots maintained their genetic stability under tissue culture conditions which is an advantageous proposition, especially when chemically characterized medicinal plant is under consideration. Liquid medium was better suited for increasing shoot length and leaf size but vitrification occurred after prolonged culturing. In *P. kurroa,* vitrification of shoots in cytokinin supplemented medium has been reported earlier (Upadhyay et al., 1989). Production of vitrified shoots with callus formation at the base of microshoots at high BAP concentrations is on record. However, regeneration of normal shoots from the base of vitrified shoots upon transfer to medium containing lower levels of cytokinins or a combination of cytokinin and auxin was observed (Chandra et al., 2006). Reversal of such vitreous shoots to normal development upon transfer to agar medium occurred as reported earlier in *Petunia* (Zimmerman et al., 1991). Beneficial or adverse effects of different cytokinins at varying concentrations for shoot multiplication in *P. Kurroa* (Lal et al., 1988; Upadhyay et al., 1989; Chandra et al., 2006) could be attributed to genotype specific response of different plant species (Mohapatra and Greshoff, 1982).

Rooting is a prerequisite for any micropropagation protocol. In the present study, use of PGR free medium for rooting of *P. kurroa* microshoots is recommended.

Callus free rooting was observed on PGR free medium whereas, callusing was observed at the base of shoots prior to rooting in 2.5 µM IBA by Chandra et al. (2006). In earlier studies, rooted microshoots were formed with 1.0 µM NAA (Upadhyay et al., 1989), 1.0 to 2.5 µM IBA (Chandra et al. 2006) and 3 mg L^{-1} IBA (Sood and Chauhan, 2009a).

Increased survival of plantlets incubated under low temperature (almost similar temperature prevailing under natural habitat) could be correlated with thickened leaves

with thick cuticle and well differentiated tissues (palisade and spongy parenchyma). At low temperature, these tissues were reported to be rich in hemicellulose which increases the leaf strength and helps the plant to cope with external environment conditions (Atkins et al., 1996; Werf et al., 1994). Higher survival rate of cold acclimated plants was earlier reported in *Cineraria saxifraga* (Burchett et al., 2002) *Rheum emodi* (Malik et al., 2009), and *P. kurroa* (Sood and Chauhan, 2009a). Besides higher survival percentage, incubation of cultures at low temperature and light also helps in upregulation of two regulatory genes of the picrosides biosynthesis pathway through increased carbon partitioning towards terpenoid metabolism resulting in higher picrosides content in *P. kurroa* (Kawoosa et al., 2010).

Seed progenies were raised under *ex situ* conditions and higher germination in the presence of sucrose was recorded. Beneficial effect of sucrose during seed germination is attributed to the fact that it is essential for growth and differentiation. Sucrose also serves as storage compound, maintains osmotic pressure and act as signaling molecule in the regulation of germination and seedling development (Teixeira et al, 2005; Xu et al., 2010). In the absence of sucrose, high germination (39%) in Hoagland indicated the preference of these seeds for high concentrations of macro-elements such as calcium nitrate but low concentrations or absence of potassium and ammonium nitrate. Higher germination (29%) in distilled water without sucrose as compared to MS0 (26%) further indicated that the seeds do not require medium enriched with high salt concentrations. Generally, seeds are rich in mineral reserves and do not require enriched medium for germination.

Highest germination (95%) was recorded in potting mix II, that is, sand and soil (1:1) at pH 5.6 to 5.8. *Picrorhiza* seeds have been reported to prefer specific soil pH and texture for their germination and seedling establishment. Sandy clay textured soil with porous soil layers favors germination and seedling establishment because this facilitates horizontal spreading of its rhizomes and sprouting of new aerial sprouts from the nodal buds (NMPB, 2008). A pH range of 5.0 to 5.7 has been reported to favor seed germination of some high altitude plants as colonization of arbuscular mycorrhizal fungi is facilitated at this pH range (Singh et al., 2008). This improves seedling growth. Preference for particular potting mix can be explained by the fact that soil surface characteristics govern seedling establishment by holding both the soil and seed in place and providing surface stabilization (Chambers et al., 1990).

Germination (98%) in seeds pretreated with water heated to 40 and 45°C for 30 and 60 s can be explained by the fact that, localized and momentary melting of snow due to intense heat of sunrays at certain patches of the soil surface at higher altitude of mountain environment provides the requisite stimulus to alpine seeds for germination (Körner, 2003). Germination was high in freshly harvested seeds. However, the viability of seeds has been reported to reduce to 60% after six months (NMPB, 2008). This duration of seed viability is sufficient for the seeds to tide over the adverse winters and establish in the following favorable season (snow melt). As in case of most alpine plants, *P. kurroa* appears to exhibit the opportunistic behavior in completing its phase of growth and reproduction well within the seasons of favorable temperatures (Körner, 2003). In nature, thus flowering and fruiting occur during June to August but during early February to May end under Palampur conditions. This difference in the time of flowering between the *in situ* and *ex situ* conditions may be attributed to the milder climatic temperatures of Palampur. While the favorable temperature regime is initiated during spring (March end) in the natural habitats of *Picrorhiza*, such conditions prevail much earlier in Palampur.

The seeds are covered by a hyaline reticulate sac which is expected to play the dual functions of seed dispersal through wind and facilitate germination by absorption of moisture. Raina et al. (2010) also stated that the hyaline reticulose sac ensures efficient seed dispersal in high wind zones and avoids overcrowding around the seed-setting parent. Seeds germinated within 20 weeks of their development under Palampur conditions. The availability of longer duration of favorable temperatures at Palampur may have facilitated germination and establishment within a span of 5 months. In nature, however, the capsules are formed by September but the seeds require one year for attaining complete maturity. These finally germinate in September of the following year (NMPB, 2008). Alpine Himalayan seeds prefer to germinate and establish only after the rains because they suffer heavy mortality due to prolonged and intense rains. This explains *P. kurroa*'s particular preference for the month of September for seed germination and establishment in nature.

Our study showed that the seeds of *P. kurroa* possess several features that ensure its adaptability to alpine environments. Therefore, the processes such as reproductive behavior and seed germination under milder climatic conditions of Palampur differed from that of alpine conditions of its natural habitats. The seed structure supporting its anemophilous behavior, higher germination in response to wet heat, avoidance of unfavorable temperatures by maturing and germinating only during favorable conditions; all indicate adaptive features for their survival in harsh alpine climates.

Conclusion

Many of the high value Himalayan medicinal plants are rare, threatened, endangered or even extinct. Therefore, conservation and cultivation of chemically characterized medicinal plants is urgently required to avoid the risk of

overexploitation and destruction of natural sites. In this regard, characterized germplasm of *P. kurroa* were conserved employing *in vitro* methods using leaf explants which ensured sustainable availability and better utilization of high yielding accessions without destroying the source plant. Besides maintaining identified high yielding germplasm, genetic diversity of heterogeneous populations were raised through seeds and domesticated for future use. The reproductive cycle also provided an insight into the behavior of *P. kurroa* under different altitudes.

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