

***In Vitro* Regeneration of *Phyllanthus amarus* Schum. and Thonn.: An Important Medicinal Plant**

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Abstract

An efficient *in vitro* plant regeneration protocol was developed for the medicinally potent plant species *Phyllanthus amarus* Schum. and Thonn. (Euphorbiaceae) using nodal segment as explant. Maximum multiplication of shoots (15.275 ± 0.96) was achieved on Murashige and Skoog's medium supplemented with BAP (0.5 mg/l) after 3-4 weeks of inoculation. The shoots were separated from cluster and subcultured for their elongation on the same medium. *In vitro* flowering was also observed on the elongated shoots after 3-4 weeks of sub culturing on the shoot elongation medium. *In vitro* rooting was obtained on half strength MS medium supplemented with IBA (0.5 mg/l). Regenerated plants were successfully hardened and acclimatized, 80 % of plantlets survived well under natural conditions after transplantation.

Key words: *In vitro* regeneration, multiple shoots, nodal segments, *Phyllanthus amarus*

Introduction

Phyllanthus amarus Schum. and Thonn. commonly known as Bhui amla, kidney cleansing herb or stone breaker, is an herbaceous medicinally potent plant belonging to family Euphorbiaceae. This plant is distributed in all the tropical regions of the planet including India, Sri Lanka, China, Bahamas etc. It is a common kharif weed occurs both in cultivated field and wastelands. It has been traditionally used in the treatment of a variety of ailments including jaundice, asthma, ulcer, hepatitis, tuberculosis, malaria, dysentery, gonorrhea, syphilis, cough, diarrhea, vaginitis and urinary diseases and other hepatic disorders (Bharatiya, 1992; Unander, 1998). This plant is a favorite choice of the rural people because of its immense medicinal properties like antidote, against liver diseases, antiviral

properties, antioxidant, hepatoprotective, anti inflammatory and strong inhibitory effect against neurogenic (Thyagarajan *et al.*, 1998; Kiemer *et al.*, 2003; Chattopadhyay *et al.*, 2006). These all medicinal properties are due to the presence of several natural compounds especially phyllanthin, hypophyllanthin, quercetin and β -sitosterol (Ishimaru *et al.*, 1992; Calixto *et al.*, 1998).

Overexploitation and population pressure have resulted large scale destruction of this plant from natural habitat. This is a seasonal plant due to which the continuous supply of this plant to the pharmaceutical companies is uncertain. Therefore, there is an urgent need to develop quick regeneration protocol to provide plant material for phytochemical and pharmacological analysis by various pharmaceutical companies. In the present

research paper, we are providing a rapid and an efficient regeneration protocol of this potent medicinal plant.

Abbreviation: BAP-6-benzylaminopurine, IBA-indole-3-butyric acid, 2,4-D-2,4-dichlorophenoxyacetic acid, Kn-6-furfurylaminopurine, IAA-indole-3-acetic acid

Materials and methods

Plant material

Nodal segments (0.5-1.5 cm. long) were procured from 1-2 months old healthy mother plant growing in the Botanical garden, University of Rajasthan, Jaipur.

Sterilization

The picked nodal segments were kept under running water for about 15-20 minutes to remove soil particles and then rinsed with liquid detergent (Teepol 1%) (v/v) for 5-10 minutes. Then they were rinsed with sterile double distilled water at least thrice to get rid of teepol. Prior to inoculation, these explants were subsequently surface sterilized in the laminar air flow chamber with 0.1% mercuric chloride (HgCl₂; w/v) for 2 minutes followed by repeated rinsing with sterile double distilled water.

Culture media

The surfaced sterilized explants were then aseptically inoculated on sterile MS medium (Murashige and Skoog, 1962) comprising 3% sucrose as carbon source and 0.8% agar as solidifying agent. The medium was also supplemented with various growth regulators [auxins- IAA (indole-3-acetic acid), IBA (indole-3-butyric acid), NAA, 2, 4-D (2, 4-dichlorophenoxyacetic acid) etc. and cytokinins- BAP(6-benzylaminopurine), Kn (6- furfurylaminopurine)] and pH of the medium was adjusted to 5.8 before

autoclaving at pressure 1.06 Kg/cm⁻³. For multiple shoot induction different concentrations (0.5, 0.75, 1.0, 1.25, 1.50, 1.75, 2.0 mg/l) of BAP and Kn alone or in combination were used. However, various auxins such as NAA, 2, 4-D, IBA, IAA at different concentrations (0.5 to 8.0 mg/l) were examined for *in vitro* root induction.

Culture conditions

The cultures were incubated at 25±2°C under cool, white and fluorescent light of 2000-2500 lux and relative humidity of about 55±5%. 16/8 hours photo and dark period were maintained in growth chamber, respectively. For each treatment, eight replicates were used and every experiment was repeated at least thrice. Data on multiple shoot induction; elongation and rooting were taken and statistically analyzed. Observations were recorded periodically.

Induction of Multiple shoots

The aseptic nodal segments were cultured on MS medium supplemented with 0.5, 0.75, 1.0, 1.25, 1.50, 1.75, 2.0 mg/l of BAP and Kn alone and/or in combination. Physiological conditions and number of shoots per explants were observed periodically. Maximum multiplication of shoots was noticed on MS medium congealed with BAP (0.5 mg/l). *In vitro* elongation was also attained on the same medium. *In vitro* flowering was also observed on MS medium supplemented with BAP (0.5 mg/l) on the shoots obtained prior to root induction.

Establishment of Root cultures, Hardening and Acclimatization

In vitro elongated shoots (6-7 cm.) with at least 3-4 nodes were taken out from the

culture vials and transferred to half strength MS medium with different concentration (0.5-8.0 mg/l) of auxins like IBA, IAA, 2,4-D, NAA for root induction. The plantlets were taken out from culture vessel without damaging the delicate root system and rinsed with distilled water to remove adhering agar and then transferred to polycups containing vermicompost and autoclaved soil (1:3). Polycups were covered with inverted glass beakers to maintain high humidity and kept in culture chamber. They were gradually exposed from artificial environmental conditions to natural conditions for their acclimatization.

Results and discussion

During the present research investigation, maximum number of shoots (15.275 ± 0.96) was obtained when nodal explants inoculated on MS medium supplemented with BAP (0.5 mg/l) after 3-4 weeks (Figure 1). Further, increase in the concentrations of BAP decreased the number of multiple shoots (Table 1). Similar observations were reported in other plant species such as *Phyllanthus urinaria* (Kalidass and Mohan, 2009), *Codiaeum variegatum* (Nasib *et al.*, 2008), *Dictyospermum ovalifolium* (Thoyajasksha and Ravishankar, 2001), *Cunila galioides* (Fracaro and Echeverrigaaray, 2001), *Strawberry* (Indra and Uppendra, 2000), *Gymemma sylvestre* (Komalavalli and Rao, 2000) and *Vitex negundo* (Sharma *et al.*, 2006). Whereas, in contrast to the present results, Catapan *et al.* (2002) observed that Kn was proved to be the best for maximum shoot proliferation in *Phyllanthus urinaria*.

These multiple shoots were separated from the clump and subcultured on the same medium for their elongation in *in vitro* conditions. Elongation of shoots were also

reported in other plant species like *Costus speciosus*, (Robinson *et al.*, 2009) and *Baliospermum montanum* (Sasikumar *et al.*, 2009) on the same medium in which they were initiated. However, in contrast to the multiplication medium, Datta *et al.* (2007) reported elongation of shoots on MS medium having different growth regulators in *Jatropha curcas*. After elongation, *in vitro* flowering was also observed in few shoots on the same medium incorporated with BAP (0.5 mg/l) (Figure 2).



Figure 1. Multiplication and elongation of *Phyllanthus amarus* shoot on MS medium supplemented with BAP 0.5 mg/l.

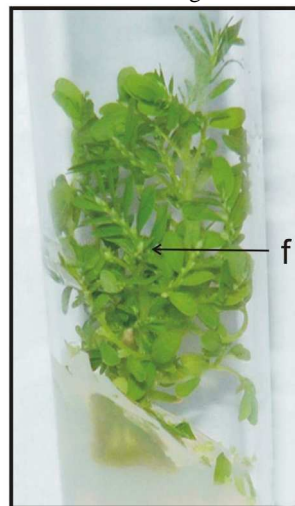


Figure 2. *In vitro* flowering on the same medium after 3-4 weeks of sub culturing (f-flower).

Table 1. Effect of cytokinin(s) on axillary bud proliferation in *Phyllanthus amarus* Schum. and Thonn.

Growth regulators (mg/l)	Mean number of shoots produced/ explants (*Mean \pm t _{0.05} S.E.)
Control (0)	Nil
BAP	
0.50	15.275 \pm 0.96
0.75	8.850 \pm 2.78
1.0	6.650 \pm 1.02
1.25	3.275 \pm 0.189
1.50	2.600 \pm 2.88
1.75	1.38 \pm 0.08
2.0	1.03 \pm 0.37
Kn	
0.50	3.525 \pm 1.17
0.75	2.975 \pm 2.88
1.0	2.600 \pm 2.88
1.25	1.875 \pm 0.83
1.50	1.325 \pm 0.89
1.75	1.00 \pm 2.16
2.0	0.110 \pm 0.12
BAP + Kn	
0.5 + 0.50	2.325 \pm 2.92
0.5 + 0.75	1.625 \pm 0.85
0.5 + 1.0	1.250 \pm 0.18
0.5 + 1.25	0.750 \pm 0.88
0.5 + 1.50	0.580 \pm 0.13
0.5 + 1.75	0.5 \pm 0.20
0.5 + 2.0	Nil

*Values are 95% confidence limits for Mean and mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.05% probability level

Table 2. Effect of various auxins on root induction in *Phyllanthus amarus* Schum. and Thonn.

Auxin conc. (mg/l)	Rooting response (%)	Root length (*Mean \pm t _{0.05} S.E.)	Remarks
Control	0		Nil
IBA			
0.5	95	3.30 \pm 0.21	Well developed root system, roots were healthy, branched, white in color and fast growing having root hairs
1.0	65-70	2.30 \pm 0.09	
1.5	40-50	1.82 \pm 0.05	
2.0-8.0	Nil		
2,4-D			
0.5	30-40	1.25 \pm 0.05	Roots developed were short, thin and without root hairs.
1.5	20-30	1.08 \pm 0.02	
2.0-8.0	Nil		
IAA & NAA			
0.5- 8.0	Nil		No response

*Values are 95% confidence limits for Mean and mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.05% probability level

In vitro root induction was obtained when the elongated shoots were transferred on half strength of MS medium fortified with IBA (0.5 mg/l) (Table 2, Figure 3). IBA is more important plant growth hormone for the root induction in tissue culture. Omran *et al.* (2008) also noticed *in vitro* rooting on IBA in *Lins culinaris* Medik., which is in consonance to the present research work. In contrast to this, Catapan *et al.* (2000) reported that, NAA and IAA enhanced root proliferation in *Phyllanthus caroliniensis*.

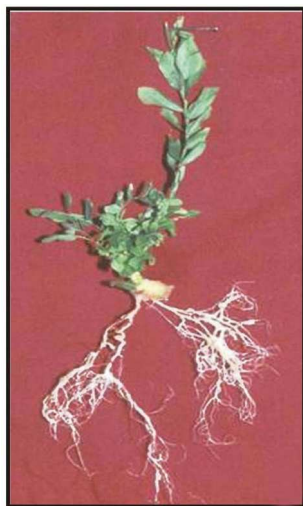


Figure 3. *In vitro* rooting on half strength MS medium containing 0.5 mg/l of IBA



Figure 4. Hardening and acclimatization of regenerated plants

In vitro raised plantlets were taken out from the culture bottle and hardening and acclimatization was done by the method described in “Materials and Methods” (Figure 4).

The present paper presents the protocol for large scale clonal propagation of *P. amarus* without any seasonal influences. However, other reports are also available on regeneration of this plant species, but they are not much efficient and taking more time as compared to the present protocol.

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