BIOLOGIA PLANTARUM 55 (1): 169-172, 2011

BRIEF COMMUNICATION

In vitro regeneration of an endangered medicinal plant *Picrorhiza scrophulariiflora*

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Abstract

A reproducible *in vitro* regeneration system for Nepalese kutki (*Picrorhiza scrophulariiflora* Pennell) was developed from *in vitro* leaf derived callus. Induction of more than seven shoot buds per explant was achieved on Woody plant medium (WPM) supplemented with 0.53 μM α-napthaleneacetic acid (NAA) and 0.23 μM kinetin (KIN). The shoots were elongated on WPM supplemented with 0.44 μM 6-benzylaminopurine (BAP) and rooted on WPM supplemented with 5.3 μM NAA within 2 weeks. The random amplified polymorphic DNA (RAPD) analysis indicated genetic uniformity of the micropropagated plants with its donor plants.

Additional key words: auxins, callus induction, cytokinins, picroside.

The Nepalease kutki (*Picrorhiza scrophulariiflora* Pennell), family *Scrophulariacea,* is a small herbaceous, endangered medicinal plant generally found in the alpine zone of Sikkim, Nepal and China within the altitude of 3000 - 4000 m (Hara *et al*. 1982, Bantawa *et al.* 2009a). The rhizomes are used in Tibetan and Chinese traditional medicines as well as pharmaceutical industries. It is a rich source of irridoid glycosides such as picroside I, II, III and kutkoside (*e.g*. Jia *et al.* 1999). *P. scrophulariiflora* contains an additional glycosides which are absent in *Picrorhiza kurroa* (Li *et al*. 1998, Smith *et al.* 2000) and total picroside content is much higher than in *P. kurroa* (Bantawa *et al.* 2009b). Conventional propagation methods through seed and root tubers for cultivation of this plant are too slow. Therefore a rapid multiplication will be the best alternative for conservation of this species.

A micropropagation of *P. kurroa* has been previously reported (Lal *et al.* 1988, Upadhyay *et al.* 1989, Pandey *et al.* 2002, Chandra *et al.* 2006). Our group have established a reproducible micropropagation protocols (Bantawa *et al.* 2009b, 2010) and somatic embryogenesis (Bantawa 2009) of *P. scrophulariiflora.* However, there is no report on organogenesis of this plant, , which is the prerequisite for any genetic manipulation and for conservation of this endangered medicinal plant. Thus, an attempt has been made to standardize the regeneration protocol.

We used the *in vitro* leaves from previously established *in vitro* culture for producing callus (Bantawa *et al.* 2009b). In present experiments, young 2-week-old *in vitro* leaves were inoculated on Woody plant medium (Lloyd and McCown 1980; WPM) with various concentration of 2,4-dichlorophenoxyacetic acid (2,4-D), α-napthaleneacetic acid (NAA), kinetin (KIN) or 6-benzylaminopurine (BAP), 3 % sucrose and 0.8 % *Difco* agar (*M/S Himedia Laboratories*, Mumbai, India). The pH of the medium was adjusted with 0.1 M KOH to 5.8 ± 0.1 before autoclaving the medium at 121 °C at 1.06 kg cm^{-2} for 15 min. Cultures were then kept at temperature of 24 ± 2 °C, air humidity of 55 % and a 12-h photoperiod (irradiance of 125 μmol m^{-2} s^{-1}; cool florescent tubes *LIFEMAX-A 73*, *Philips*, Mumbai, India).

Individual microshoots were aseptically excised from the culture bottle and transferred to WPM medium

Received 7 May 2009, *accepted* 15 November 2009.

Abbreviations: BAP - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; KIN - kinetin; NAA - α-napthaleneacetic acid; WPM - Woody plant medium.

Acknowledgments: The authors are thankful to Department of Biotechnology and Department of Science and Technology, Government of India for financial assistance, Mr. Bijoy Gurung for his help to conduct the survey and Mr. Kamal Das for his assistance.

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supplemented alone with various concentration of IBA, IAA or NAA for *in vitro* rooting. The survival percentage was recorded after 60 d.

For genetic fidelity test, genomic DNA was isolated from the leaves of donor as well as *in vitro* grown plants and isolation was performed following the CTAB technique. The isolated DNA was subjected to PCR to generate fingerprinting patterns using a total of 6 most polymorphic decamer primers (*M/S Operon Technologies*, Alameda, CA, USA) (for detail see Bantawa *et al.* 2009d). The DNA amplification was performed in a thermal cycler (*iCycler*, *BioRad*, Hercules, USA). The reaction mixture (0.025 cm^3) contained 10× PCR buffer, 2 mM $MgCl_2$, 100 mM dNTP (*Bangalore Genei*, Bangalore, India), 200 mM primers, 25 ng of template DNA and 0.5 U Taq DNA polymerase (*Bangalore Genei*). The PCR reaction conditions were as follows: one initial cycle of 4 min at 94 °C, followed by 30 amplification cycles of 1 min at 94 °C, 1 min at 35 °C and 2 min at 72 °C, then a final extension step of 10 min at 72 °C. Around 0.015 cm^3 sample of each reaction mixture was then subjected to 1.4 % agarose gel electrophoresis and stained with ethidium bromide. The 1 kb ladder (*Bangalore Genei*) was used as the DNA marker and the amplified fragments were visualized under UV radiation and documented using the *Gel Doc* equipment (*BioRad*). The PCR analyses were repeated at least twice to check the reproducibility.

The experiments were set up in a randomized block design and data were analyzed using analysis of variance (*ANOVA*) to detect significant differences between means (Sokal and Rohlf 1987). Means differing significantly were compared using Duncan's multiple range test (DMRT) at $P \leq 0.05$ with *Statistica* software *ver. 5.0* (*INC StatSoft* 1995).

For the induction of callus, explants were inoculated on WPM medium supplemented with various concentrations of auxins and cytokinins. While callus formation was observed 4 weeks from the cut end of the leaf tissue in all the treatments (Table 1, Fig *1A*) but a continuous sub-culturing leads to a varying degree of response such as rhizogenesis, browning *etc*., apart from the organogenesis. However, callus induced from 2,4-D or NAA alone failed to produce any regeneration, a similar kind of observation was made by Naika and Krishna (2008). The callus appeared hard and white with little red colour which perhaps is an indication of anthocyanin formation. Where as callus produced in presence of cytokinin such as either KIN or BAP was green and friable (Fig *1A*). Auxin such as NAA is known to induce

Table 1. Effect of different combinations and concentrations of plant growth regulators on callus formation and regeneration of shoots from *in vitro* leaf derived explants. Means ± SE, $n = 5$. Means followed by the same letter do not differ significantly according to DMRT ($P \leq 0.05$).

PGRs	Concentrations [μM]	Callus formation [%]	Shoot induction [%]	Shoot number [explant^{-1}]
NAA + KIN	0.53 + 0.23	88.88 ± 0.22 d	35.88 ± 0.22 b	7.8 ± 2.22 a
	2.65 + 0.46	89.88 ± 0.28 c	38.22 ± 0.22 a	4.2 ± 1.30 b
	5.30 + 0.46	88.08 ± 0.84 d	21.00 ± 0.26 c	3.5 ± 1.16 b
	10.60 + 0.46	86.88 ± 0.84 e	5.00 ± 0.88 g	1.2 ± 1.23 c
NAA + BAP	0.53 + 0.22	100.00 a	12.00 ± 0.64 e	3.2 ± 2.37 b
	2.65 + 0.44	100.00 a	15.00 ± 0.45 d	1.2 ± 2.24 c
	5.30 + 0.44	100.00 a	8.00 ± 0.22 f	1.2 ± 3.22 c
	10.60 + 0.88	98.00 ± 0.22 b	0 h	0 d

Table 2. *In vitro* rooting of micropropagated shoots of *Picrorhiza scrophulariiflora.* Means ± SE, $n = 5$. Means followed by the same letter do not differ significantly according to DMRT ($P \leq 0.05$).

Auxins	Concentrations [μM]	Rooting [%]	Root number [explant^{-1}]	Time to emergence [d]
Control	0	26.0 g	2.6 ± 0.15 e	18 - 24
IAA	2.53	30.0 f	3.8 ± 0.25 d	15 - 18
IAA	5.07	69.0 d	5.4 ± 0.22 b	15 - 18
IAA	10.14	29.0 g	2.9 ± 0.18	12 - 17
NAA	2.69	68.0 e	3.6 ± 0.08 cd	15 - 18
NAA	5.37	96.0 a	6.7 ± 0.22 a	10 - 14
NAA	10.74	79.0 b	4.9 ± 0.36 b	10 - 14
IBA	2.46	68.0 e	3.4 ± 0.33 bcd	11 - 18
IBA	4.92	75.0 c	4.1 ± 0.15 bc	14 - 18
IBA	9.84	66.0 d	2.8 ± 0.15 d	14 - 21

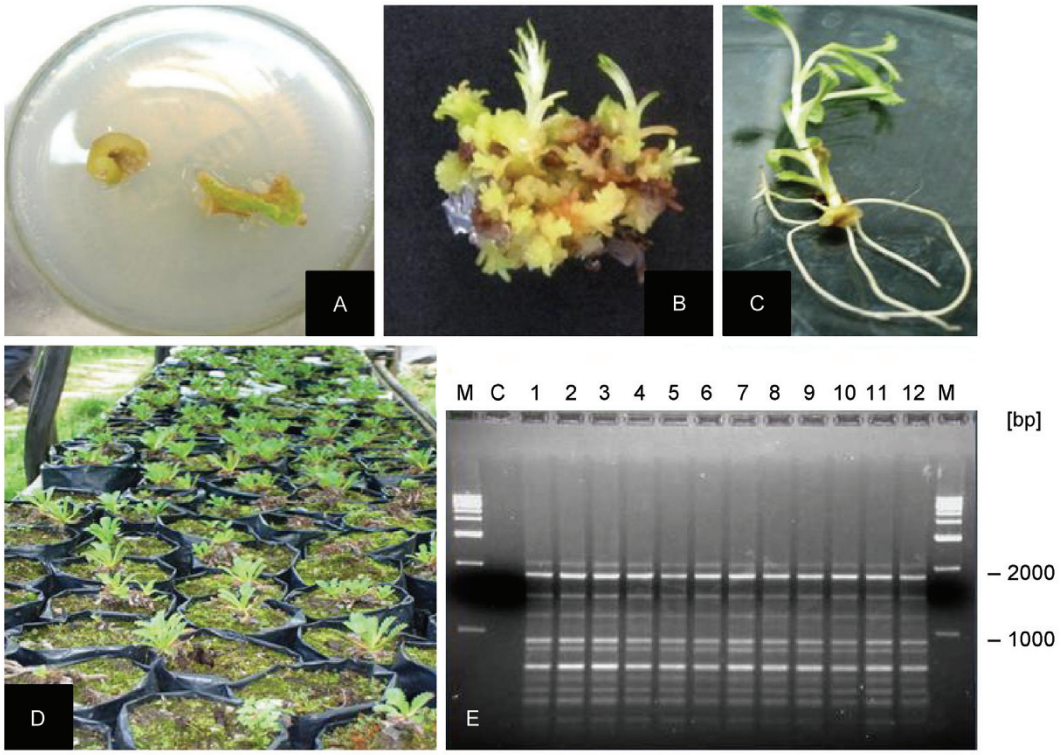

Fig. 1. Plant regeneration from *in vitro* leaf derived callus. *A* - Initiation of callus from *in vitro* grown leaf. *B* - Organogenesis from the callus after 8 weeks. *C* - *In vitro* rooting after 8 weeks on WPM with 5.3 µM NAA. *D* - Hardened plants after 8 months. *E* - DNA fingerprinting patterns generated with primer OPE 17 among micropropagated plants and donor plant (M - 1 kb ladder, C - positive control, 1 - donor plant, 2 - 12 - regenerated plants).

good quality callus and organogenesis in a number of medicinal plants (Sivakumar and Krishnamurthy 2000, Saha *et al.* 2003). However, our result showed that NAA alone had no effect on callus induction and shoot initiation of *P. scrophulariiflora* which concur with the observation of Alagumanian *et al.* (2004). When NAA (0.53 - 10.6 µM) along with either KIN or BAP were used, a wide range of regeneration was found (Table 1). The result showed that callus induction was higher when BAP as compared to KIN was used together with NAA. Subsequently, more shoots were produced in KIN containing media, although no significant difference was noticed in the number of explants producing callus, NAA (0.53 µM) along with KIN (0.23 µM) was the best combination for organogenesis (from 38 % of explant with an average of 7 - 8 shoot buds per explants; Fig. *1B*).

Individual shoots (3 cm) regenerated from callus were separated and transferred to elongation media, *i.e.*, WPM with 0.44 µM BAP (Bantawa *et al.* 2009b) for another 4 weeks which occasionally gives multiple shoots. Then, individual shoots were put on the rooting medium consisting of WPM with various auxins (Table 2). Complete plantlets were formed on WPM with 5.3 µM NAA. The initiation of root primordial was visible after 7 d, prominently visible after 14 d. Within 8 weeks, a maximum of 6 - 7 roots (4 - 6 cm long) per shoot (Fig 1*C*) was produced. Survival rate of plants subsequently transferred for hardening was 90 %. After another 10 months the well hardened plants were distributed to the local farmers for planting (Fig *1D*).

DNA fingerprinting using RAPD technique has been applied to many plant species to evaluate clonal fidelity and genetic stability among tissue culture-grown plants and donors (Mondal and Chand 2002, Das and Pal 2005, Huang *et al.* 2009). In *P. scorphulariiflora*, fingerprinting profiles of both the regenerates and the respective donor plants were generated using a total of 6 polymorphic decamer primers. Monomorphic RAPD profiles yielded a total of 54 alleles, with an average of 9 fragments, ranging from 300 to 2000 bp per primer (Fig *1E*), indicating homogeneity among the culture regenerates and genetic uniformity with that of the donor plants. Characterization of *P. scorphulariiflora* plants at the genetic level indicates that the protocol followed in our studies is capable of generating large numbers of clonal plants throughout the year, which is prerequisite for the conservation of the germplasm.

In conclusion, we describe here a simple, reproducible and affordable organogenesis protocol of plant production in *P. scorphulariiflora*. The complete protocol requires 15 - 20 weeks to produce a large number of genetically true-to-type plants. Keeping in view the enormous potential of this age-old medicinal plant, our aim is to use the *in vitro* leaf explants for genetic manipulation.

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