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Rapid In Vitro Production of Cloned Plants of *Uraria picta* (Jacq.) DC—A Rare Medicinal Herb in Long-Term Culture

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Abstract An efficient in vitro process for rapid production of cloned plants of *Uraria picta* has been developed employing nodal stem segments taken from field-grown plants. Explants showed bud-break followed by regeneration of shoots with restricted growth within 12 days on modified Murashige and Skoog's medium supplemented with 0.25 mg l^{-1} each of 6-benzylaminopurine and indole-3-acetic acid and 25 mg l^{-1} adenine sulfate. Normal growth of shoots with good proliferation rate was achieved by reducing the concentrations of 6-benzylaminopurine and indole-3-acetic acid to 0.1 mg l^{-1} each and incorporating 0.5 mg l^{-1} gibberellic acid in the medium in which, on an average, 19.6 shoots per explant were produced. Further, during successive subcultures, increased concentrations of adenine sulfate (50 mg l^{-1}) and gibberellic acid (2 mg l^{-1}) along with the addition of 20 mg l^{-1} DL-tryptophan were found conducive to control the problem of necrosis of shoots. In this treatment, several "crops" of shoots were obtained from single culture by repeated subculturing of basal portion of stalk in long-term. Isolated shoots rooted 100% in 0.25 mg l^{-1} indole-3-butyric acid. In vitro-raised plants after hardening in inorganic salt solution grew normally in soil and came to flowering. Genetic fidelity of in vitro-raised plants was ascertained by rapid amplified polymorphic DNA (RAPD) markers. Also, quantitative estimation of two isoflavonones in their root extracts further confirmed true-to-type nature of plantlets.

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Introduction

The genus *Uraria* is represented by more than 11 species known from India, of which *Uraria picta* is well known for its medicinal properties. It is a perennial herb, distributed throughout Bangladesh, India, Sri Lanka, Tropical Africa, Malay Islands, and the Philippines [1–3]. The whole plant is medicinally important and is used by certain Adivasi and native tribes [4]. Traditionally, the plant is used as an antidote to the venom of a dangerous Indian snake, *Echis carinata* [2]. The fruits and pods are effective against oral sores in children, and the roots are used against cough, chills, and fever [2, 3]. Two isoflavonones isolated from *U. picta* have antimicrobial activity [5]. The plant is also known for healing fractures, as it has the property of accumulation of phosphorous and deposition of calcium. The species is increasingly becoming rare due to overexploitation by various pharmaceutical industries as well as local tribes for medicine and trade coupled with poor seed viability[6] (personal observation). Hence, there is an urgent need to develop in vitro methods for rapid production of cloned plants of *U. picta* for providing uniform raw material for medicinal purposes as well as for its rehabilitation in natural habitat for conservation and sustainable utilization. In the present communication, we report an in vitro method for rapid production of cloned plants of *U. picta*, employing nodal stem segments, in long-term culture.

Materials and Methods

Establishment of Aseptic Shoot Cultures

Growing twigs from fresh flush of shoots were collected from a 3-year-old plant growing in the Herbal Garden of Deendayal Research Institute, Chitrakoot, Madhya Pradesh. The explants, comprising single node stem segments of 2–3 cm in size, were washed thoroughly in running tap water for 30 min, pretreated with 5% v/v Labolene (GlaxoSmithKline Pharmaceuticals Ltd., Mumbai, India) solution containing Tween 20 (one drop per 100 ml of solution) for 5 min and surface-sterilized with 0.1% $HgCl_2$ solution for 8 min. The surface-sterilized explants were washed thoroughly with sterilized distilled water four to five times, trimmed at the cut ends, and cultured in the nutrient medium.

Culture Media and Conditions

The modified Murashige and Skoog [7] (MS) medium was used in the present investigation, which differed from the original MS medium in having the following changes: $MgSO_4.7H_2O$ 450 mg l^{-1}, $(NH_4)_2SO_4$ 250 mg l^{-1}, thiamine–HCl 0.2 mg l^{-1}, pyridoxine–HCl 0.1 mg l^{-1}, ascorbic acid 10 mg l^{-1}, and glycine 3 mg l^{-1}. The pH of all the media was adjusted to 5.8 before adding 0.75% (w/v) Qualigens agar (GlaxoSmithKline Pharmaceuticals Ltd., Mumbai, India) and autoclaved at 121 °C (1.08 kg cm^{-2}) for 15 min. The cultures were incubated under 25 μmol m^{-2} s^{-1} quantum flux density for 15 h/day at 27±1 °C temperature and 70±5% relative humidity (RH).

Bud-break and Shoot Multiplication

For bud-break, nodal stem segment explants were cultured in modified MS medium supplemented with 0.25 mg l^{-1} 6-benzylaminopurine (BAP), 0.25 mg l^{-1} indole-3-acetic acid (IAA), and 25 mg l^{-1} adenine sulfate (AdS). The regenerated shoots having two nodes were excised and subcultured in the same medium for induction of multiple shoots. For improving the restricted growth of regenerated shoots having condensed internodes and tiny leaves and augmenting the rate of shoot multiplication, the effect of different concentrations of gibberellic acid (GA; 0.1, 0.25, 0.5, 0.75, 1.0, 1.10, and 1.25 mg l^{-1}) along with 0.1 mg l^{-1} each of BAP and IAA was monitored. In addition, for circumventing the severe problem of necrosis and browning of shoots, particularly at the central region during successive subculturing, the effect of different inorganic salts, Na_2SO_4 (100 mg l^{-1}) and K_2SO_4 (100 mg l^{-1}), and ascorbic acid (25 mg l^{-1}) and amino acids, L-glutamine (25 mg l^{-1}) and DL-tryptophan (20 mg l^{-1}), as well as increased concentrations of AdS (50 mg l^{-1}) and GA (1 mg l^{-1}) in various combinations were seen.

Rooting of Shoots

For root induction, the well-developed isolated shoots (3–4 cm in size), excised from cultures of proliferating shoots, were cultured on half-strength MS medium supplemented with different auxins, namely, IAA, indole-3-butyric acid (IBA), indole-3-propionic acid (IPA), α-naphthaleneacetic acid (NAA), and α-naphthoxyacetic acid (NOA) at the concentration of 0.25 mg l^{-1}. In case of IBA, the effect of other concentrations (0.1, 0.5, 0.75, 1.0, and 1.25 mg l^{-1}) was also seen besides 0.25 mg l^{-1}.

Acclimatization of In Vitro-Raised Plantlets

The rooted shoots were taken out from the culture tubes; their root system washed off under running tap water to remove traces of nutrient agar and were first hardened in half strength Knop's solution [8] fortified with trace elements and iron of MS medium for 30 days in a hardening chamber under higher humidity (99% RH), which was gradually reduced to 70% RH during a period of 30 days and thereafter they were transplanted to potted Soilrite for 60 days. The hardened plants were finally transferred to potted soil (3 soil/1 leaf mould) and kept in a glasshouse.

Molecular Analysis

Total genomic DNA was extracted from young leaves of mother plant and in vitro-raised plants of *U. picta* by cytyl trimethyl ammonium bromide (CTAB) procedure [9]. Ten arbitrary decamer primers (Bangalore Genei, India) were used for polymerase chain reaction (PCR). PCR was performed in 20-ml reaction mixture containing 5 ng template DNA, 1 unit of Taq DNA polymerase, 100 μM dNTPs, 1 μM primer, 2.5 mM $MgCl_2$, 10 mM Tris–HCl (pH 9), 50 mM KCl, and 0.01% gelatin. PCR amplification was performed using a PTC-100 Peltier Thermal Cycler (MJ Research, Waltham, MA) under the following conditions: preheating for 4 min at 94 °C; 45 cycles of 15 s at 94 °C, 45 s at 36 °C, and 1.5 min at 72 °C, and elongation was completed by a final extension of 4 min at 72 °C. After amplification, the PCR product was resolved by electrophoresis in 1% agarose gel with 19 Tris–borate–EDTA (TBE) buffer. Bands were visualized by staining with ethidium bromide (0.5 μg/ml) under UV light and photographed.

Quantification of Isoflavonones 5,7-Dihydroxy-2-Methoxy-3,4-Methylendioxyisoflavonones (1) and 4,5-Dihydroxy-2,3-Dimethoxy-7-(5-Hydroxy Oxychromen-7yl)-Isoflavonone (2) in Mother Plant and In Vitro-Raised Plants

Plant Materials

The roots collected from the in vitro-raised plants as well as from the mother plant of *U. picta* were dried under shade and stored at room temperature. The dried roots were powdered and used for extraction of isoflavonones.

Extraction and Isolation Of Isoflavonones

One hundred grams of powdered roots of mother plant and of in vitro-raised plants was separately extracted first with petroleum ether (60–80 °C) then chloroform and finally with methanol in a Soxhlet apparatus to obtain residues 1, 2, and 3, respectively. Four grams of the residue 3 of both the types of materials was fractionated by flash column chromatography over a silica gel (250–400 m mesh) by using chloroform and methanol in increasing polarity for the isolation of pure isoflavonones. Seventy-five milligrams of compound (1) was collected from fractions 43–55, and 25 mg of compound (2) was collected from fractions 60–75.

High-Performance Liquid Chromatography and Quantification Of Isoflavonones (1) and (2) in Different Samples

The purity of isoflavonones was confirmed by high-performance liquid chromatography (HPLC). The stock solutions of 1 mg/ml isoflavonones (1) and (2) were prepared in MeOH. From the stock solutions, dilution of 2–20 μl of each sample was taken in triplicate and analyzed independently by HPLC, and the standard curve was plotted between concentration and peak area.

Fifteen microliters of each sample of mother plant as well as of in vitro-raised plants was applied in triplicate for quantification of isoflavonones (1) and (2) by using regression equation of calibration curve.

A Merck Chromolith performance RP-18 column (100 mm×4.6 mm ID) and the solvent methanol–water containing 1% trifluoroacetic acid (20:80) were used at constant flow rate of 1 ml/min. PDA detector set at wavelength of 265 nm. All the analyses were performed in triplicate.

The purified compound was characterized on the basis of UV, ^{1}HNMR, ^{13}CNMR, and ESI-MS data.

Chemicals Used

Trifluoroacetic acid and the solvents used for extraction and chromatography were of analytical grade purchased from Merck, Mumbai, India. Before use, the solvents were filtered through a 0.45-μm Millipore membrane (Bangalore Genei) after sonication for 15 min.

Statistical Analysis

The data were collected after 4 weeks of culture for shoot regeneration as well as rooting in isolated shoots. There were 10 replicates per treatment, and the experiments were repeated

thrice. The results are expressed as a mean±SE of three experiments. The data were analyzed by Student's unpaired *t* test, and treatment mean values were compared at $P \leq 0.05$–0.001. All statistical analyses were performed using InStat Statistical Software package.

Results and Discussion

Nodal stem segment explants cultured on modified MS medium supplemented with 0.25 mg l^{-1} each of BAP and IAA along with 25 mg l^{-1} AdS showed more than 90% bud-break followed by shoot regeneration within 12 days of culture, but the regenerated shoots remained stunted with restricted growth of internodes and had poorly developed leaves. To achieve optimum shoot proliferation with normal shoot growth and foliage development, the concentrations of BAP and IAA were reduced to 0.1 mg l^{-1} each, and GA at different concentrations (0.1, 0.25, 0.50, 0.75, 1, 1.10, and 1.25 mg l^{-1}) was incorporated in nutrient medium. Among different concentrations of GA, 0.5 mg l^{-1} concentration was found most effective. In this treatment, on an average, 19.6 shoots having an average of 5.3-cm height and 8.5 leaves per shoot were formed (Table 1). The stimulatory effect of GA on internodal elongation is well documented in literature [10–13]. It is pertinent to mention that in our study, the rate of regeneration of shoots is quite higher, i.e., 19.6 shoots per explant as compared with only 7.16 shoots per nodal explant as reported earlier [6]. However, by employing callus, they could get 17.35 shoots cm^{-2} callus, but in this case, the resultant plants may not be clonal as the callus has been reported to be genetically unstable [14, 15] and thereby defeating the very purpose of cloning. However, the proliferating shoots during successive subcultures exhibited a severe problem of excessive browning and necrosis, mostly at the central region. To overcome this problem, different inorganic salts (Na_2SO_4 and K_2SO_4), ascorbic acid, amino acids (L-glutamine and DL-tryptophan), as well as increased concentrations of AdS and GA in various combinations were incorporated in the culture medium, and the data are presented in Table 2. Among all the treatments, a combination of 20 mg l^{-1} DL-tryptophan, 50 mg l^{-1} AdS, and 1 mg l^{-1} GA was found optimum in which very good proliferation of healthy shoots with good foliage development and virtually no browning and necrosis at the central region was achieved. The beneficial

Table 1 Effect of different concentrations of GA on shoot regeneration in nodal stem segments of *U. picta* (0.1 mg l^{-1} IAA, 0.1 mg l^{-1} BAP and 25 mg l^{-1} AdS were common to all the treatments).

Segment no.	GA (mg l^{-1})	% Regeneration of explants	No. of shoots per explant (mean±SE)[a]	Length of shoots (mean±SE; cm)[a]	No. of leaves (mean±SE)[a]
1	0.10	20	3.4±0.34*	1.7±0.52*	3.4±1.03**
2	0.25	40	7.5±0.48**	1.2±0.39***	3.6±1.33***
3	0.50	100	19.6±2.69**	5.3±1.13***	8.5±1.38**
4	0.75	80	10.8±1.02**	2.1±1.03*	4.9±1.18*
5	1.00	55	7.9±0.64*	1.9±0.64***	4.6±1.05**
6	1.10	65	8.1±1.49***	2.4±0.58**	3.8±1.22***
7	1.25	25	3.70±0.55*	1.3±0.33**	3.1±1.09*

[a] Values represent mean±standard error of 10 replicates per treatment in three repeated experiments. Significantly different at *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, respectively, according to Student's unpaired *t* test

Table 2 Effect of inorganic salts, ascorbic acid, amino acids, AdS, and GA on necrosis of proliferating shoots of *U. picta* (0.1 mg l^{-1} BAP, 0.1 mg l^{-1} IAA, 25 mg l^{-1} AdS, and 0.5 mg l^{-1} GA were common to all the treatments except mentioned otherwise).

Segment no.	Treatment (concentration in mg l^{-1})	% Extent of necrosis of shoots (mean±SE)
1	Control	80±2.50**
2	Na_2SO_4 (100)	98±1.164**
3	K_2SO_4 (100)	99.2±2.81**
4	Ascorbic acid (25)	80±2.98*
5	L-Glutamine (25)	90±0.41*
6	DL-Tryptophan (20)	60±2.88**
7	DL-Tryptophan (20), AdS (50)	9.9±1.04*
8	DL-Tryptophan (20), AdS (50), GA (1)	0.3±0.21**

Values represent mean±SE of 10 replicates per treatment in three repeated experiments. Significantly different at $*P \leq 0.05$, $**P \leq 0.001$, respectively, according to Student's unpaired *t* test. Only significant treatments are recorded in the table

role of tryptophan in enhancing vegetative growth by foliar application has been reported in in vivo studies also in *Ocimum basilicum* [16], *Pelargonium graveolens* [17], and *Philodendron erubescens* [18]. The effectiveness of AdS for improved multiplication of shoots is also reported by many workers [19–21]. In the aforesaid treatment, sustained rate of shoot proliferation along with normal growth of regenerated shoots has been maintained for the last 5 years, observed so far (Fig. 1a). In this process, each culture of proliferating shoots served as a source to obtain several "crops" of shoots by repeated subculturing after every 25 days of the basal portion of differentiating stalk, on the same morphogenetic medium, remained after clipping off developed shoots each time for inducing rooting in them to produce plantlets. In this way, continuous rapid production of enormous number of clonal plantlets of *U. picta* through long-term culture of proliferating shoots could be facilitated.

For root induction in excised shoots, among different auxins used at the concentration of 0.25 mg l^{-1}, IBA was found more effective in comparison to other auxins, as 70% rooting was obtained in IBA, whereas only 50%, 30%, and 20% rooting was induced in IAA, IPA, and NAA, respectively, and no rooting in NOA was observed. Hence, for improving the rooting percentage, different concentrations of IBA (0.1, 0.25, 0.5, 0.75, 1, and 1.10 mg l^{-1}) were employed, and the data are presented in Table 3. Among these, 0.5 mg l^{-1} concentration of IBA was found optimum as in this treatment 100% rooting in isolated shoots was achieved (Fig. 1b). Furthermore, the number of roots formed per shoot without intervening callusing was also maximum, i.e., average 16.20, which resulted in more than 90% ex vitro survival of plantlets. The effectiveness of IBA for root induction in legumes has been reported by many workers [22–24]. The in vitro-raised plants after being acclimatized in liquid culture survived 100% on transplantation first in Soilrite and thereafter in soil, where they registered uniform luxuriant growth and came to flowering after approximately 5 months of transplantation (Fig. 1c, d). In the present study, no differences were observed between mother plant and plantlets regenerated from nodal stem segment explants by RAPD analysis. Out of 20 primers screened, 8 primers produced clear and scorable amplification products. Each primer produced a unique set of amplification products ranging in size from 400 to 3,500 bp with primer OPA 9: 3′GGGTAACGCC 5' (Fig. 1e). The number of bands for each primer varied from three in OPA 4 to eight in OPA

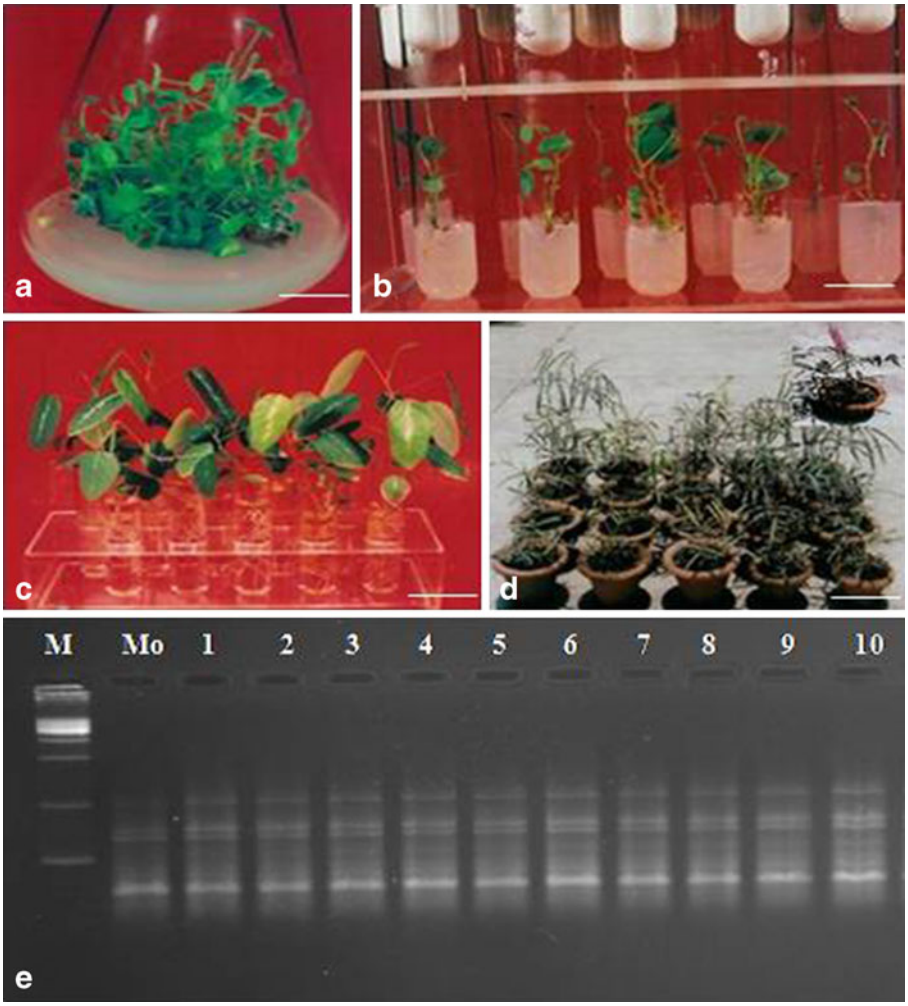

Fig. 1 **a–e** Cultures of *U. picta*. (**a**) Proliferating shoots as seen after 5 years of culture (bar, 1.28 cm). (**b**) Rooting of isolated shoots (bar, 2.78 cm). (**c**) Acclimatization of in vitro-raised plantlets in liquid culture (bar, 5 cm). (**d**) In vitro-raised potted plants showing good growth; in *inset*, a potted plant in flowering is shown (bar, 10 cm). (**e**) RAPD marker analysis of in vitro-raised field-grown plants and mother plant; *lane M*, 500 bp DNA size markers, *Mo*, DNA from mother plant; *lanes 1–8*, DNA from randomly selected regenerated plantlets

9. RAPD fingerprints have been shown to be useful to confirm the genetic fidelity of plantlets regenerated through in vitro culture [25–27].

Apart from molecular analysis, the quantitative estimation of two isoflavonones, 5,7-dihydroxy-2-methoxy-3,4-methylendioxyisoflavonone (1) and 4,5-dihydroxy-2,3-dimethoxy-7-(5-hydroxy oxychromen-7yl)-isoflavonone (2) isolated from the roots of mother plant as well as from the in vitro-raised plants of *U. picta*, showed no significant difference in the concentration. The data are presented in Table 4. The purity (99%) of estimated compounds was further confirmed by comparison with published data of standard

Table 3 Effect of different concentrations of IBA as well as 0.25 mg l^{-1} of IAA, IPA, NAA, and NOA on rooting of in vitro regenerated shoots of *U. picta* using half-strength MS medium.

Auxin (mg l^{-1})					Percentage rooting	No. of roots/shoot (mean±SE)[a]
IAA	IBA	IPA	NAA	NOA		
0.25					50	5.43±1.45*
	0.10				60	12.33±3.53**
	0.25				70	14.23±2.52**
	0.50				100	16.20±2.44***
	0.75				60	9.50±3.50*
	1.00				40	8.50±7.50*
	1.10				20	2.75±1.00*
		0.25			30	3.15±0.34*
			0.25		20	4.30±1.50*
				0.25	00	00

[a] Values represent mean±standard error of 10 replicates per treatment in three repeated experiments. Significantly different at $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, respectively, according to Student's unpaired *t* test

isoflavonones (1) and (2) by UV, ^{1}HNMR, ^{13}CNMR, and ESI-MS [5, 28] and was found identical. This study further ensures the genetic fidelity of plantlets regenerated through in vitro culture.

Conclusion

The present investigation provides a complete in vitro process which is simple, reproducible and efficient for rapid production of cloned plants of an important rare and threatened medicinal herb, *U. picta* in long-term culture. The process can be exploited for catering enough raw material to various pharmaceutical industries as well as for providing large number of cloned plants for its rehabilitation in natural habitat for conservation and sustainable utilization.

Table 4 Concentrations of isoflavonones (1) and (2) found in root extracts of the in vitro-raised plants and mother plant of *U. picta* as measured by HPLC.

Isoflavonones	Concentration (mg l^{-1})[a]									
	Mother plant	10 in vitro-raised plants								
		1	2	3	4	5	6	7	8	9	10
(1)	0.8±0.1	0.83±0.2	0.78±2.4	0.76±1.4	0.79±1.0	0.86±3.3	0.82±2.3	0.78±0.2	0.79±1.4	0.84±1.6	0.85±3.1
(2)	0.69±1.4	0.7±1.0	0.65±0.1	0.68±1.2	0.75±2.2	0.75±1.0	0.71±3.1	0.67±0.1	0.73±3.2	0.7±1.4	0.68±1.4

[a] Values are mean±SE of isoflavonones content

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