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Bangladesh J. Sci. Ind. Res. **46(2)**, 205-210, 2011

BANGLADESH JOURNAL OF SCIENTIFIC AND INDUSTRIAL RESEARCH

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In Vitro Shoot Proliferation and Plant Regeneration of *Phyllanthus fraternus* Webster (B. Bhuiamla), a Seasonal Medicinal Herb

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Abstract

An efficient system was developed for shoot proliferation and large scale plant regeneration of a seasonal multipotent medicinal herb, *Phyllanthus fraternus* Webster through *in vitro* culture. Shoot tips and nodal explants of young sprouts from selected plants were used as explants. Best shoot induction was observed on MS basal medium supplemented with 0.5 mg/l BAP + 0.1 mg/l GA_3, in which 88% of nodal explants responded to produce maximum number (16.8 ± 0.95) of shoots per culture. *In vitro* raised shoots rooted on half strength MS medium with 0.5 mg/l IBA. For acclimatization and transplantation, the plantlets in the rooting culture tubes were kept in normal room temperature for 7 days before transplanting in pots where plantlets were reared for three weeks. The survival rate of regenerated plantlets was 82%.

Key words: *Phyllanthus fraternus*, Medicinal plant, Shoot proliferation, Regeneration, Acclimatization

Introduction

Phyllanthus fraternus Webster (syn. *Phyllanthus niruri* Hook. f.) commonly known as 'Bhui-amla' belongs to the family - Euphorbiaceae, a small, seasonal herb with alternate compound leaves of small leaflets and axillary, fascicled, short-pedicelled small flowers, grows wild as a weed throughout Bangladesh (Ghani, 2003). It is a popular and valuable traditional medicinal herb used for the treatment of various ailments such as flu, dropsy, diabetes and jaundice (Unander and Blumberg, 1991). The plant is used as a diuretic in dropsical affections and in gonorrhoea, leucorrhoea, dyspepsia, colic, diarrhea and dysentery; it is also regarded as deobstruent, stomachic, febrifuge and anticeptic; leaves are used as poultice on swellings and ulcers; tender shoots are used in curing chronic dysentery; fresh roots are beneficially used in jaundice (Ghani, 2003). The latex is also applied to offensive sores ulcers and mixed with oil it is used in ophthalmia (Anonymous, 1969).

Interest in this plant was further enhanced with reports of its anti-tumor and anti-carcinogenic activity and its potential as a remedy for hepatitis B viral infection (Rajeshkumar *et al.,* 2002). *Phyllanthus fraternus* Webster was also found to have anti-oxidant and hepatoprotective properties and anti-inflammatory potential (Kiemer *et al.,* 2003).

In recent years, there has been an increased interest in *in vitro* culture techniques which offer a viable tool for mass multiplication and germplasm conservation of rare, endangered and threatened medicinal plants (Ajithkumar and Seeni 1998; Prakash *et al*. 1999). Commercial exploitation and elimination of natural habits consequent to urbanization has led to gradual extinction of several medicinal plants. Micropropagation is an effective approach to conserve such germplasm. *In vitro* propagation has proven as a potential technology for mass scale production of medicinal plant species (Azad *et al.* 2005; Lui and Li, 2001; Martin 2002, 2003; Wawrosch *et al.* 2001). It is important, therefore to develop an efficient micropropagation technique for *Phyllanthus fraternus* Webster for rapidly disseminate superior clones. There have been few reports to date on micropropagation of *Phyllanthus fraternus* Webster using shoot tip and nodal explants (Liang and Keng, 2006; Rajasubramaniam and Saradhi, 1997). However, in Bangladesh, there is no report on the establishment of a micropropagation protocol through callus culture for *Phyllanthus fraternus* Webster. The present study was therefore undertaken to develop a technique for shoot proliferation and large scale plant regeneration of this seasonal multi-important medicinal herb through *in vitro* culture.

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Materials and Methods

Phyllanthus fraternus Webster (F. Euphorbiaceae, B. Bhuiamla) grown in BCSIR Campus, Dhaka was used as a source of explants. Shoot tip and nodal explants with a single axillary bud were used for this purpose. The explants were washed thoroughly under running tap water, presoaked in liquid detergent for about 30 min, wiped with cotton and dipped in 70% (v/v) ethanol for 1 min. They were then surface-sterilized with 0.1% (w/v) mercuric chloride for 5 min, followed by rinsing with sterile distilled water for 5 minutes under laminar air flow cabinet. The surface-sterilized explants were sized to 1.0-1.5 cm length containing a single node with an axillary bud or a shoot tip with an apical bud. The explants were placed vertically on the culture medium. The new shoots induced from the *in vitro* cultures were further used as an explants for shoot regeneration.

MS (Murashige and Skoog 1962) basal medium was used for shoot proliferation and adventitious shoot regeneration and half strength MS media was used for *in vitro* root induction. All media were supplemented with 30 g/l sucrose, 7 g/l agar (Difco) and dispensed into 15x150 mm culture tubes and 250 ml conical flasks. The pH of the media was adjusted to 5.8 before autoclaving at 1.9 kg/cm^2 pressure at 121^{o}C for 20 min. The cultures were incubated for a 16 h photoperiod at 24 ± 2^{o}C under 1200 lux/m^2 fluorescent light.

Shoot proliferation from shoot tip and nodal explants was obtained in two separate sets of experiments. In the first experiment 0.5-2.0 mg/l BAP were incorporated into MS media to select the best cytokinin for the response of shoot induction. In the second set, combination of BAP (0.5-2.0 mg/l) with NAA (0.1-0.5 mg/l), BAP (0.5-2.0 mg/l) with IAA (0.1-0.5 mg/l) and BAP (0.5 mg/l) with GA_3 (0.1mg/l) were assessed for shoot multiplication. Number of new shoot proliferation of each culture was recorded after every week of inoculation.

For *in vitro* rooting, individual shoots (3-5 cm) were excised from the proliferated shoot cultures and implanted onto half strength MS media with different concentrations and combinations of NAA, IBA and IAA.

The rooted plants were taken out from the culture tubes, washed to remove agar gel adhered to the roots and transplanted to plastic pots with soil and compost (1: 1) for hardening. The plantlets were kept in a polychamber at 80% relative humidity, 32 ± 2^{o}C temperatures for a 12 h photoperiod under 1500 lux/m^2 sun light for acclimation. Established

Table I: Effect of growth regulators in MS media on morphogenic response of *Phyllanthus fraternus* Webster shoot tips and nodal explants

Growth regulators (mg/l)				shoot tips		nodal explants	
BAP	NAA	IAA	GA_3	% of explants forming shoots	Mean No. of Shoot/explant	% of explants forming shoots	Mean No. of Shoot/explant
0.5				71.2±2.58	12.0± 0.59	84.4±2.10	16.4± 0.91
1.0				63.4±1.57	10.6± 0.45	71.4±2.38	15.6± 0.72
1.5				57.6±2.16	9.4± 0.72	67.6±2.16	13.4± 1.18
2.0				34.8±2.58	7.8±0.76	33.6±1.84	12.4±0.82
0.5	0.1			61.4±2.87	9.2± 0.59	68.6±1.70	13.6± 1.14
1.0	0.2			42.6±0.87	7.6±0.77	43.6±0.51	12.6±0.91
1.5	0.5			28.2±1.66	6.0± 0.63	41.2±2.47	10.4± 0.66
2.0	0.5			22.2±1.96	4.4± 0.45	32.2±0.66	8.8± 0.95
0.5		0.1		48.8±1.77	8.0± 0.39	56.8±2.14	12.2± 0.76
1.0		0.2		26.6±1.66	6.2± 0.65	47.6±2.10	10.2± 0.51
1.5		0.5		21.0±1.14	5.4± 0.45	32.6±1.63	8.4± 0.91
2.0		0.5		16.2±0.86	3.4± 0.45	18.4±0.93	7.4± 0.66
0.5			0.1	72.4±2.89	12.4±0.76	88.2± 2.80	16.8± 0.95

Results are mean ± SE of three experiments with 15 replications.

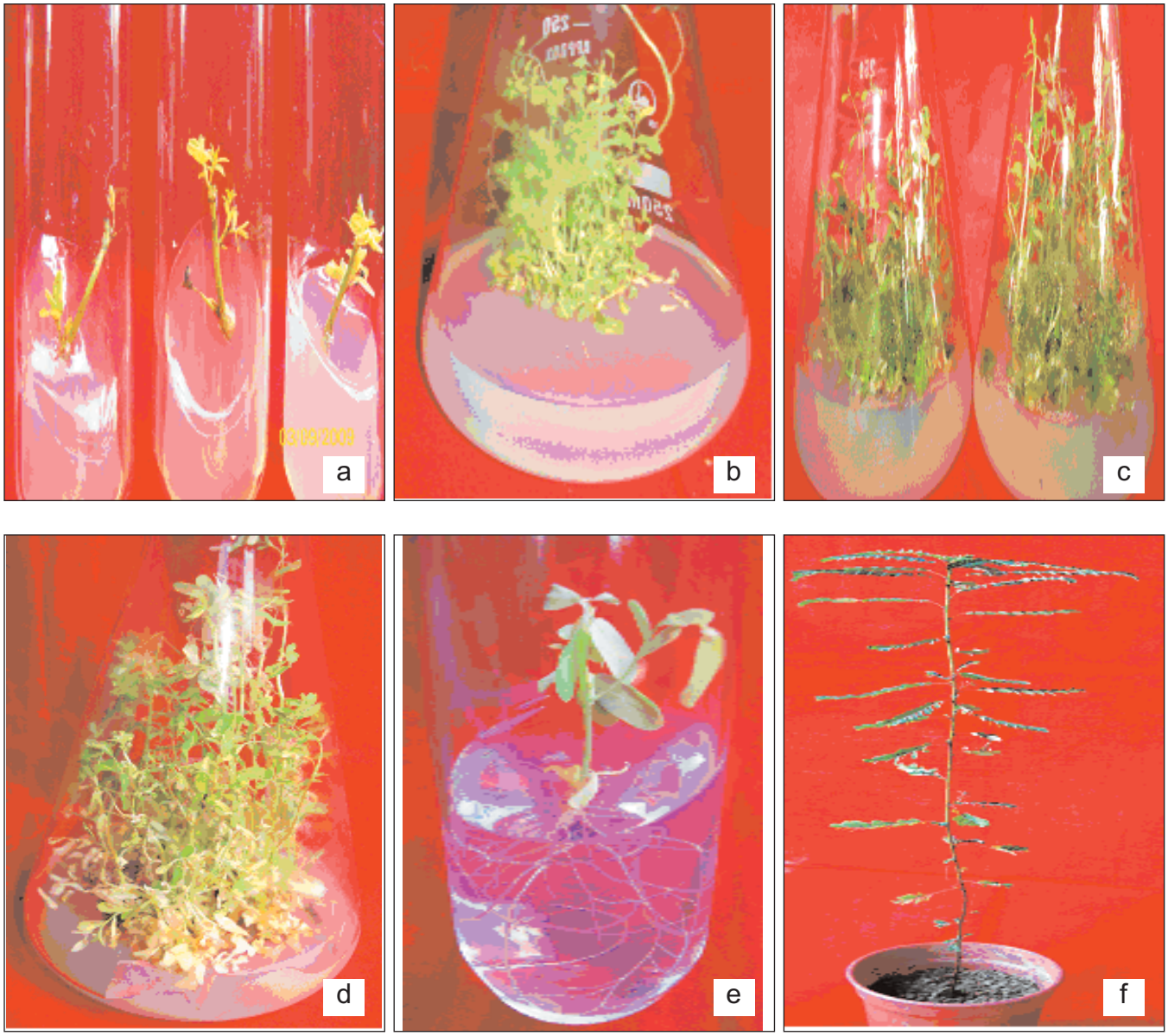

Fig. 1: *In vitro* regeneration of *Phyllanthus fraternus* Webster from nodal explants

(a) Induction of shoots in three weeks of culture on MS + 0.5 mg/l BAP + 0.1 mg/lGA_3 from nodal explants.
(b) Development and multiplication of shoots on MS + 0.5 mg/l BAP + 0.1 mg/lGA_3 from nodal explants after six weeks of culture.
(c) Development and multiplication of shoots on MS + 0.5 mg/l BAP + 0.1 mg/lGA_3 from nodal explants after nine weeks of culture.
(d) Development and multiplication of shoots on MS + 0.5 mg/l BAP + 0.1 mg/lGA_3 from nodal explants after twelve weeks of culture.
(e) Rooting of *in vitro* regenerated shoots cultured on half strength MS + 0.5 mg/l IBA in third weeks.
(f) Acclimatized regenerated plants of three months old.

Key Words : Heavy metals, Adsorption, Single component system, Multicomponent system.

plants were transplanted in earthen pots under natural conditions and the survival rate was recorded.

Results and Discussion

Shoot tip and nodal explants of *Phyllanthus fraternus* Webster were cultured on MS media supplemented with four concentration of BAP alone and its some concentrations with NAA, IAA and GA_3 for multiple shoot regeneration. The explants were found to be swollen and they produced three to four shoots within three weeks after inoculation (Fig.1a) on MS media containing BAP alone but the number of shoots increased up to 12 when the explants were cultured in MS media with 0.5 mg/l BAP + 0.1 mg/l GA_3 (Table I, Fig.1b). Both the explants responded in the same medium but highest numbers of micro shoots were observed to be induced from nodal explants (Fig.1c). Combinations of BAP with NAA or IAA were not found to be suitable than BAP with GA_3 for shoot induction (Table I) Newly initiated shoots were separated and sub cultured repeatedly in fresh MS media with 0.5 mg/l BAP + 0.1 mg/l GA_3, where the number of shoots increased up to 16.8 ± 0.95 per culture (Fig. 1d). Nearly same response of microshoot were obtained in MS medium fortified with 0.5 mg/l BAP + 0.1 mg/l GA_3 and 0.5 mg/l BAP alone but healthier shoots were found in the former medium content. Liang and Keng (2006) reported that the aseptic nodal segments cultured on MS medium supplemented with 1.0 mg/l BAP produced an average of 6.6 shoots from each explant in *Phyllanthus fraternus* Webster. It was also observed that multiple shoots were found by using different concentration of cytokinin with auxins in different species of *Phyllanthus* (Bhattacharya and Bhattacharya, 2001; Captain *et al.* 2000, 2002, 2009; Rajasubramaniam and Saradhi, 1997).

Table II: Effect of auxin(s) on root induction in regenerated shoots of *Phyllanthus fraternus* Webster on half strength MS media

Growth regulators (mg/l)			% of shoots producing roots(±SE)	No. of roots/shoot (±SE)
IBA	NAA	IAA		
0.5			82.4±0.64	11.8±0.59
0.75			67.2±1.53	9.8±0.65
1.0			63.2±1.46	09.2±0.76
	0.5		71.0±0.10	09.6±0.72
	0.75		57.8±1.85	09.2±0.76
	1.0		54.2±1.53	08.0±0.63
0.5	0.5		82.0±0.71	9.8±0.59
1.0	1.0		59.4±1.08	08.2±0.71
0.5		0.5	65.2±1.16	09.8±0.65
1.0		1.0	61.4±0.75	08.6±0.96
0.5	0.5	0.5	62.6±0.93	09.6±0.72
1.0	1.0	1.0	56.2±1.34	09.2±0.65

Data were recorded after four weeks of culture. Results are mean ± SE of 15 replications.

82.4% regenerated shoots rooted (Fig.1e) when cultured individually on root inducing medium consisted of half-strength MS medium with 0.5 mg/l IBA (Table II). Use of auxins singly or in combination for rooting was also reported by different authors (Gawde and Paratkar 2004; Hassan and Roy 2005; Hassan *et al*. 2009; Sahoo and Chand 1998; Sivakumar and Krishnamurthy 2000).

After four weeks the rooted shoots were transferred to pots. None of the plantlets were survived when directly transferred from rooting medium to the pot under natural conditions. About 85 percent of the transplanted plants of *Phyllanthus fraternus* Webster survived if the plants in the rooting culture tubes were kept in normal room temperature for seven days before transplantation in pots and reared for three weeks. The plantlets were reared under semi-controlled temperature (30±2^{o}C) and light (1500 lux) in a chamber with 80 percent humidity. During this period of acclimation shoots were elongated, leaves were also expanded and turned deep green and healthier (Fig.1f).

After three weeks, plants were transferred to an open place and gradually acclimated to outdoor conditions, where 85 percent plants were survived. The technique described here appears to be readily adaptable for large scale clonal propagation and plantation for sustainable use.

Acknowledgement

The authors are indebted to the Professor Dr. S. M. Imamul Huq, Chairman, BCSIR, Dhaka. Grateful acknowledgement is also due to Professor Samir Ali, Vice-Principal, Savar College, Savar, Dhaka for their kind permission, sincere suggestions and moral support during research work.

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Received : June 14, 2010;
Accepted : October 10, 2010