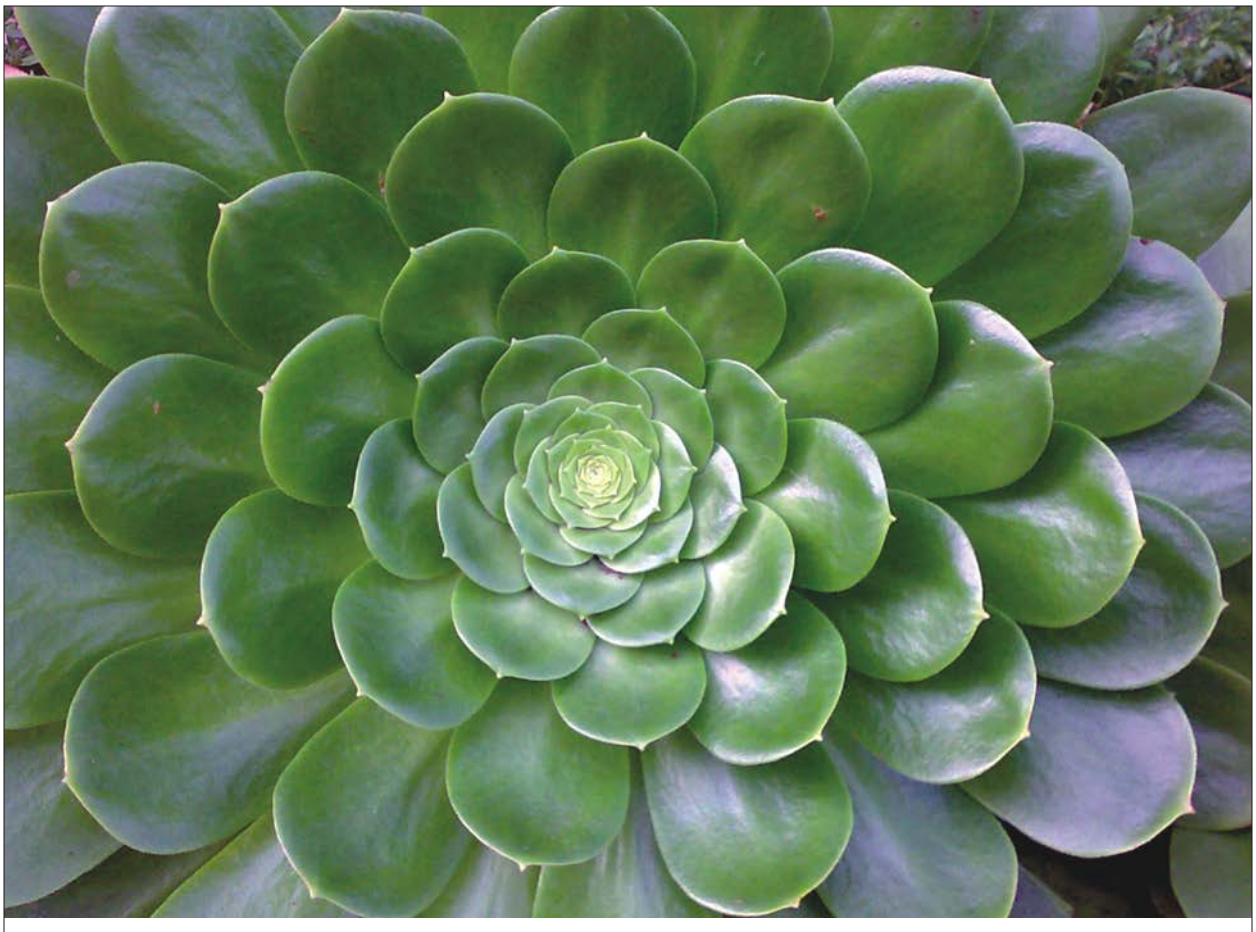

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Development of a Low Cost Micropropagation Technology for an Endangered Medicinal Herb (*Picrorhiza kurroa*) of North-Western Himalayas

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Abstract: The current study was undertaken with the objective that tissue culture conditions need to be optimized for obtaining vigorous shoot growth coupled with modifications in the nutrient medium so as to reduce the cost of nutrient medium. Axillary shoot tips cultured on MS +IBA (2 mg L^{-1}) + KN (3 mg L^{-1}) + sucrose 3% (w/v) + agar-agar 0.8% (w/v) was the best medium for multiple shoot formation with 86.3% shoot apices forming multiple shoots. The sucrose was replaced with table sugar and agar-agar was omitted completely. Out of 6 low-cost media combinations tested, MS liquid medium supplemented with Indole-3-Butyric Acid (IBA) (2 mg L^{-1}) + kinetin (KN) (3 mg L^{-1}) + table sugar 3% (w/v) was found to be the best with 27 shoots/explant. Seventy percent shoots formed roots on half strength MS salts supplemented with IBA (3 mg L^{-1}) + table sugar 3% (w/v) + agar-agar with an average of 5.6 roots per shoot. The study has resulted in the identification of a low-cost medium combination for rapid multiplication of *P. kuura* with a potential that the technology can be up-scaled to a large-scale production.

Key words: Low-cost medium, micropropagation, *Picrorhiza kurroa*

INTRODUCTION

Medicinal plants have been used as an important source of drugs for thousands of years. Even today, the World Health Organization estimates that up to 80% of people still rely mainly on traditional remedies such as herbs for their medication. *Picrorhiza kurroa* Royel ex Benth (Family:Scrophulariaceae) is a perennial herb also known as kutki or karu mainly found in the North-Western Himalayas at altitudes of 3000-4300 m. *Picrorhiza kurroa* is a well-known herb in the Ayurvedic system of medicine and has traditionally been used to treat disorders of the liver and upper respiratory tract, reduce fevers and to treat dyspepsia, chronic diarrhea, scorpion sting, etc. The active constituents are obtained from the dried roots and rhizomes. The pharmacological importance of *P. kurroa* has been demonstrated to be due to rich source of hepatoprotective picrosides, Picroside-I and Picroside-II and other metabolites like Picroside-III, Picroside-IV, Apocynin, Androsin, Catechol, Kutkoside, etc. (Weinges *et al*., 1972; Stuppner and Wanger, 1989). The pharmacological properties of *P. kurroa* have been demonstrated and validated in modern system of medicine like hepatoprotective (Chander *et al*., 1992), antioxidant (particularly in liver) (Ansari *et al*., 1988), antiallergic and antiasthamatic (Dorch *et al*., 1991), anticancerous activity particularly in liver (Joy *et al*., 2000) and immunomodulatory (Gupta *et al*., 2006). A commercial formulation named as Picroliv prepared from *P. kurroa* extracts containing picroside 1 and kutkoside was launched as a hepatoprotective drug after clinical testing (Ansari *et al*., 1991). Picroliv has also been shown to have immunostimulating effect in hamsters and helps to prevent infections (Gupta *et al*., 2006).

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The plant is self-regenerating in nature, however, unregulated over-harvesting of roots and rhizomes has reduced its populations in the natural habitat to a level that it has been categorised as threatened plant species nearing to extinction. The endangered status of *P. kurroa* warrants its rapid multiplication on a large scale and subsequent plantation in its natural habitat. There are fragmentary reports on micropropagation of *P. kurroa* (Lal *et al*., 1988; Upadhay *et al*., 1989; Trivedi and Pandey, 2007; Chandra *et al*., 2006) and induction of hairy roots (Verma *et al*., 2007). However, none of those studies were aimed at developing a low-cost micropropagation technology for *P. kurroa*. Moreover, in previous reports the tissue culture plantlets of *P. kurroa* were very thin and slender (Lal *et al*., 1988) with a problem of vitrification (Upadhay *et al*., 1989), thereby reducing the survival rate of tissue cultured plants in the field conditions.

There are reports on tissue culture of other plant species, wherein the components of tissue culture media have been modified or replaced with low cost substitutes such as sucrose with table sugar (Kaur *et al*., 2005), omission of agar-agar (Mehrotra *et al*., 2007) and use of sunlight and tubular skylight (Kodym *et al*., 2004). The use of shake cultures utilizing liquid culture medium alone (Weathers and Giles, 1988) or in combination with solid culture medium (Debergh and Maene, 1981; Aitken-Christie and Jones, 1987) have also been tried as low-cost alternatives by various workers (Earle and Langhans, 1975; Takayama and Misawa, 1981; Takayama, 1991; Paque *et al*.,1992; Chu *et al*., 1993).

There is no information as of today on optimization of tissue culture conditions for rapid multiplication of *P. kurroa*, wherein the components of nutrient media have been modified or substituted with low-cost alternatives so as to reduce the cost of per liter medium. The current study is first of its kind, which explored the feasibility of reducing the cost of *in vitro* multiplication of *P. kurroa* in such a way that the quality of planting material remains comparable to standard media with the feasibility that the technology can be up-scaled with ease to a commercial scale. The identification of low-cost micropropagation technology is expected to go a long way in providing sufficient planting material of *P. Kurroa* for reclaiming its populations in the natural habitat. The current study reports a low-cost nutrient medium with vigorous shoot growth for large-scale multiplication of *P. kurroa* and for their better survival in the field conditions.

MATERIALS AND METHODS

Selection of Plant Material and Establishment of Axenic Cultures

The *P. kurroa* plants were procured from the Himalayan Forest Research Institute, Panthaghati, Shimla, H.P., India and planted in a pot in a polyhouse at the experimental area of the Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat. All the experiments on different aspects of current study were done during the last years (August, 2006 to January 2009) in the tissue culture laboratory of the Department of Biotechnology and Bioinformatics at the Jaypee University of Information Technology, Waknaghat, Solan, H.P., India. Axillary shoot tips (0.5-1.5 cm long) excised from pot grown plants were washed in 2% (v/v) detergent solution Teepol (Qualigen, India) and surface sterilized for 2-3 min in 0.5% (w/v) Bavistin (BASF, India, Ltd.) and for 30 sec in 0.1% (w/v) Mercuric Chloride (Merck, India) followed by 4-5 washings in sterile distilled water. The sterile shoot apices were cultured on MS medium supplemented with different concentrations and combinations of auxins and cytokinins.

Preparation of Media and Incubation of Cultures

Modifications of MS (Murashige and Skoog, 1962) media supplemented with different concentrations and combinations of IBA (indole-3-butyric acid), KN (kinetin) and BA (6-benzyladenine) were prepared, pH adjusted to 5.7 using 0.1 N HCl and 0.1 N NaOH and solidified

with agar-agar (Glaxo, Bacteriological Mediaid) 0.8% (w/v) as a gelling agent. The media were autoclaved at 121°C and 15 lb in^{-2} pressure for 15-20 min in 150 mL Erlenmeyer flasks (Borosil, India) by dispensing 40 mL molten media in each flask plugged with non-absorbent cotton wrapped in one layer of muslin cloth. The cultures were incubated at 15±1°C in plant tissue culture chamber with 70% relative humidity under 16 h photoperiod provided by cool fluorescent light (3000 lux). Data were collected on days to multiple shoot formation, per cent shoot apices with multiple shoots and number of shoots per explant. The data were analyzed for test of significance. The cultures were subcultured after every 4 weeks on shoot proliferation media for 2 months so as to obtain good growth.

Cost-Effective Media for Micropropagation

Once a MS medium supplemented with auxins and cytokinins was found suitable for *in vitro* multiplication of *P. kurroa*, modifications in replacing the sucrose (Glaxo, ExcelaR) with table sugar and omission of agar-agar were tried to see the effect on shoot proliferation and growth. Data were recorded for all parameters as given above and statistically analyzed.

Low cost medium (LCM)	Medium composition (g L^{-1})
LCM1	MS + table sugar (2%) + agar-agar (0.8% w/v)
LCM2	MS + table sugar (2%) without agar-agar
LCM3	MS + table sugar (3%)+ agar-agar (0.8% w/v)
LCM4	MS + table sugar (3%) without agar-agar
Standard medium (STM)	
STM 1	MS + sucrose (2%) +agar-agar (0.8% w/v)
STM 2	MS + sucrose (2%) without agar-agar
STM 3	MS + sucrose (3%) +agar-agar (0.8% w/v)
STM 4	MS + sucrose (3%) without agar-agar

Induction of Roots in Shoots, Hardening and Acclimatization of Plantlets

Individual shoots were excised from the parent cultures and transferred onto half strength MS media supplemented with different concentrations and combinations of auxins such as IBA, IAA and NAA for root induction in culture tubes (25×150 mm) containing 10 mL of medium. The pH of medium was adjusted to 5.7 prior to autoclaving. The cultures were incubated under the same photoperiod conditions as mentioned above. Data for days to root initiation, number of roots, average root length and per cent rooting were recorded and analyzed for test of significance. The rooted shoots were gently removed from the culture vessels, washed under running tap water and transferred to pots containing sand: soil: vermiculite (1:1:1) in the greenhouse conditions for acclimatization and hardening.

Relative Cost Estimation of Low-Cost Media

The cost of MS medium per liter was worked out with and without modifications in its components. The cost of sucrose used in the standard medium was compared with the cost of table sugar with and without the use of agar-agar to find out reduction in cost of per liter medium (Table 1, 2). The comparative cost of medium required for the production of one plantlet was worked out for low-cost versus standard media by culturing defined number of explants on one liter medium and then following the entire micropropagation protocol for shoot multiplication and root induction by sub-cultures on shooting or rooting media. The total amount of medium used and time duration required for the completion of micropropagation cycle resulting in the production of tissue cultured plantlets were worked out (Fig. 1).

Table 1: Relative cost (in India Rupees) of components used in low cost media (LCM) and standard media (STM)

Media component	Cost kg^{-1} (Rs.)	Amount used L^{-1} medium (g)	Component cost L^{-1} medium (Rs.)
Agar-agar (Glaxo,India)	3,950	8	31.6
Sucrose ExcelaR (Glaxo, India)	640	30 or 20	19 or 12.8
Table Sugar	20	30 or 20	0.60 or 0.40

Table 2: Comparative expenditure on one liter of low cost media (LCM) versus standard media (STM)

LCM	Expenditure L^{-1} (in Indian Rs.)	STM	Expenditure L^{-1} (in Indian Rs.)
1	32.00	1	44.40
2	0.40	2	12.80
3	32.20	3	50.60
4	0.60	4	19.00

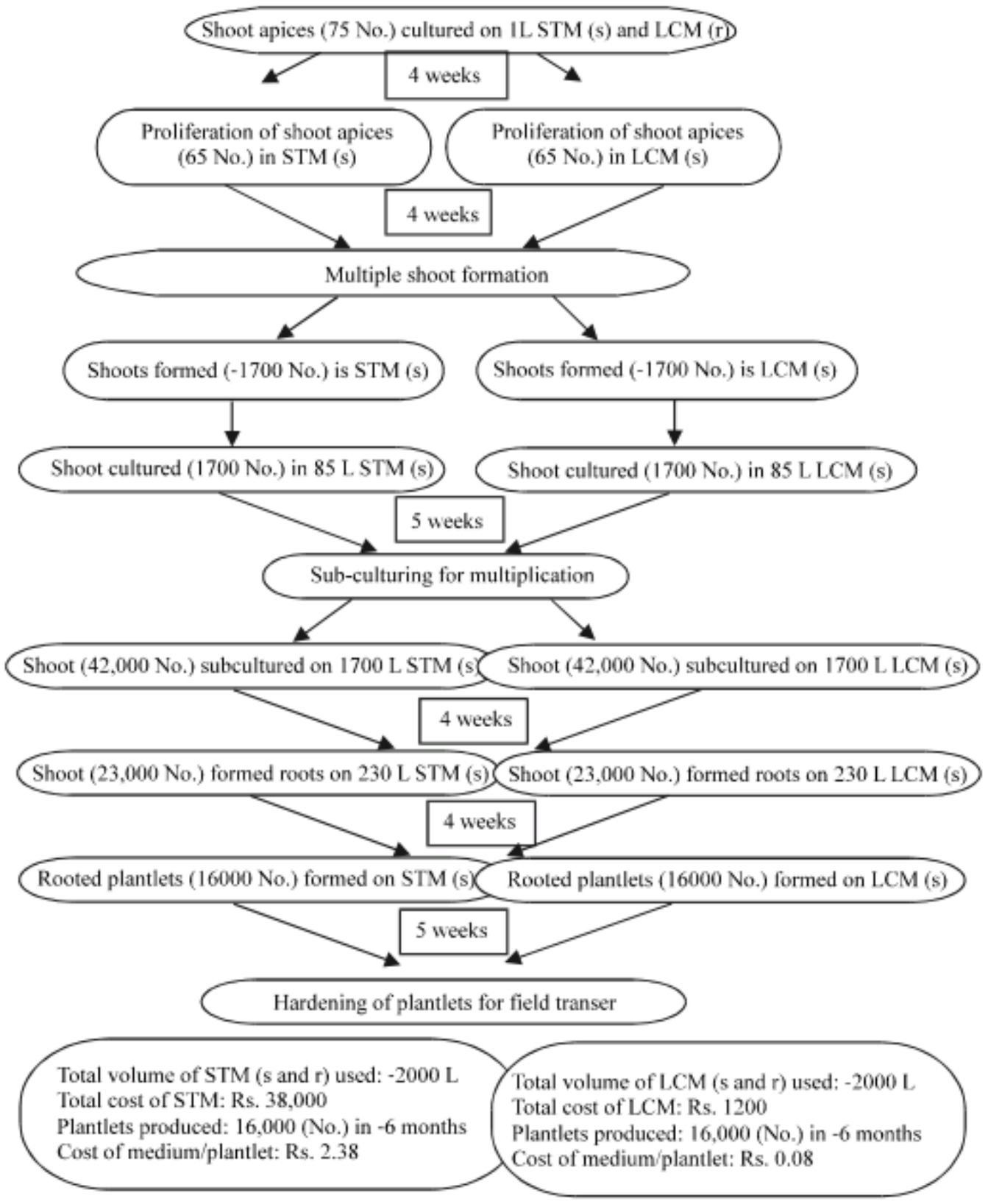

Fig. 1: Flowchart of micropropagation technology for *Picrorhiza kurroa* w.r.t. cost of medium/plantlet and amount of medium used for the production of N number plantlets in a defined duration of time; STM (s): Standard medium for shoots; STM (r): Standard medium for roots, LCM (s): Low cost medium for shoots, LCM (r): Low cost medium for roots

RESULTS

Establishment of Multiple Shoots

The surface sterilized auxiliary shoot tips were cultured on MS media containing BA (0.5-2 mg L^{-1}), IBA (0.5-3 mg L^{-1}) and KN (1-4 mg L^{-1}) in different combinations. Out of 15 different media combinations tested for multiple shoot formation from shoot apices, MS medium containing KN (3 mg L^{-1}) + IBA (2 mg L^{-1}) + sucrose 3% (w/v) + agar-agar 0.8% (w/v) was found to be the best with 86.3% of shoot apices proliferating into multiple shoots within 5-6 days of inoculation (Table 3; Fig. 2a-c). The same medium was found suitable for obtaining maximum shoots (25.3) per explant (Fig. 2d). With the addition and increase in concentration of BA in the medium, the shoot proliferation increased but the shoots were thin and slanderous with small leaves when cultures were incubated at 25±1°C (Fig. 3a, b).

The shoot apices were cultured on MS + KN (3 mg L^{-1}) + IBA (2 mg L^{-1}) and incubated at low temperatures (10±1, 15±1 and 25±1°C) with the same photoperiod conditions as mentioned above in order to see whether better shoot growth can be obtained. The shoot apices proliferated into multiple shoots within 5-6 days of culture with significant differences for leaf size and shoot biomass yield at 15±1°C. The leaves were ~10x longer and ~5x wider in shoots formed at 15±1°C compared to shoots at 25±1°C. Similarly the total shoot biomass yield and per cent survival were significantly higher in shoots grown at 15±1°C compared to at 25±1°C (Table 4; Fig. 3a-d).

Induction of Roots in *in vitro* Grown Shoots

The shoots formed from *in vitro* grown cultures were transferred onto half strength MS media supplemented with different concentrations and combinations of IBA, IAA and NAA for root

Table 3: Effect of different MS media combinations on *in vitro* shoot multiplication from shoot apices of *P. kurroa*

MS + Cytokinins (mg L^{-1})			Days to multiple shoot formation	Percent shoot apices with multiple shoots	Shoots explant^{-1}	Shoots forming roots (%)
BA	IBA	KN		(*Mean±SE)		
0.0	0.0	0	18-20	16.3±0.5	6.3±0.5	16.3±0.5
0.5	0.5	0	9-10	26.3±0.5	12.3±0.5	32.3±0.5
1.0	0.5	0	9-10	33.3±0.3	13.6±0.3	40.6±0.5
1.0	1.0	0	8-9	45.4±0.5	18.2±0.6	46.6±0.5
1.0	2.0	0	8-9	46.3±0.3	18.3±0.3	50.0±0.5
0.0	2.0	1	6-8	56.5±0.5	21.3±0.3	55.3±0.3
0.0	3.0	2	6-8	62.6±0.3	20.6±0.3	58.1±0.6
0.0	2.0	3	5-6	86.3±0.3	25.3±0.3	54.2±0.5
0.0	2.0	4	5-6	79.6±0.4	21.6±0.3	56.3±0.3
1.0	0.0	3	6-7	68.3±0.6	20.2±0.2	44.7±0.5
2.0	0.0	3	6-7	69.4±0.5	21.6±0.5	45.6±0.3
2.0	3.0	2	5-7	70.3±0.3	22.2±0.2	56.9±0.5
1.0	2.0	3	5-7	74.3±0.3	20.2±0.5	46.5±0.2
1.0	3.0	4	5-6	75.6±0.3	23.3±0.3	57.6±0.5
2.0	3.0	4	5-6	74.6±0.4	23.6±0.3	57.3±0.6

*Data represents mean of 20 replicates treatment^{-1} in three repeated experiments

Table 4: Effect of different incubation temperatures on *in vitro* plantlets formation in *P. kurroa*

Temperatures	Shoots/explant	Shoot length (cm)	Leaf size (L×W) (mm)	Roots/shoot	Root length (cm)	Shoot biomass (g)	Percent survival
	(*Mean±SE)						
25±1°C	25.3±0.3	7.03±0.9	5×3±0.49	5.60±0.5	8.90±0.6	2.03±0.3	43.3±0.4
15±1°C	21.5±0.5	8.63±0.3	50×15±0.67	5.01±0.3	8.01±0.5	3.73±0.5	80.2±0.8
10±1°C	18.1±0.3	4.70±0.3	12×8±0.35	3.73±0.2	5.40±0.3	1.33±0.3	49.5±0.3

*Mean of twenty replicates per treatment; repeated three times

Fig. 2: Micropropagation protocol for *P. kurroa* at low temperature (15±1°C), (a) field grown plant as a source of shoot apices, (b) shoot apex, (c) multiple shoot formation, (d) shoot proliferation and growth, (e) root induction, (f) rooted plantlet, (g) hardening of plantlets and (h) plants in pots

induction. Data were recorded for days to root initiation, percent shoots forming roots, number of roots/shoot and root length. Root induction occurred in 9-10 days of culturing in all media combinations with highest root induction (70%) on MS medium containing IBA (3 mg L^{-1}) followed by 66.6% on MS+ IBA (3 mg L^{-1}) and NAA(2 mg L^{-1}) (Table 5; Fig. 2e, f). The same medium was found suitable for more number of roots per shoot and maximum root length/shoot.

Optimization of a Low-Cost Medium

After the standardization of a suitable MS medium for *in vitro* shoot multiplication, the major components of nutrient medium such as carbon source used in the form of sucrose was replaced with table sugar (2-3%) and the agar-agar was omitted completely. Four different modifications of low cost-media (LCM1-LCM4) were compared with the standard media (STM1-STM4), which were found suitable for *in vitro* shoot multiplication (Table 6). All 4 low cost media (LCM1-LCM4) showed comparable response corresponding to standard media (STM1-STM4) in terms of various parameters of *in vitro* shoot multiplication and growth (Fig. 4 a-d). On the similar lines, root induction media were also modified for the same components i.e., substitution of sucrose with table sugar and omission of

Fig. 3: Comparative shoot growth and leaf development in *in vitro* shoots formed at (a, b) low temperature (15±1°C) and (c, d) Optimum temperature (25±1°C)

Table 5: Effect of different concentrations of auxins on root induction in *in vitro* grown shoots of *P. kurroa*

1/2 MS + Auxins (mg L^{-1})						
IBA	IAA	NAA	Percent shoots forming roots	Roots/shoot	Root length (cm)	Days to rooting
			(*Mean±SE)			
0	0.0	0	0	0	0	0
1	0.0	0	50.1±0.5	3.6±0.3	3.9±0.2	11-12
2	0.0	0	61.2±0.3	3.7±0.2	5.4±0.3	12-15
3	0.0	0	70.1±0.5	5.6±0.5	8.9±0.6	8-10
0	0.5	1	43.3±0.3	5.3±0.6	5.9±0.3	11-12
0	1.0	2	51.1±0.6	3.6±0.3	5.7±0.4	11-12
2	0.0	1	52.4±0.4	4.1±0.2	6.2±0.3	11-13
3	0.0	2	66.6±0.6	5.3±0.7	8.1±0.5	9-10
3	1.0	0	50.1±0.5	4.3±0.3	6.7±0.2	9-10
3	2.0	0	53.3±0.3	5.0±0.5	6.9±0.2	9-10

*Mean of 20 replicates per treatment; repeated three times

Table 6: *In vitro* shoot multiplication in *P. kurroa* on low-cost media

Type of medium		Days to multiple shoot formation		Shoot apices forming multiple shoots		Shoots per explant	
Low-cost medium (LCM) (g L^{-1})	Standard medium (STM) (g L^{-1})	LCM	STM	LCM	STM	LCM	STM
				(*Mean±SE)			
LCM1	STM1	6-9	6-7	78.0±0.5	80.3±0.3	20.0±0.5	22.3±0.3
LCM2	STM2	5-9	4-7	80.3±0.6	82.4±0.6	21.0±0.5	23.0±0.5
LCM3	STM3	7-8	5-6	82.2±0.6	86.3±0.3	25.0±0.5	25.3±0.3
LCM4	STM4	4-7	4-6	84.6±0.6	85.6±0.6	26.4±0.3	26.5±0.7

*Mean of 20 replicates per treatment; repeated three times

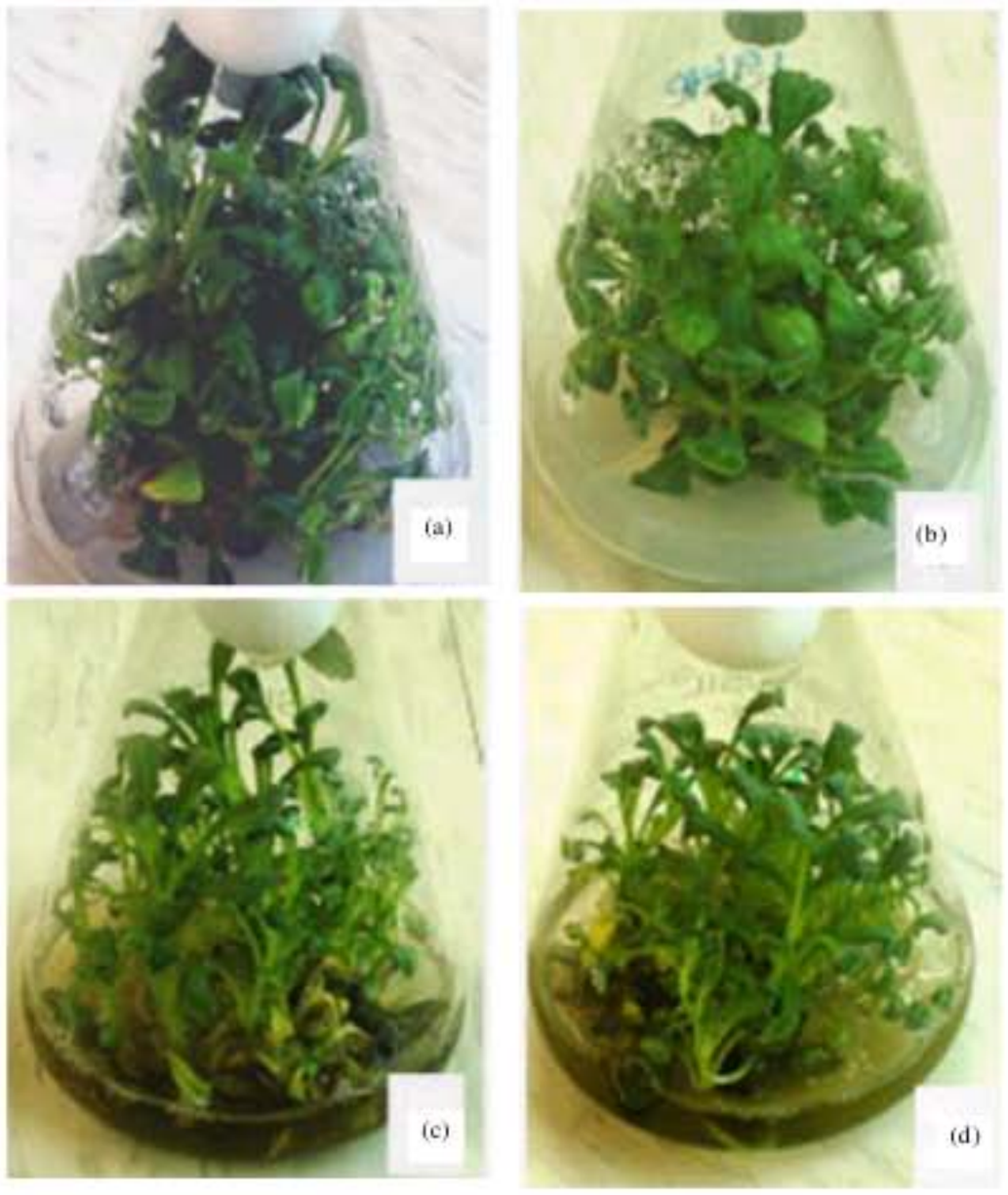

Fig. 4: Comparative response of low cost versus standard media for multiple shoot formation; (a, b) LCM 3 vs. STM 3 with agar-agar; (c, d) LCM 4 vs STM 4 without agar-agar

Table 7: Root induction in *in vitro* shoots of *P. kurroa* on low-cost media

Type of medium		Shoots forming roots		Roots/shoot		Root length (cm)	
Low-cost medium (LCM) (g L^{-1})	Standard medium (STM) (g L^{-1})	LM	ST	LM	ST	LMST	
		(*Mean±SE)					
LCM1	STM1	65.0±0.5	66.6±0.6	4.2±0.6	4.5±0.6	7.5±0.5	7.9±0.6
LCM2	STM2	65.9±0.6	68.0±0.5	4.4±0.6	4.9±0.6	7.9±0.6	8.0±0.6
LCM3	STM3	68.6±0.6	70.1±0.5	5.2±0.6	5.6±0.5	7.9±0.6	8.9±0.6
LCM4	STM4	68.9±0.6	69.9±0.6	5.6±0.6	5.9±0.6	8.2±0.6	8.6±0.6

*Mean of twenty replicates per treatment; repeated three times

agar-agar. The root induction response was also comparable between low cost media (LCM1-LCM4) corresponding to standard media (STM1-STM4; Table 7; Fig. 5a-d). The same medium was found suitable for more numer of roots per shoot and maximum root lenght/shoot.

Hardening of *in vitro* Plantlets

Well rooted plantlets derived from various experiments were transferred to pots containing autoclaved potting mixture consisting of sand, soil and vermiculite (1:1:1) in the greenhouse for hardening. Initially for 10-15 days, the plantlets were covered with poly bags to provide sufficient humidity and to avoid desiccation till the plantlets showed new growth (Fig. 2g, h). During the hardening process, poly bags were taken off every day for 1-2 h so as to acclimatize the plantlets to external environment. Large numbers of well rooted plantlets were regenerated and transferred to field conditions after proper hardening.

Fig. 5: Comparative response of low cost versus standard media for root induction. (a, b) LCM 3 vs. STM 3 and (c, d) LCM 4 vs. STM 4

The total cost of low-cost medium has been reduced significantly because the expenditure of one liter medium for low-cost media, LCM 2 and LCM 4 comes to Rs. 0.40 and Rs. 0.60, respectively compared to Rs. 12.80 and Rs. 19.00 for one liter of standard media, STM 2 and STM 4, respectively.

DISCUSSION

The identification of MS medium supplemented with KN (3 mg L^{-1}) + IBA (2 mg L^{-1}) for better shoot multiplication in *P. kurroa* is not very different from previous studies by Lal *et al.* (1988), who have identified kinetin with another auxin (IAA) as the best medium combination for *in vitro* shoot multiplication. Though we had to use higher amounts of kinetin and IBA compared to previous studies, which can be attributed to the differences in genotypes of *P. kurroa* used. Another important refinement in the micropropagation of *P. kurroa* in our study was that there was absolutely no occurrence of vitrified and fasciated shoots, which were reported in previous studies on tissue culture of *P. kurroa* (Upadhay *et al.*, 1989).

The current study reports for the first time wherein a low-cost micropropagation technology has been developed for an endangered medicinal herb endemic to North-Western Himalayas of India. The low-cost micropropagation technology has certain distinctive features over the previous reports of low-cost tissue culture alternatives in different plant species. For example, our technology has not only replaced some components of the nutrient medium with low-cost alternatives as reported in previous studies (Kaur *et al.*, 2005; Patra *et al.*, 1998), but also have worked out a complete process outline which depicts that what amount of nutrient medium is required for producing a defined number of *P. kurroa* plants in a particular period of time. This analysis would be of great practical us in setting up a industrial plant tissue culture units not only for micropropagation of *P. kurroa* but also other plant species. More importantly the tissue culture process described in this study has rectified the problem of poor or low survival of tissue cultured plants of *P. kurroa* in the field conditions as reported in previous studies (Upadhay *et al.*, 1989; Lal *et al.*, 1988). The modifications in MS media by changing the concentrations and combinations of auxins and cytokinins did not help in obtaining shoots with good growth and well developed leaves. Since, *P. kurroa* grows at high altitudes where temperatures are low, the incubation of auxiliary shoot tip cultures at low temperatures (15±1°C) helped significantly to provide better shoot growth with well developed leaves. The low-cost medium composition identified in the current study holds great promise in not only rapid multiplication of *P. kurroa* for its reclamation in natural habitat but also in rapid multiplication of genetically superior high phytopharmaceutical content strains of *P. kurroa* at a relatively lower cost compared to what has been reported so far.

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