J. Sci. Trans. Environ. Technov. 2009, 2(3): 121-125

Regeneration of plantlets from leaf disc and internode explants of *Phyllanthus amarus* Schum. & Thonn.

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Abstract

A procedure is outlined for indirect organogenesis of *Phyllanthus amarus* using leaf discs and internodes. Profuse callusing of leaf discs and internodes were obtained on Murashige and Skoog's basal medium supplemented with α–Napththalene Acetic Acid (NAA) and 2, 4-D-Dichlorophenoxy Acetic Acid. The callus thus obtained was repeatedly sub-cultured at two weekly intervals for two cycles. Complete plantlets were obtained when the callus was sub-cultured on MS medium supplemented with Benzyl Amino Purine (BAP) (2.0 mgl^{-1}) and Gibberlic acid (0.5 mgl^{-1}). Rooting (87.09 %) of the shoots was best achieved on half strength MS medium supplemented with Indole-3-Butyric acid (0.5 mgl^{-1}) and Indole-3-Acetic acid (0.5 mgl^{-1}). Regenerated plants were successfully transferred to soil after acclimatizing them in the plant growth chamber.

Keywords : callus, internode, leaf disc, micropropagation, *Phyllanthus amarus*

INTRODUCTION

Phyllanthus amarus (Euphorbiaceae), expounded vastly in Indian Pharmacopoeia (Kamboj, 2000), has been traditionally used in India in the treatment of a variety of ailments including hepatic disorders (Nadkarni, 1976) dropsy, diarrhoea, dysentery, intermittent fevers, diseases of urino-genital system, scabies, ulcers and wounds (Thagarajan *et al.,* 1982). The plant is bitter, astringent, cooling, diuretic, stomachic, febrifuge and antiseptic.

The conventional method of propagation of this species is through seeds. However, poor germination potential restricts their multiplication and as such micropropagation technique could be an alternative method for the cloning of these plants (Unander, 1991 and Santos *et al.* 1994). We describe the micropropagation of *P.amarus* from leaf discs and internodes and successful establishment of plantlets so obtained in soil.

MATERIALS AND METHODS

Two month old plants of *P.amarus* which were raised from seeds and maintained in the green house of 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 leaf discs (0.5-1.0 cm) and internodes (1.0 -1.5 cm) were initially washed with teepol for 2 minutes and then under running tap water for 3 minutes. Then they were treated with 0.1% mercuric chloride for 2 minutes, washed 4-5 times with sterile distilled water and inoculated on basal medium consisting of Murashige and Skoog's (1962) salts and vitamins, 3% sucrose and 0.8% agar. Basal medium was supplemented with various concentrations of α-Napthalene Acetic Acid (NAA) (0.1-0.5 mgl^{-1}) and 2,4-D-Dichlorophenoxy Acetic Acid (2, 4-D) (1.0-5.0 mgl^{-1}) for callus induction. The regeneration medium consisted of MS basal medium supplemented with Benzyl Amino Purine (BAP) (1.0-5.0 mgl^{-1}) and Gibberlic Acid (GA_3) (0.5 mgl^{-1}). Half strength MS medium was supplemented with Indole-3-Acetic Acid (IAA) (0.25-1.25 mgl^{-1}) and Indole-3-Butyric Acid (IBA) (0.25-1.25 mgl^{-1}) for rooting of *in vitro* plants. The media were adjusted to pH 5.8 and dispensed in 25 x 150 mm culture tubes before autoclaving at 121^0C for 15 min. All cultures were maintained at 25±2^0C, under 16 h photoperiod provided by cool white fluorescent light (35 μEM-2S-1) with 70% relative humidity. Each experiment was performed thrice with total number of 20 inoculated explants per treatment. The leaf discs and internodes were inoculated on Murashige and Skoog's basal medium supplemented with NAA and 2, 4-D. Four weeks later, profuse callusing of leaf discs and internodes were obtained. The callus thus obtained was repeatedly sub-cultured at two weekly intervals for two cycles. Relative growth of callus was scored after four weeks using the following scale (Narmda and Vijayakumar, 2000) a) Poor callusing – 1; b) Slight callusing - 2; c) Moderate callusing - 3; d) Profuse callusing – 4.

Callus index (CI) was calculated in the following way.

CI = Percentage culture forming callus X Relative growth of callus.

After four weeks of callusing, callus clumps were transferred to regeneration medium. Multiple shoots, developed after 30 days of first sub-culture, were

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dissected out individually for further multiplication and this process was continued repeatedly every 20 days for two cycles. Finally the cluster shoots developed were transferred to basal medium additionally supplemented with 0.5 mgl^{-1} GA_3 for elongation before transferring to rooting medium. The rooted plantlets were transferred to plastic pots containing sterilized sand and vermiculite (1:1). The plants were kept in hardening chambers with high relative humidity (80%) and low light intensity. The survival percentage was determined after 20 days in pots. One month later, when the plants had acclimatized well, they were transferred to the field.

RESULTS AND DISCUSSION

Indirect organogenesis of *P. amarus* was successfully obtained from leaf discs (Fig. 1) as well as from internodes (Fig. 2) with multiple shoots derived from callus (Fig. 3). High callusability (CI=325.62) of internodes was achieved on MS medium with 2, 4-D (4.0 mgl^{-1}) and NAA (0.4 mgl^{-1}) (Table 1). Earlier Haicour (1974) was successful to obtain callus from leaf disc and internodes of *Phyllanthus urinaria*. Among the different explants used, internodes showed maximum response. Similar observation was made by Unander (1991) as well for *Phyllanthus amarus*.

The possibility of callus mediated regeneration which could be brought through the manipulation of hormone balance is a known factor (Rossini, 1969). The level of auxin and cytokinin alone or in combination were reported to decide the efficiency of callusability and organogenesis (Kohlenbach, 1977). In the present study leaf disc and internode derived callus showed better regeneration capacity of shoots in the medium containing cytokinin (2.0 mgl^{-1} BAP) along with GA_3 (0.5 mgl^{-1}) (Table 2). The effective and efficient role played by the BAP in the shoot proliferation of vari-

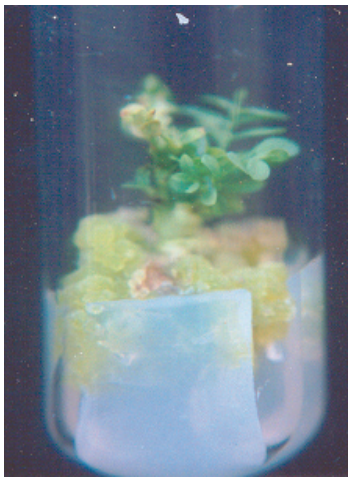

Figure 1. Indirect organogenesis of *P. amarus* from leaf disc

Figure 2. Indirect organogenesis of *P. amarus* from internode

Figure 3. Multiple shoots derived from callus

Figure 4. *P. amarus* grown *in vitro*

Table 1. Effect of 2,4-Dichlorophenoxy Acetic Acid (2, 4-D) and a-Napthalene Acetic Acid (NAA) on callus induction from internodes and leaf discs of *P.amarus* (See "Materials and Methods" section for details on the calculation of relative growth and callus index)

Treatment (Concentration of 2,4-D + NAA) (mgl^{-1})	Internodes				Leaf discs			
	Cellusability (%)	Relative growth	Callus Index	Days taken for callusing	% of forming callus	Relative growth	Callus Index	Days taken for callusing
T_1 (1.0 + 0.1)	29.97	1.33	40.24	56.33	20.00	1.21	24.23	59.16
T_2 (2.0 + 0.2)	21.66	1.65	37.33	58.16	40.00	1.20	48.90	57.66
T_3 (3.0 + 0.3)	73.33	2.88	211.61	52.00	62.22	2.60	166.72	51.77
T_4 (4.0 + 0.4)	84.99	3.83	325.62	46.66	84.99	3.77	320.90	49.16
T_5 (5.0 + 0.5)	20.25	1.16	23.14	56.83	21.10	1.48	30.14	58.50
Mean	46.04	2.17	127.63	54.00	45.66	2.04	118.18	55.25
SE	6.97	0.19	17.97	1.95	5.07	0.37	31.14	0.88
CD (0.05)	15.54	0.42	40.50	4.35	11.31	0.83	69.40	1.98

SE: Standard Error; ***CD*** : Critical Difference

Table 2. Effect of Benzyl Amino Purine (BAP) and Gibberlic acid (GA_3) on multiple shoot induction from leaf discs and internodes of *P.amarus*

Treatment (Concentration of BAP + GA_3) (mgl^{-1})	Survival percentage	Days taken for shooting	No. of shoots per calli	Length of shoot (cm)
T_1 (1.0 + 0.5)	67.81	24.29	4.66	1.41
T_2 (2.0 + 0.5)	82.04	15.35	5.08	1.72
T_3 (3.0 + 0.5)	63.01	28.64	3.98	1.23
T_4 (4.0 + 0.5)	56.90	29.66	3.09	1.23
T_5 (5.0 + 0.5)	52.83	28.99	3.60	1.07
Mean	64.52	25.39	4.08	1.33
SE	1.132	0.310	0.318	0.089
CD (0.05)	2.522	0.690	0.709	0.200

SE – Standard Error ***CD*:** Critical Difference

Table 3. Effect of Indole-3-Acetic Acid (IAA) and Indole-3-Butyric Acid (IBA) on rhizogenesis in *P. amarus*

Treatment (Concentration of IAA + IBA) (mgl^{-1})	Percentage response to rhizogenesis	Days taken for rooting	Number of roots per plant	Length of root (cm)
T_1 (0.25 + 0.25)	50.63	20.21	2.88	1.94
T_2 (0.50 + 0.50)	87.09	15.31	4.69	5.34
T_3 (0.75 + 0.75)	71.18	18.30	3.34	3.34
T_4 (1.00 + 1.00)	68.23	21.20	5.77	4.04
T_5 (1.25 +10.25)	42.30	21.21	2.03	2.25
Mean	63.89	19.25	3.74	3.83
SE	0.896	0.565	0.337	0.176
CD(0.05)	1.997	1.258	0.751	0.393

SE – Standard Error; ***CD***- Critical Difference

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

Medium	Number of plantlets transferred	Survival percentage		
		After 1 week	After 2 week	After 3 week
Sand & vermiculite (1:1)	50	90.00	80.00	80.00
Sand, soil & leaf mould (1:1:1)	50	30.00	20.00	16.66

ous medicinal crops were emphasized earlier by Harikrishnan and Hariharan (1999) in *Acorus calamus*, Arockiasamy *et al*. (1999) in *Datura metel*, Al-Wasel (1999) in *Atropa belladonna*, Chetia and Handique (2000) in *Plumbago indica*, Selvakumar and Balakumar (2000) in *Acalypha fruiticosa,* Muthuram *et al.* (2000) in *Scoparia dulsis* and by Jagatram *et al*. (2003) in *Madhuca latifolia.*

GA_3 is known to have stimulatory effect on stem elongation in different plants (Ganga and Balakrishnamoorthy 1998). In the present study, when lower concentration of GA_3 was supplemented to the MS basal media the response was more with regard to survival percentage, no. of shoots per calli and shoot length (Table 2). Similar observations were made by Pattnaik and Chand (1996) in *Ocimum sanctum*. However, above the optimal level (T_2), a negative relationship was found between the concentration of GA_3 and the response of shoot elongation (Table 2).

IBA and IAA at lower concentrations (0.5 mgl^{-1}) gave good response in rooting (87.09%). Ray and Jha (2002) reported that the rooting was highest in microshoots of *Withania somnifera* with 0.5 mgl^{-1} IBA as it induced 80 % rooting. Soniya and Das (2002) also found elongated shoots of *Piper longum* in MS medium supplemented with 2.46 µM IBA. However, in the present study addition of IBA (1.25 mgl^{-1}) and IAA (1.25 mgl^{-1}), even though gave good response to rooting, the rooting was only 15 per cent and yellowing of leaves was also observed (Table 3). This might be due to overdose of auxin. The root elongation phase is very sensitive to auxin concentration and might be inhibited by high concentration as reported by Thimmann (1977).

The rooted plantlets were then transferred to hardening chamber. Two types of media *viz*., sand, soil & leaf mould (1:1:1) and sand & vermiculite (1:1) were tried. Survival and establishment was higher in sand & vermiculite medium (Table 4). When plantlets got better established in about 25 days, they were transferred to pots (Fig. 4) with soil, sand, and farmyard manure (1:1:1) from where they can be taken for field planting.

In conclusion, the protocols used for the successful multiplication of *P.amarus* through *in vitro* routes in the present study could be useful for conservation as well as biotechnological improvement of this pharmacologically important plant species.

REFERENCES

Al-Wasel, A.S. 1999. *In vitro* multiplication of *Atropa belladonna* L. using nodal segments. *Alexandria J. Agri. Res*., 44: 263-274.

Arockiasamy, D.I., Muthukumar, B., Britto, S.J. 1999. *In vitro* plant regeneration from internodal segments of *Datura metal* L. *Adv. in Plant Sci*., 12: 227-231.

Chetia, S. and Handique, P.J. 2000. High frequency *In vitro* shoot multiplication of *Plumbago indica*, a rare medicinal plant. *Curr. Sci*., 78:1187-1188.

Ganga, M. and Balakrishnamoorthy, G. 1998. *In vitro* propagation of *Rosa spp*. M.Sc (Hort) thesis submitted in Tamil Nadu Agricultural University, Coimbatore.

Haicour, R. 1974. Comparaison chez *Phyllanthus urinaria* L. de I' activite antibact erienne desd'ecoctions de diverses portions de la plante et al, de cultures de tissue qui en proviennent. *CR Seances Acad Sci Paris S'er D*. 278: 3323 – 3325.

Harikrishnan, K.N. and Hariharan, M. 1999. *In vitro* clonal propagation of sweet flag (*Acorus calamus*) – a medicinal plant. *In: Plant tissue culture and biotechnology emerging trends.* Jan : P. 29-31.

Jagatram, M.C., Surendran, M., Parthiban, K.T. and Paramathma. 2003. *In vitro* propagation of *Madhuca latifolia. Indian J. For.,* 26: 445-448.

Kamboj, V.P. 2000. Herbal Medicine. *Curr. Sci.,* 78: 35-39.

Kohlenbach, H.W. 1997. Basic aspects of différentiation and plant regeneration from cell and tissue cultures. *In: Plant tissue culture and its biotechnology application*. W.Berg, E. Rainard, M.H. Zenk, (Eds.). Springer Venlarg, Berlin.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tabacco tissue cultures. *Physiol. Plant*, 15: 437-497

Muthuram, G., Brindha, P. and Lokeswari, T.S. 2000. *In vitro* propagation of *Scoparia dulcis* Linn. A medicinal herb. *In:* Recent Trends in Spices and Medicinal Plants Research. Amit Krishna De (Ed.). Associated Publishing Co., New Delhi: P. 100 – 105.

Nadkarni, K.M. 1976. *Indian Materia Medica*. Popular Prakashan Ltd., Bombay. Vol.1: P. 947 – 949.

Narmda, D.S. and Vijayakumar, M. 2000. Micropropagation of *Gymnema sylvestre*. M.Sc (Hort) thesis submitted in Tamil Nadu Agricultural University, Coimbatore.

Pattnaik, S. and Chand, P.K. 1996. *In vitro* propagation of the medicinal herbs *Ocimum americanum* L.syn. *Ocimum canum* Sims. and *Ocimum sanctum* L. *Plant Cell Rep*., 15: 846-850

Ray, S. and Jha, S. 2002. Regeneration of *Withania somnifera* plants. *J. Trop. Med. Plants,* 3: 89-95.

Rossini. 1969. One nogevelle method de culture in vitro de cellules parenchymatessus separess de fevilles de *Calystegia sepium* L. CR. Acad. Sci., Paris, *In: Plant tissue culture and its biotechnology application*. W.Berg, E. Rainard, M.H. Zenk, (Eds.). Springer Venlarg, Berlin.

Santos, A.R.S., FilhoNiero, V.C.R., Viana, A.M., Moreno, F.N., Campos, M.M., Yunes, R.R. and Calixto, J.B. 1994. Analgesic effects of callus culture extracts from selected species of *Phyllanthus* in mice. *J. Pharmacol*., 46: 755-759.

Selvakumar, V. and Balakumar, T. 2000. *In vitro* induction of axillary branching in the medicinal plant *Acalypha fruiticosa* Forsk. *In: Recent Trends in Spices and Medicinal Plants Research*. Amit Krishna De (Ed.). Associated Publishing Co., New Delhi: P. 96-97.

Soniya, E.V. and Das, M.R. 2002. *In vitro* micropropagation of *Piper longum*-an important medicinal plant. *Plant Cell Tiss. Org. Cult.,* 70: 325-327.

Thagarajan, S.P., Thiruneelakantan, K., Subramaniam, S. and Sundaravelu, T. 1982. *In vitro* inactivation of HBsAg by *Eclipta alba* and *Phyllanthus niruri* Linn. *Ind. J. Med. Res*., 76: 124-130.

Thimmann, K.V. 1977. *Hormone action in the whole life of plant*. University of Massachusetts Press, Amherst.

Unander, D.W. 1991. Callus induction in *Phyllanthus* spp. and inhibition of viral enzymes by callus extracts. *Plant Cell*