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Standardization of *in vitro* Multiplication Protocol for *Picrorhiza kurroa* (Royle Ex. Benth)

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A B S T R A C T

An *in vitro* protocol for plant regeneration through organogenesis was established for *Picrorhiza kurroa* Royle Ex. Benth (Scrophulairaceae). The plant is a principle source of glycoside that is Picroside-I, Picroside-II and Kutkoside. Callus was induced on Murashige and Skoog basal medium supplemented with cytokinin (Kn or BA) and auxin (2,4-D/IBA/NAA) using leaf, petiole and stem explants. Shoots were regenerated on MS media with BA (1.5 to 2.5 mgl^{-1}) combined with auxin (IBA or NAA) in the range of 0.5 to 2.5 mgl^{-1}. Rooting was initiated with full or half media supplemented with 0.5 to 1.5 mgl^{-1} of IBA or NAA. *In vitro* regenerated plantlets were successfully transferred to the soil. Pots having garden soil with organic compost and regular irrigation of half strength MS solution at initial stages are desirable for higher survival of plantlets.

Introduction

Picrorhiza kurroa Royle ex. Benth commonly known as Kutki, is an important temperate medicinal plant species found in western Himalayas, a fast depleting medicinal value plant belonging to family Scrophulairaceae. It is perennial herb and also the principle source of glycoside that is Picroside-I, Picroside-II and Kutkoside which are extracted from dried rhizomes and roots of 3 year old plants (Rastogii *et al.*, 1949, Jia *et al.*, 1999). Other identified active constituents are apocynin, drosin, and nine cucurbitacin glycosides (Weinges *et al.*, 1972, Stuppner and Wagner 1989).

It is extensively used in both modern as well as traditional systems of medicine for its invaluable medicinal properties being stomachic, cathartic, cholagogue, blood purifier and useful in treating jaundice, asthma, flatulence, cardiac complaints, antifungal and chronic hepatitis as its active

constituents are apocynin, drosin, and nine cucurbitacin glycosides (Weinges *et al.*, 1972, Stuppner and Wagner 1989 and Hussain 1984). Now a day's researches are more focused on evaluating its hepatoprotective, anticholestic antioxidant and immune modulating activity (Atal *et al.*, 1986, Subedi 2000). In the past few decades there has been a resurgence of interest in the study and use of medicinal plants in health care and in recognition of the importance plants to the health system (Hoareau and DaSilva 1999).

The natural resurgence of *Picrorhiza kurroa* is through rhizomes and seeds, however their cultivation rate is very poor. The poor cultivation coupled with over exploitation for pharmaceutical use has depleted the species from natural habitat. The Indian system of medicine is predominantly dependent upon the use of plant based raw materials in most of their preparations and formulations, thereby, widening the gap between demand and supply and thus, putting further pressure on the species. In fact *P. kurroa* is now listed at one of the endangered plant species of India (Nayar and Shastry, 1990).

Owing to these factors, the species is at the verge of extinction. It is essential for the conservation of *P. kurroa* to encourage *ex-situ* plantation which require large scale planting material. In view of the problems of conventional propagation and high demand of planting material, the large scale multiplication of this species can only be met efficiently and economically in a sort span of time by *in vitro* propagation. Therefore, an efficient *in vitro* propagation system for producing this plant is required to further clarify its potential medicinal values and germplasm conservation. Plant regeneration from *P. kurroa* has earlier been reported using shoot tips (Nanda and Ahuja 1996). Our finding are at variance from the earlier report in tissue culture of this species.

Materials and Methods

Picrorhiza kurroa leaf, petiole and stem explants collected from the Division of Medicinal and Aromatic Plants Block College of Horticulture VCSG, UUHF, Bharsar, Pauri Garhwal, India. Initially the plant materials were washed with tap water followed by washing with 1% (v/v) labolene detergent for 15 minutes and then in running tap water for 30 minutes. The explants were surface sterilized with an aqueous solution of 0.1% $HgCl_2$ (w/v) for 2-3 minute. The explants are then rinsed several times with sterilized double distilled water. Damage parts were aseptically trimmed with sterilized surgical blade. All the explants were separately inoculated in the MS basal medium (Murashige and Skoog, 1962) supplemented with growth regulators such as cytokinin (Kn or BA) and auxin (2,4-D/IBA/NAA). Coconut Milk (CM), incorporated into basal medium in varying concentrations. The pH of the media was adjusted to 5.8 ± 0.1 using 0.1 NaOH or 0.1 HCL. The medium was autoclaved at 1.06 kg cm^2 at 121^0 C for 25 to 30 minutes. Cultures were incubated at $24^0C \pm 2^0C$ and 60% relative humidity with 16: 8 hours light and dark photoperiod.

To induce root formation, regenerated shoots were transferred to MS medium supplemented with full or half strength mineral salts and different concentrations of auxins (0.5 to 1.5 mgl^{-1} IBA or NAA). Rooted plantlets were transferred to thermocol cups having (1) sterilized mixture of soil and organic compost manure in the ratio of 1:1 (v/v), (2) sterilized mixture of sand and soil in the ratio of 1:1 (v/v), (3) sterilized forest soil. The thermocol cups were kept in the Growth chamber at $18^0C \pm 2^0C$ temperature and 60% relative humidity with 16:8 hours light: dark photoperiod. Plantlets were transferred to earthenware pots containing different soil types. They were transferred in shade for two weeks and

watered regularly. All the potted plants were then transferred to sun gradually increasing the periodicity of exposure.

Results and Discussion

Callusing response in the form of callus biomass was measured at the interval of 25 days up to 100 days using leaf, petiole and stem explants (Table 1). MS medium was supplemented with cytokinin (Kn or BA) and auxin (2,4-D/IBA/NAA) in varying concentration and combinations. 15m/l CM was added with 2,4-D (1.5 – 2.0 mg/l^{-1}) and cytokinin (0.5 -1.5 mg/l^{-1}) in another set of experiment. MS medium without any cytokinin and auxin failed to induce callusing in all the explants. Highest callus biomass (fresh weight) was recorded in 2.0: 2.5 mg/l^{-1} of Kn: 2,4-D combination for leaf and 2.5 mg/l^{-1} Kn with 3.0 mg/l^{-1} 2,4-D as well as 3.0 mg/l^{-1} Kn with 2.5 mg/l^{-1} 2,4-D for stem explants (Plate 1 and 3).

BA: IBA (1.5 mg/$l^{-1:}$ 2.0 mg/l^{-1}) combination produced maximum callus biomass in petiole explants (Table 1 and Plate 2). Second highest values were recoded in 2.5: 2.5 mg/l^{-1} (Kn: 2,4-D) combination in leaf and stem, 2.0: 2.0 mg/l^{-1} (BA: IBA) and 1.0: 1.5 + CM (BA: 2,4-D) combination in petiole explants. Kn and 2,4-D combination induced better calli in leaf and stem explants than BA with other auxin. However, BA: IBA and BA: 2,4-D + CM combination were observed to be better in petiole explants as reflected by the biomass values.

Earlier study on *in vitro* propagation of *Picrorhiza kurroa* reveals that regeneration of shoot bud from *ex vitro* leaves on Kn (3.48µM) after 15 days of inoculation while, shoot bud primordial from callus was produced on TDZ (0.5 µM) Patial *et al.,* (2012). NAA 0.1 and 1.0 mg/l^{-1} alone in the medium or fortified with 0.01 and 1.0 and 1.0 mg/l^{-1} BA resulted into callusing as well as higher proliferation rate of calli in *Swertia pseudochinensis* while, in *Swertia japonica* successful growth of calli was obtained with the addition of 1.0 mg/l^{-1} 2,4-D or 1.0 mg/l^{-1} IAA or NAA using seedling root or hypocotyle as explants (Miura 1991). The variation in the concentration of cytokinin and auxin for callus induction in the present study may be due to explants used and physiological age of the explants. Physiological age of explants is an important factor determining the morphogenetic response (Ammirato 1996). In general explants derived from micropropagated shoots have an early and greater capacity for morphogenesis than tissues excised from field plants (George 1996). Maximum number of shoots (23.95 ± 2.76) was produced after 90 days using BA (1.5 mg/l^{-1}) and IBA (1.0 mg/l^{-1}) combination (Plate 6 and 7). In the second set maximum shoots appeared in 1.5: 1.0 mg/l^{-1} concentration followed by 1.0: 0.5 mg/l^{-1} and 2.0: 1.5 mg/l^{-1} combination of BA and NAA at 90 days stage. Shoots were induced from callus on White's medium fortified with 1 mg/l^{-1} BA and 2g/l^{-1} casein hydrolysate in *Swertia pseudochinensis* by Miura (1991). Root induction was studied in MS medium supplement with 0.5 to 1.5 mg/l^{-1} of auxin in separate sets of experiment in the well developed shoots that were transferred in the fresh medium for rooting purpose. Number of roots periodically recorded at the interval of 15 days (Plate 8).

Requirement of 0.5 mg/l^{-1} auxin in MS full for the development of higher number (9.33 ± 1.13) of roots were recorded (Plate 9). However, slightly lesser number of roots (8.12 ± 1.29) was noticed by using half of the MS medium with 1.0 mg/l^{-1} of auxin. Half strength B5 basal medium containing 1µg/l^{-1} NAA has been reported to be suitable for root development in *S. psedochinensis* by Kitamura *et al.,* (1987).

Plate.1 Callus formation in leaf explants of *Picrohiza kurroa* with 2.5: 2.5 mgl^{-1} of Kn and 2,4-D. **Plate.2** Callus formation in petiole explants of *Picrohiza kurroa* with 1.5: 2.0 mgl^{-1} of Kn and 2,4-D + 15mgl^{-1} coconut milk. **Plate.3** Callus formation in stem explants of *Picrohiza kurroa* with 2.5: 2.5 mgl^{-1} of Kn and 2,4-D. **Plate.4 and 5** Shoot bud differentiation from callus of *Picrohiza kurroa* with 1.5: 1.0 mgl^{-1} of BA and IBA. **Plate.6 and 7** Regenerated shoot from callus. **Plate.8 and 9** Rooting response of *Picrohiza kurroa* with MS Full + 0.5mgl^{-1} IBA

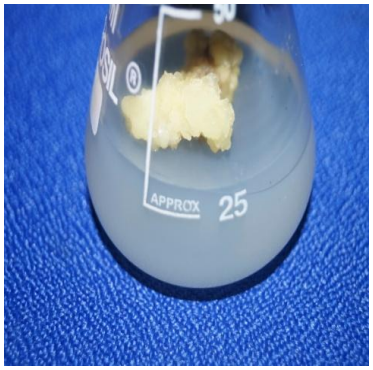

Plate 1

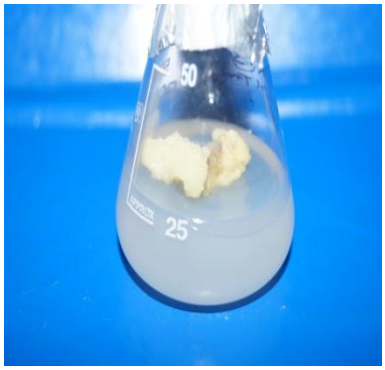

Plate 2

Plate 3

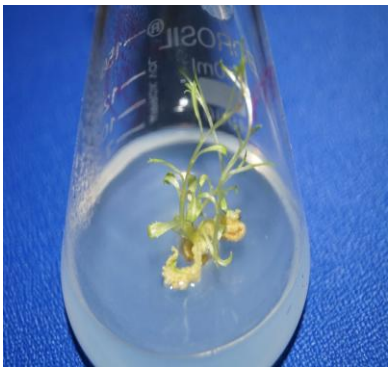

Plate 4

Plate 5

Plate 6

Plate 7

Plate 8

Plate 9

Table.1 Maximum callus biomass* (mg.) of different explants of *Picrohiza kurroa* on MS media supplemented with cytokinin, Auxin and Coconut milk (CM) after 100 days of inoculation

Cytokinin (mgl^{-1})	Auxin (mgl^{-1})	**Explants**		
		Leaf	**Petiole**	**Stem**
		Mean ±SD	Mean ±SD	Mean ±SD
Kn	2,4-D\			
0	1.5	-	-	10.20 ± 1.32
2.0	2.5	11.36 ± 1.57	-	10.75 ± 0.95
2.0	3.0	10.20 ± 1.10	-	-
2.5	2.5	11.25 ± 1.15	-	10.97 ± 1.10
2.5	3.0	10.15 ± 1.20	-	11.12 ± 1.20
3.0	2.5	-	10.90 ± 0.90	10.12 ± 1.20
BA	IBA			
1.5	2.0	10.70 ± 1.18	11.27 ± 1.17	9.40 ± 0.87
2.0	2.0	10.45 ± 1.37	10.98 ± 1.40	9.72 ± 0.99
BA	NAA			
1.5	1.5	10.08 ± 0.90	-	-
1.5	2.0	-	9.20 ± 0.88	10.48 ± 1.22
2.0	1.5	10.60 ± 1.20	-	-
2.0	2.0	-	10.65 ± 1.26	9.82 ± 1.07
Kn	2,4-D + $15ml^{-1}$CM			
0.5	1.5	11.05 ± 1.22	-	-
1.5	1.5	-	10.35 ± 0.90	-
1.5	2.0	10.80 ± 1.25	10.62 ± 1.10	-
BA	2,4-D + $15ml^{-1}$CM			
1.0	1.5	10.60 ± 1.44	10.98 ± 0.90	-
1.5	2.0	10.82 ± 1.10	10.40 ± 1.07	-

*Biomass on fresh weight basis, - Poor response (very low biomass) hence not mentioned.

Ten plantlets were kept at 25°C in growth chamber for studying the effect of temperature. 8 – 9 plants died and in another experiment ten plantlets were kept at 18°C and 5 – 6 plants survived. Three sets of 10 plantlets, each were kept at 18°C in Growth chamber. First and second set was MS medium and third set with water only. 5 -6 plants survived in first and second set while one plant survived in third set. The experiments were repeated thrice. Ten plantlets at two leaf stage were gradually exposed, initially for 1hr. than 2, 3, 4 hrs. to shade and then gradually exposed to sun. Sixty per cent survival was noticed. These plants were then transferred to field condition. Higher percentage of survival could be achieved by keeping the plantlets under controlled conditions (Growth chamber) for a longer period by supplying adequate nutrients (held of quarter MS). MS medium irrigation with water only increased the mortality rate reflecting the requirement of nutrients at initial stages. It was important to maintain low temperature of (18° to 20°) because at 25°± 2°C most of the plantlets died.

Similar, finding has been reported for *Valeriana wallichii* (Mathur *et al.,* 1987) and *Centella asiatica* (Banerjee *et al.,* 1999). Field transfer trails made in the present study indicate that garden soil with organic compost potted plants kept in controlled environment for a longer duration, irrigation with half or quarter strength MS solution and gradual exposure to shade and sun are the desirable conditions to achieve high survival percentage for *S. angustifolia*.

Kn (2.0 mg/l^{-1}) and 2, 4-D (2.5 mg/l^{-1}) combination is the best for callus proliferation while, 1.5: 1.0 mg/l^{-1} of BA: NAA is most suitable for shooting and MS medium with 0.5 mg/l^{-1} of auxin for rooting. Keeping the plantlets under controlled conditions for longer period with supply of half or quarter MS at low temperature (18 - 20° C) resulted into the higher survival rate.

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