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In Vitro Organogenesis in *Phyllanthus amarus* Schum. and Thonn.

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ABSTRACT

A procedure is outlined for organogenesis of *Phyllanthus amarus* using nodal segments and shoot tips. Multiple shoots were induced from both explants on Murashige and Skoog medium (MS) supplemented with benzyl amino purine (BAP) and kinetin (Kin). MS + BAP (4.0 mgl^{-1}) + Kin (4 mgl^{-1}) resulted in maximum multiple shoots from shoot and nodal segments. Among the explants, nodal segments produced more shoots (5.94 shoots/explant) than shoot tips (5.22 shoots/explant). The shoot tip-derived plants (95%) showed a higher survival percentage than nodal segments (88%). The addition of gibberellic acid (GA_3) to the medium enhanced shoot length. Sub-culture of multiple shoots onto MS medium supplemented with BAP (2.0 mgl^{-1}) and GA_3 (0.5 mgl^{-1}) resulted in a 7-fold increase in shoots. Rooting (87%) of the shoots was best achieved in ½ MS + IBA (0.5 mgl^{-1}) + IAA (0.5 mgl^{-1}). The plantlets were hardened in a mixture of sand and vermiculite (1: 1) and successfully established in natural soil, where they grew and matured normally.

Keywords: bhumyamalaki, mass production, micropropagation, nodal segment, shoot tip

INTRODUCTION

Phyllanthus amarus Schum. and Thonn. (Euphorbiaceae), known popularly in the Indian system of medicine as "Bhumyamalaki", has been traditionally used in the treatment of a variety of ailments including hepatic disorders (Bharatiy 1992; Unander 1998). The plant is being used as one of the important ingredients in many indigenous polyherbal formulations and other ayurvedic preparations. This plant is a favorite choice of rural people because of its immense medicinal properties: antidote, against liver diseases, antiviral, antioxidant, heptoprotective, and anti-inflammatory properties and strong inhibitory effect against neurogenic disorders (Kiemer *et al*. 2003; Chattopadhyay *et al.* 2006).

The conventional method of propagation of these species is through seeds. However, poor germination potential restricts their multiplication. Micropropagation offers an alternative method for cloning these plants (Unander 1991; Santos *et al*. 1994). This research describes the *in vitro* propagation of *P. amarus* from nodal segments and shoots tips and successful establishment of plantlets in soil.

MATERIALS AND METHODS

Two month-old plants of *P. amarus* raised from seeds and maintained in the greenhouse of the Medicinal Plant Conservatory of the Botanical Garden, Horticultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore were used as the source of explants. Excised nodal segments (1.0-1.5 cm) were initially washed with teepol for 2 min and then under running tap water for 3 min. This was followed by treatment with 0.1% mercuric chloride for 3 min. After 4-5 washes with sterile distilled water, explants were inoculated on basal medium consisting of Murashige and Skoog (1962) salts and vitamins, 3% sucrose and 0.8% agar. Basal medium was supplemented with various concentrations of kinetin (Kin; 1.0-5.0 mg l^{-1}), benzyl amino purine (BAP; 1.0-5.0 mg l^{-1}) and gibberellic acid (GA_3; 0.5 mg l^{-1}) for multiple shoot formation, indole-3-acetic acid (IAA; 0.25-1.25 mg l^{-1}) and indole-3-butyric acid (IBA; 0.25-1.25 mg l^{-1}) for rooting.

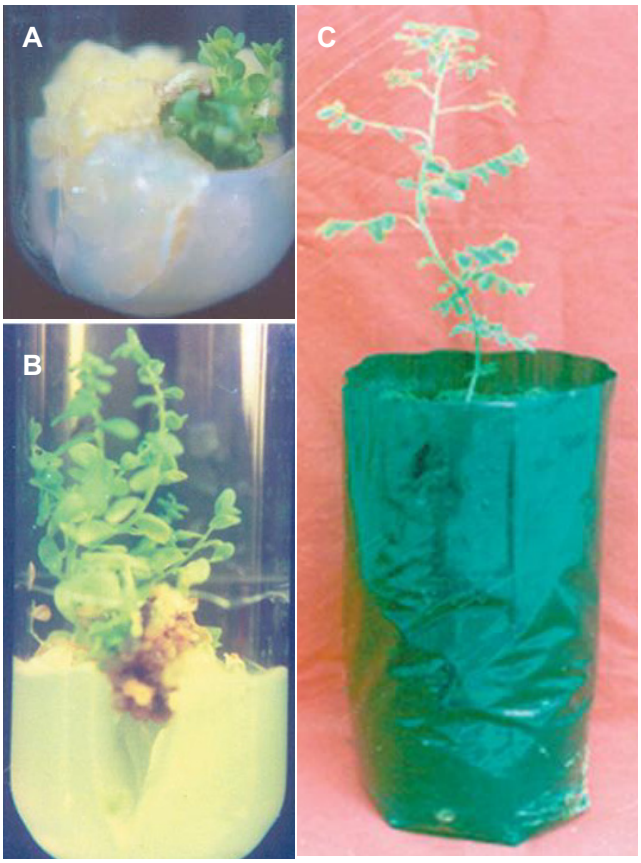

Fig. 1 (**A**) Regenertion of shoots from nodal segments of *P. amarus*. (**B**) Multiple shoots of *P. amarus*. (**C**) Acclimatized *in vitro* plant of *P. amarus.*

The medium was buffered to pH 5.8 and dispensed in 25 × 150 mm culture tubes before autoclaving at 121°C for 15 min. All cultures were maintained at 25 ± 2°C, under a 16-h photoperiod provided by cool white fluorescent light (35 μEM^{-2} S^{-1}) with 70%

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Short Communication

Table 1 Effect of BAP and kinetin on multiple shoot induction from nodal segments and shoot tips of *P. amarus*.

Concentration of growth regulators (mg l^{-1})		Shoot tip				Nodal segment			
BAP	KIN	Survival %	Days taken for shooting	No. of shoots per explant	Length of shoots (cm)	Survival %	Days taken for shooting	No. of shoots per explant	Length of shoots (cm)
1.0	1.0	23.33	39.00	2.77	1.15	33.32	36.88	1.49	1.06
2.0	2.0	75.00	31.50	4.38	1.52	65.59	31.80	2.94	1.28
3.0	3.0	95.00	30.02	6.05	1.73	88.88	29.55	5.94	1.56
4.0	4.0	70.55	33.55	5.22	1.40	55.55	33.11	5.05	1.33
5.0	5.0	55.00	37.88	3.83	1.26	38.59	34.44	2.38	1.18
SEd		4.76	0.81	0.52	0.19	5.94	0.86	0.36	0.07
CD (0.05)		10.62	1.82	1.15	0.41	13.24	1.92	0.80	0.17

relative humidity. Each experiment was performed in triplicate with a total of 20 explants inoculated per treatment.

Axillary shoots, which developed after 30 days of culture, were dissected out individually for further multiplication and this process was repeated continuously every 20 days. Finally, cluster shoots developed and were transferred to basal medium supplemented with 1.0 mgl^{-1} GA_3 for elongation before transferring to ½-strength MS medium containing IBA/IAA for rooting. The rooted plantlets were transferred to plastic pots containing sand and vermiculite (1: 1), humidity being maintained by covering with plastic bags. The survival percentage was determined after 20 days in pots. Statistical parameters like mean, standard error and critical difference for all the observations were assessed by adopting standard methods of analysis as suggested by Panse and Sukhatme (1978).

RESULTS AND DISCUSSION

Multiplication and of axillary shoots was achieved on MS medium with BAP and Kin (3.0 mg l^{-1} each) (**Table 1**) and elongation with 2.0 mg l^{-1} BAP and 0.5 mg l^{-1} GA_3 (**Table 2**). Of these, BAP in combination with both GA_3 and Kin was the most effective in inducing multiple shoots (**Fig. 1B**) from both shoot tips and nodal segments. These results indicate that BAP, a cytokinin, played an important role in induction of multiple shoot formation and was very effective in shoot proliferation. However, BAP at higher concentrations not only reduced the number of shoots formed but also resulted in their stunted growth.

When Kin was added to the culture medium along with BAP, a remarkable effect was seen in the induction of multiple shoots. This was in agreement with the reports suggested by Vincent *et al.* (1992), who found that use of Kin with BAP in the culture medium enhanced multiple shoot induction in *Kaempferia galanga*. Similar effects have already been documented in *Gymnema elegans*, where Kin in the culture medium enhanced bud break in explants (Komalavalli and Rao 1997). Anilkumar *et al*. (2005) reported that maximum multiple shoots in *Ocimum basilicum* L. were obtained in MS medium supplemented with 5 mgl^{-1} BAP and 10 mgl^{-1} Kin.

GA_3 is known to have stimulatory effect on stem elongation in different plants. The same effect was seen in the present study when GA_3 was supplemented to the MS basal media at a lower concentration responded well to elongation (an average length of 1.83 cm) (**Table 2**). This was supported by the findings of Pattnaik and Chand (1996) in *Ocimum sanctum* and Chitra *et al*. (2009) in *Phyllanthus amarus*.

IBA and IAA at a lower concentration (0.5 mg l^{-1}) gave good rooting response (93.63%). Ray and Jha (2002) reported that rooting was highest in *Withania somnifera* microshoots with 0.5 mg l^{-1} IBA inducing 80% rooting.

Table 2 Effect of BAP and GA_3 on multiple shoots development of *P. amarus*.

Concentration of growth regulators (mg l^{-1})		Survival percentage	Days taken for shooting	No. of shoots per explant	Length of shoot (cm)
BAP	GA_3				
1.0	0.5	77.57	21.99	4.55	1.34
2.0	0.5	83.91	14.49	5.15	1.83
3.0	0.5	74.84	28.63	4.77	1.26
4.0	0.5	64.66	30.37	3.75	1.61
5.0	0.5	57.10	30.08	3.38	1.31
SEd		0.74	0.80	0.31	0.26
CD (0.05)		1.64	1.78	0.69	0.38

Table 3 Effect of IAA and IBA on rhizogenesis of *P. amarus*.

Concentration of growth regulators (mg l^{-1})		Percentage response to rhizogenesis	Days taken for rooting	No. of roots per explant	Length of root (cm)
IAA	IBA				
0.25	0.25	78.63	21.20	3.34	3.43
0.50	0.50	93.63	15.31	5.77	5.37
0.75	0.75	89.44	18.30	4.69	2.67
1.00	1.00	83.69	21.21	2.03	2.40
1.25	1.25	74.13	20.21	2.88	2.10
SEd		1.30	0.56	0.34	0.09
CD (0.05)		2.78	1.25	0.75	0.20

Soniya and Das (2002) confirmed that elongated shoots of *Piper longum* rooted in MS medium supplemented with 2.46 µM IBA. Further, a slightly higher concentration of IAA (1.25 mg l^{-1}) and IBA (1.25 mg l^{-1}) also gave good response to rooting, however the percentage of rooting was only 74.13% and yellowing of leaves was also observed (**Table 3**). This might be due to an overdose of auxin. The root elongation phase is very sensitive to auxin concentration and will be inhibited by high concentrations (Thimmann 1977).

The rooted plantlets were transferred to a hardening chamber. Two types of media *viz*., sand, soil and leaf mould (1: 1: 1) and sand and vermiculite (1: 1) were attempted. Survival and establishment was higher in the latter medium (**Table 4**). Within 25 days, plants established better and were transferred to pots with soil, sand, and farmyard manure from where they were planted in the field (**Table 4**). In conclusion, a protocol for the successful multiplication of *P. amarus* through *in vitro* routes is useful for conservation as well as biotechnological improvement of this pharmacologically important plant species.

Table 4 Establishment of *P. amarus* plantlets *in vivo*.

Medium	No. of plantlets transferred	Survival percentage		
		After 1 week	After 2 weeks	After 3 weeks
Sand and vermiculite (1:1)	100	89.12	83.56	80.29
Sand, soil and leaf mould (1:1:1)	100	63.00	45.13	26.48

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