

In vitro propagation schedule of *Picrorhiza kurroa*: An endangered medicinal plant of Central Himalaya

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Abstract

Picrorhiza kurroa Royle ex Benth (Kutki) has traditionally been used to treat disorders of the liver and upper respiratory tract, fever, and to treat dyspepsia, chronic diarrhoea and scorpion sting in Ayurveda medicine owing to the presence of active principles in root and rhizomes. The plant is self-regenerating but unregulated over-harvesting has caused it to be threatened to near extinction. The current research describes a protocol of micro propagation of this important medicinal plant from establishment to hardening in field conditions. Multiple shoots were induced in apical and axillary meristems derived from mature explants on Murashige and Skoogs (1962) medium supplemented with 0.25 mg L⁻¹ 6-benzylaminopurine (BA), 0.25 mg L⁻¹ kinetin (KN), 0.5 mg L⁻¹ ascorbic acid and 3% (w/v) sucrose. Optimal rooting (86.6%) and growth of microshoots were observed on a medium containing 0.25 mg L⁻¹ indole-3-butyric acid (IBA) with 2% (w/v) sucrose. Micropropagated plantlets were acclimatized and successfully grown in soil.

Key words: *Picrorhiza kurroa*, axillary bud, *In vitro* multiplication, micropropagation

Introduction

Picrorhiza kurroa Royle ex Benth (Scrophulariaceae) commonly known as kutki is an important medicinal plant which occurs at higher altitude, between 3000-4500m, in Himalayas. It is a perennial herb with elongated stout and creeping stolon (Semwal *et al.*, 1983). It secretes a large quantity of glucosidal bitter principle named kutkosides and picosides which are the constituents of kutkin (Rastogi *et al.*, 1949). The drug *Picrorhiza* is obtained from dried stolon and roots. Its herbal preparation is antiperiodic, stomachic, laxative in small doses and cathartic in large doses and useful in dropsy (Kirtikar and Basu, 1933). However there are many problems that restrict its multiplication on a large scale.

Propagation through seed is unreliable because of disease and pest problems, short viability and heavy rains during the seeding season in the natural habitat. Pharmaceutical companies largely depend upon materials procured from naturally occurring stands raising concern about possible extinction and providing justification for development of *in vitro* techniques for mass propagation of *P. kurroa*. Conservation of genetic stability in germplasm collections and micropropagation of elite plants is of the utmost importance and propagation of plants through apical or axillary meristem culture allows recovery of genetically stable and true to type progeny (Hu and Wang, 1983). The present study describes an optimized protocol upto hardening and acclimatization in field conditions in its natural habitat for mass propagation of homogenous and elite material of *P. kurroa* for increased production and improving the socio economic status of hill farmer community.

Materials and methods

Plant material and explant source: Actively growing young plants of *P. kurroa* were collected from greenhouse as well as

field grown plants from Herbal Research and Development Institute, Mandal, Gopeshwar in the month of July, 2003. These plants were established in earthen pots at controlled environment containment facility, College of Basic Sciences and Humanities, GBPUA&T, Pantnagar. After establishment, explants (axillary and apical shoot tips 4-5cm in length) were collected at weekly intervals from two elite survived plants. These explants (4-5) were washed with 2% (v/v) detergent 'Tween 20' and rinsed several times with running tap water. The explants were surface sterilized in 0.1% (w/v) aqueous mercuric chloride solution for 15 min followed by four washings with sterile distilled water. The apical and axillary meristems were inoculated on media (25-30 mL) containing different sets of phytohormones with single explant in each culture vessel (Jam Bottle of 500 mL capacity) for *in vitro* establishment.

Culture medium and growth conditions: The meristems (apical and axillary) were placed on semi-solid basal MS (Murashige and Skoog, 1962) medium supplemented with different concentrations and combinations of 6-benzylaminopurine (BA: 0.0, 0.25, 0.5 and 1.0 mg L⁻¹), kinetin (KN: 0.0, 0.25, 0.5 and 1.0 mg L⁻¹), indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) (0.0, 0.10, 0.25 and 0.5 mg L⁻¹) for shoot proliferation and multiplication. The pH of the media was adjusted to 5.8 using 0.1N NaOH or 0.1N HCl before autoclaving at 121°C and 1.06 kg cm⁻² pressure for 20 min for sterilization. The cultures were maintained at 25±2°C under 14h photoperiod (55 mmol m⁻²s⁻¹) from cool, white fluorescent tube lights. The cultures were maintained by regular subcultures at 4-week intervals on fresh medium with the same compositions. To avoid blackening, the medium was supplemented with 0.5 mg L⁻¹ ascorbic acid.

Induction of rooting and acclimatization: For root induction, excised microshoots (1-2 cm length) were transferred to MS basal medium supplemented with different concentrations of IAA or IBA (0.0, 0.1, 0.25 and 0.5 mg L⁻¹) and 2% (w/v) sucrose. One

excised shoot was placed in each culture vessel having 25-30 mL of the culture media. All the cultures were incubated at 25±2°C under 16h photoperiod with cool, white fluorescent light. Rooted micro-propagules were thoroughly washed to remove the adhering gel and planted in 2.5 cm plastic pots (250mL) containing a sterile mixture of sand, soil and cow-dung manure in the ratio of 1: 1: 1 (v/v) and kept in the greenhouse for acclimatization.

Results and discussion

Meristem proliferation and multiplication: Meristem proliferation and multiplication was initiated from apical and axillary explants of *P. kurroa* within 8-10 days of inoculation onto MS basal medium supplemented with BA, KN and 0.5 mg L⁻¹ ascorbic acid. Of the different cytokinins tested, BA + KN was the most effective for shoot proliferation and multiplication. The maximum shoot proliferation and multiplication was observed both in apical and axillary meristems cultured on MS medium supplemented with 0.25 mg L⁻¹ BA, 0.25 mg L⁻¹ KN and 0.5 mg L⁻¹ ascorbic acid within 4 weeks of culture under 14h photoperiod (Table 1). The apical and axillary shoots proliferated and elongated to 1.0-1.5 cm within 4 weeks of culture. There was no sign of shoot proliferation when explants were cultured in media devoid of cytokinin. At higher concentrations of BA or kinetin, the rate of shoot proliferation declined. Inclusion of either IAA or IBA in the culture medium did not help in proliferation and multiplication of shoot. In most of the cases, the growth was inhibited and only 1-2 shoots elongated; some produced compact callus at the base of the explants. Prolonged culture on the proliferation and multiplication media resulted in the blackening of the basal ends of the developing shoots. There were differences among the treatments for both the percentage of cultures with multiple shoots and the mean number of shoots/culture. The axillary meristems produced more number of shoots (4.57) than the apical meristems (3.25). The highest percentage of cultures with multiple shoots was observed on media containing 0.25 mg L⁻¹ BA, 0.25 mg L⁻¹ KN and 0.5 mg L⁻¹ ascorbic acid when the cultures were incubated in the continuous light for 4 weeks (Table 1). The frequency of multiple shoots per culture varied from 1.24 to 4.42 in case of the 14h photoperiod. The rate of multiplication was high and stable upto 5th subculture and declined in subsequent subcultures.

Induction of root from microshoots: Elongated shoots (1-2 cm long) were rooted on MS basal medium supplemented with various concentrations of either IAA or IBA. The rooting in the microshoots was inhibited in the medium devoid of growth regulator. Root initiation took place within 10-12 days of transfer to MS basal medium supplemented with 0.1-0.5 mg L⁻¹ IAA or IBA. However, optimal rooting (70.6%) and growth of microshoots was observed on medium containing 0.25 mg L⁻¹ IBA with 2% (w/v) sucrose (Table 2). The rooting ability was reduced with the increase in the concentration of IAA or IBA in the culture medium. The percentage of shoots forming roots and days to rooting significantly varied with different concentrations of IAA or IBA.

Acclimatization and field establishment: About 96% of the rooted plantlets established in the greenhouse within 2-3 weeks of transfer. The plant grew well and attained 6-8 cm height within 4 weeks of transfer. The whole plant regeneration and acclimatization has been depicted in Fig. 1.

Table 1. Effect of BA, kinetin and 2 mg L⁻¹ ascorbic acid on shoot growth from apical (A) and axillary (B) meristems of *Picrorhiza kurroa* after 4 weeks of culture under 14h photoperiod

MS + Growth regulators (mg L ⁻¹)		Percent of cultures with multiple shoots (Mean ± S.E.)*		Number of shoots/explant (Mean ± S.E.)*	
Kinetin	BA	A	B	A	B
0.0	0.0	0.0	0.0	0.0	0.0
0.0	0.25	38.7 ± 0.3	46.7 ± 0.3	2.72 ± 0.5	2.31 ± 0.2
0.0	0.50	40.2 ± 0.5	43.3 ± 0.7	1.25 ± 0.4	1.73 ± 0.5
0.0	1.0	17.7 ± 0.4	21.3 ± 0.6	1.87 ± 0.3	1.34 ± 0.6
0.25	0.0	31.6 ± 0.4	41.4 ± 0.3	2.40 ± 0.2	2.93 ± 0.5
0.25	0.25	61.8 ± 0.5	73.7 ± 0.3	3.25 ± 0.2	4.57 ± 0.4
0.25	0.50	50.7 ± 0.4	57.6 ± 0.5	3.15 ± 0.4	3.20 ± 0.7
0.50	0.0	41.2 ± 0.5	43.8 ± 0.6	2.19 ± 0.4	2.30 ± 0.4
0.50	0.25	51.3 ± 0.6	56.4 ± 0.3	2.20 ± 0.7	2.17 ± 0.6
0.50	0.50	40.4 ± 0.4	41.8 ± 0.5	1.80 ± 0.6	1.88 ± 0.5
0.5	1.0	20.6 ± 0.4	23.4 ± 0.5	1.17 ± 0.5	1.13 ± 0.3
1.0	0.0	28.6 ± 0.7	23.5 ± 0.4	1.12 ± 0.5	1.30 ± 0.6
1.0	0.5	22.7 ± 0.5	25.5 ± 0.4	1.17 ± 0.6	1.27 ± 0.6

* Mean of 20 cultures per treatment, repeated thrice

Table 2. Effect of IAA and IBA on rooting from excised shoots of *P. kurroa* cultured on MS basal medium supplemented with 2% (w/v) sucrose

MS + Growth regulators (mg L ⁻¹)		Percentage of rooted shoot (Mean ± S.E.)*	Days to rooting
IAA	IBA		
0.10	0.0	30.6±0.20	10-11
0.25	0.0	45.4±0.6	10-13
0.50	0.0	50.2±0.4	11-12
0.0	0.10	57.20±0.70	10
0.0	0.25	70.60±0.30	12
0.0	0.50	54.83±0.50	11

*Data represent mean of 20 cultures/treatment, repeated thrice

Present study showed that it was possible to explore the morphogenetic potential of *P. kurroa* by modification of growth regulators and light conditions. The use of different combinations of cytokinins for the induction and multiplication of shoots derived from apical and axillary meristems and the regulatory action of cytokinin and apical dominance in shoot induction and multiplication *in vitro* is well documented (Wickson *et al.*, 1958). The higher shoot induction and multiplication was observed both in apical and axillary meristems cultured on MS medium supplemented with 0.25 mg L⁻¹ BA, 0.25 mg L⁻¹ kinetin and 0.5 mg L⁻¹ ascorbic acid within 4 weeks of culture under 14h photoperiod. At higher concentrations of BA or kinetin, the rate of shoot proliferation declined. The axillary meristems produced more number of shoots than the apical meristems. The results demonstrated that inclusion of either IAA or IBA in the culture medium did not help in shoot multiplication. Prolonged culture on the proliferation and multiplication media containing IAA or IBA resulted in the blackening of the basal ends of the developing shoots. The results are consistent with earlier reports indicating cytokinins and auxins effect on shoot multiplication in other plants using shoot tip or axillary bud explants (Rout *et al.*, 1999). With the increase in the concentration of either BA or KN, the percentage of shoot multiplication declined. The results also implies that there were differences among the treatments for

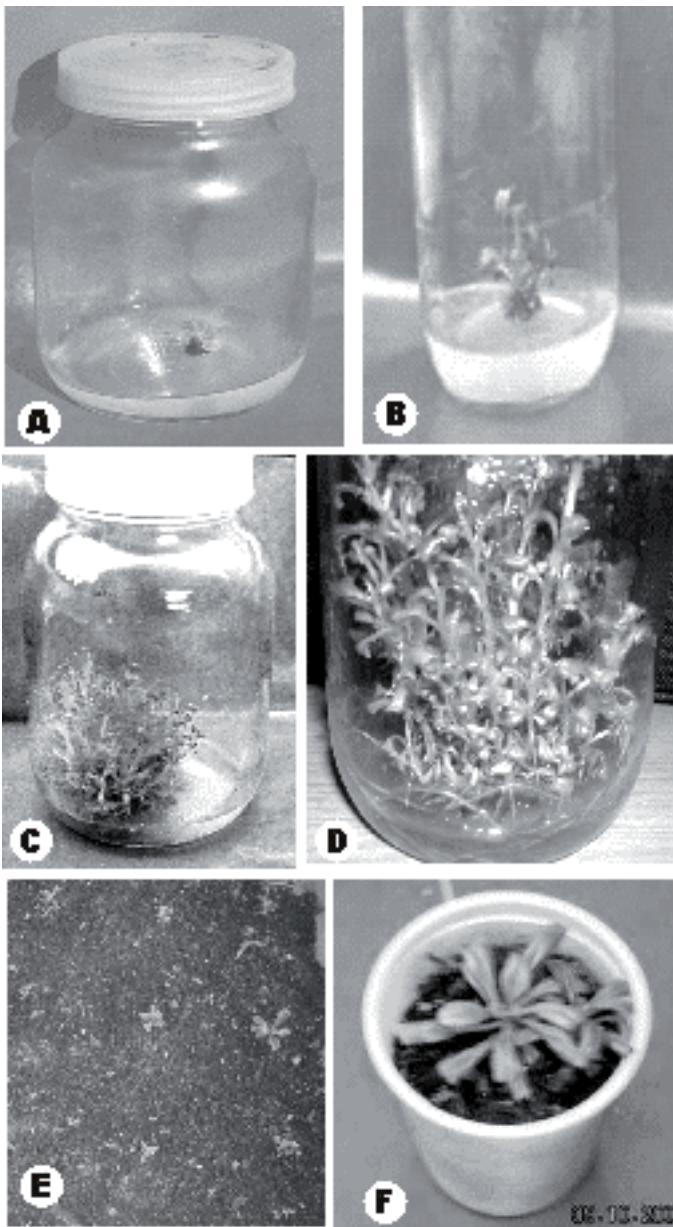


Fig. 1. Micropropagation of kutki (*P. kurroa*) (A) Establishment stage, (B & C) Shoot proliferation, (D) Rooting. Hardening and acclimatization (E) in pot (F) in field.

both the percentage of culture developing multiple shoots and the mean numbers of shoots per culture. The rate of multiplication was high and stable upto 5th subculture and declined in subsequent subcultures. This might be due to the balancing of the endogenous and exogenous growth regulators and the ionic concentration of nutrient salts as reported earlier in other plants (Zimmermann, 1985). Elongated shoots rooted better in MS basal medium supplemented with 0.25 mg L⁻¹ IBA and 2% sucrose. The rooting ability was reduced with the increase in the concentration of IAA or IBA in the medium. The percentage of shoots forming roots and days to rooting significantly varied with different concentrations of IAA or IBA. The rooted plantlets were established in the field and grew normally.

In conclusion, an attempt was made to develop an *in vitro* protocol for mass multiplication of *P. kurroa* by manipulating the nutrient salts, growth regulators and culture conditions. This investigation may be useful for conservation of this economically important medicinal plant species.

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