Somoclonal variation studies on *Phyllanthus amarus* Schum & Thonn

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Abstract
The present study was aimed at the development of a somoclonal variant and isozyme marker for *Phyllanthus amarus* Schum & Thonn using inter-nodal segments and the enzyme peroxidase. Maximum callus proliferation was obtained on Murashige and Skoog's medium supplemented (MS) with 1.0 mg/l of 2,4-Dichlorophenoxyacetic acid. Three weeks-old pale yellowish white semi-friable callus was used for organogenesis; the maximum percentage of multiple shoot formation (85%) was achieved after 4 weeks when callus was cultured on Murashige and Skoog's medium fortified with 1.0 mg/l of 6-Benzylaminopurine. The multiple shootlet formations were also achieved in the presence of the same concentration. The maximum formation of rootlets was observed on MS medium augmented with 1.0 mg/l of Kinetin and 0.5 mg/l of Naphthalene acetic acid. The banding pattern and phytochemical constituent differences were observed between mother plants, directly regenerated via nodal segments, calli, and calli mediated plants. The calli mediated somoclonal variation was confirmed through isozyme (peroxidase) and phytochemical analysis. The isoperoxidase banding profile showed a difference in calli and calli mediated plants. The phytochemical study confirmed the presence of more alkaloids, saponins, tannins and others from calli and calli mediated shoots and roots. Hence the isozyme banding patterns can be used as molecular markers in future plant breedings or genetic improvement programmes.
Keywords: Callus; Medicinal plant; Isozymes; Organogenesis; *Phyllanthus amarus*; Somoclonal variation.

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INTRODUCTION

Phyllanthus amarus Schum & Thonn is an important medicinal plant species due to its antiviral properties, and thus useful against hepatitis infection. The plant is also highly valuable in the ayurvedic system of medicine. Its application in the treatment of jaundice has been well documented (Bratati *et al*., 1990; Joy and Kuttan, 1998; Raphael *et al*., 2002). The species is also used in stomach ailments such as dyspepsia, colic, diarrhea, dysentery, dropsy, urinogential problems and also as external applications for edematous swelling and inflammation. An aqueous extract of this plant was found to inhibit the hepatitis B virus (Thyagarajan *et al*., 1998). The plant is also used as a hypoglycemic agent in traditional medicine to control non-insulin dependent Diabetis mellitus (Sivarajan and Balachandran, 1994). The accumulation of phytochemicals in plant cell cultures has been studied for more than thirty years, and the generated knowledge has helped in the realization of using cell cultures for production of the desired phytochemicals (Castello *et al*., 2002). Direct regeneration has already been achieved in our laboratory (Johnson, 2006) but there is no report yet on the production of somoclonal variants and organogenesis. Currently the various applications of genetic engineering are implemented in medicinal plants to increase the production of secondary metabolites (Nisha *et al*., 2003). Since the 1930s, electrophoresis in conjunction with the zymogram technique has been used as a tool for the study of heritable variation. Isozymes are widely used because of their relative efficiency and cost effectiveness, particularly in studies of intra- and inter-specific variation (Sabu *et*

al. 2001 and Smila *et al*. 2007). The importance of isozymes has been reviewed by Smith and Smith, (1992). The influence of peroxidase in the regulation of cell growth and differentiation has been observed by Johnson (2003). Due to its medicinal value, there is a great demand for this species in the market. In the present study, organogenesis and production of somoclonal variant using inter-nodal segments, was investigated. In addition to this, an attempt was made to produce molecular markers (isozyme) for this medicinally important plant and compared the phytochemical constituent's presence. A few reports were available for phytochemical analysis in in vitro derived cultures, based on this back ground the present study was initiated on calli production and organogenesis in Phyllanthus amarus Schum and Thonn. In addition the present study emphasized on phytochemical and biochemical comparative studies specially on somoclonal variation using isoperoxidase as tool.

MATERIALS AND METHODS

Inter-nodal segments of *Phyllanthus amarus* Schum and Thonn were collected from the young top shoot cuttings of mature plants grown in St. Xavier's College Botanic Garden, Palayamkottai, Tamil Nadu India. The explants were washed thoroughly under running tap water for 5 min, and then washed with the commercial detergent tween-20 for 3 min. This was followed by thorough washing with sterile distilled water. Surface sterilization was carried out in the presence of mercuric chloride solution (0.5% w/v) for 2 min and then washed thrice with sterile distilled water. The explants were cut into 0.7 cms pieces and cultured on MS (Murashige and Skoog, 1962) solid medium supplemented with 3% (w/v) sucrose, gelled with 0.6% (w/v) agar containing different concentrations of 2,4-Dichlorophenoxyacetic acid either alone or in combination. The pH of the medium was adjusted to 5.8 prior to autoclaving (at a pressure of 1.06 Kg/cm^2, 121°C for 15 min.). After two to three weeks, the *in vitro* proliferated calli cultures were sub-cultured onto MS medium supplemented with Benzyl-6-amino purine (BAP), Kinetin and Naphthalene acetic acid (NAA) (replicates of culture tubes) (0.1 to 2.5 mg/l), either alone or in combinations, for the purpose of organogenesis. The cultures were incubated at 25°C ± 2°C with 12/8 h photoperiods under white fluorescent tubes (1500 lux, Phillips, India). Each and every experiment was performed with 10 replicates of culture tubes and was repeated thrice. The callus cultures were maintained for a period of over 10 months by periodic sub-culturing with 2 to 4 weeks intervals onto fresh multiplication medium. Phytochemical assays were performed on the 21st day. Consequently, 21 to 28 days old calli were harvested for phytochemical analysis, kept at above 90°C for 3 to 5 minutes in a hot air oven to inactivate enzyme activity followed by continuous drying at 50 to 60°C for 60 to 72 h (Jain *et al*. 2004). Dried inter-nodes, roots and calli were homogenized to a fine powder and further exploited for extraction and phytochemical analysis. Dried whole plants and also the *in vitro* derived calli were powdered using an electric homogenizer and exhaustively extracted with 150 ml of different solvents (ethanol, chloroform, petroleum ether and acetone) , alone or combined for 8-12 h by using the soxhlet apparatus. The preliminary phytochemical screening was performed according to the Harborne (1964) method. For the electrophoresis studies, the mother plant and the young leaves of the *in vitro* grown were harvested and grounded in an ice cold mortar and pestle with phosphate buffer (0.1 M, pH 7.0). The slurry was then centrifuged at 10,000 rpm for 10 min, at 4°C. The supernatants were collected and electrophoresed. The native poly acrylamide gel electrophoresis (PAGE) was performed gel electrophoresis was performed by Anbalagan (1999) method. The gel was then stained with O-dianisidine (100mg) in acetate buffer (90ml, pH 4.2), ethanol (5ml), H_2O_2 (1ml) and distilled water (4ml) (Sadasivam and Manickam, 1992). The banding patterns were documented and Rf values were calculated using the Biogene Software. Variations in banding pattern were determined by migration from the origin towards the anode. Isozymes region were designated to define the general area on the zymogram with in which the bands migrated.

RESULTS

Callus induction was observed in the inter-nodal segments on MS medium supplemented with 2, 4-D. Based on concentration of the plant growth hormone, the callus formation frequency was varied (Table 1). The maximum percentage (85.6 ± 0.81) of callus formation was observed on MS medium augmented with

Table 1. Effect of 2, 4-D concentration on callus formation by *P. amarus.*

2,4-D concentration (mg/l)	Percentage of callus formation ± S.D.	Type of calli		
		Friable	Semi-friable	Hard/compact
0.0	0.0 ± 0.0	NIL	NIL	NIL
0.5	55.8 ± 0.31	+	+	NIL
1.0	85.6 ± 0.81	+	+	NIL
1.5	65.3 ± 0.61	+	+	NIL
2.0	60.0 ± 0.63	NIL	+	+
2.5	50.6 ± 1.3	NIL	+	+

+ Sign indicates callus formation; NIL – indicates no callus induction in the respective media containing different hormone concentrations.

1.0 mg/l of 2,4-D. Different types of calli were obtained of which, the friable, semi-friable and creamy white coloured showed high proliferation rates (Figs. 1 A, B and C). At concentrations of auxins, the callus was hard and dark yellowish brown in colour. The semi-friable callus was transferred to MS medium augmented with different concentrations of BAP, KIN, and NAA either alone or in combinations for the purpose of organogenesis. The MS medium supplemented with 1.0 mg/l of BAP produced maximum percentage and number of shootlets on the *in vitro* induced calli (Figs. 1 D and E, Table 2). The Kin and NAA supplemented media produced maximum percentage of hairy roots on the calli (Fig. 1 F).

The isozymic study produced an isozymic pattern for the wild plant species (mother plant), the *in vitro* induced calli, direct and indirect regenerated plants. Multiple regions of activity were obtained for the peroxidase system (PRX 1 to 6). Region 1 contained three bands, PRX1[1] to 3. The first two bands showed their unique presence with calli and calli mediated *in vitro* raised plantlets. The first band (PRX1[1]-P1) was unique to *in vitro* calli mediated raised plantlets. The second band (PRX1[2]-P2) was specific to the inter-nodal derived calli. The third band (PRX1[3]-P3) was common except, the inter-nodal derived calli. Region 2 contained bands in three different positions. Region 2 revealed the somoclonal variation, calli mediated plantlets showed the unique presence in two different positions (PRX2[1]-P4). The second band (PRX2[2]-P5) was common except for the calli mediated plantlets. Region 3 also illustrated the variation between the calli mediated plantlets and others. PRX3[1]-P8 was very specific to the mother plants and nodal derived plants or directly regenerated plants. The second band (PRX3[2]-P9) was restricted to the inter-nodal derived

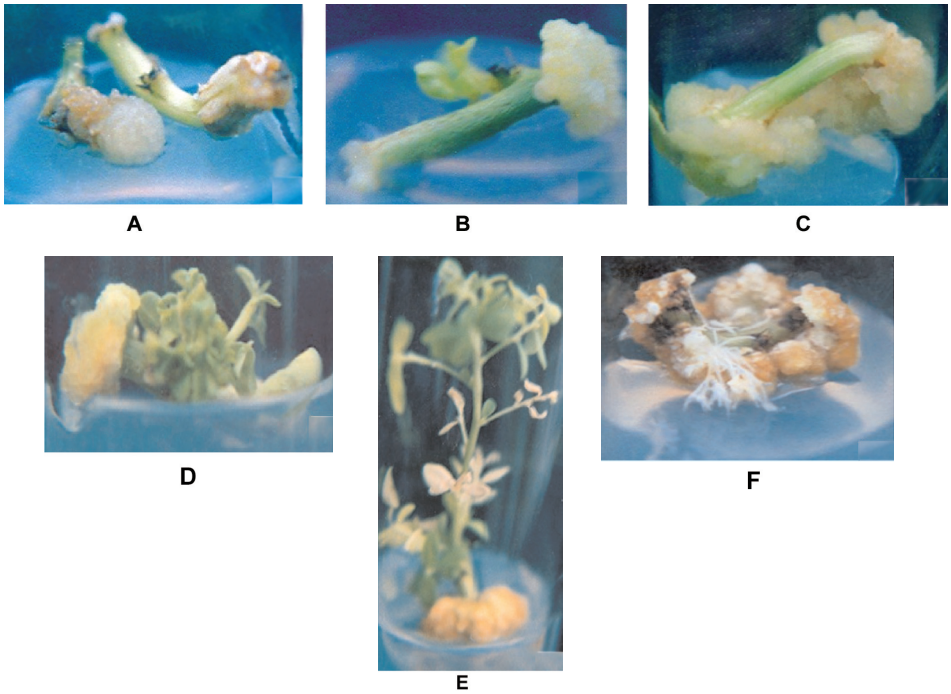

Figure 1. Organogenesis of *P. amarus* Schum & Thonn. **A.** Inter-nodal segment derived calli – Initial stage. **B.** Direct Shootlets formation from the auxiliary buds and calli formation from the wounds. **C.** Calli formation on inter nodal segments. **D.** Multiple shootlets formation from the intermodal derived calli. **E.** *In vitro* calli derived shootlet. **F.** Hairy rootlets formation from the *in vitro* derived calli. **G.** Iso – Peroxidase zymogram of the mother plant (Lane 1(L1)), nodal segment derived plantlets (Lane 2 (L2)), inter nodal derived calli (Lane 3 (L3)) and calli mediated plant – organogenesis (Lane 4 (L4)).

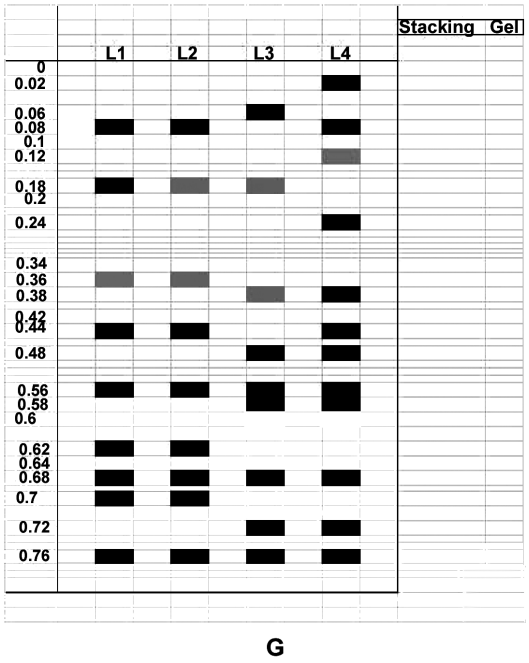

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Figure 1. Continue.

calli and calli mediated plantlets. Region 4 contained two bands in two different positions. The first band (PRX4[1]-P10) was common, except for the inter-nodal derived calli. The second band (PRX4[2]-P11) was restricted to calli mediated plants. Similar to region 4, the region 5 band profile also showed, the first band (PRX5[1]- P12) was illustrated the similarity between the mother plant, nodal derived plantlet and calli mediated plant, but this band was absent in inter-nodal raised calli. Band PRX5[2]-P13 was observed in inter-nodal derived calli and calli mediated plantlets. Region 6 contained three bands in different positions, the first (PRX6[1]-P14) and the third (PRX6[3]-P16) bands showed their iniquity in the mother plant and nodal mediated plantlets (directly regenerated). The second band was common to all. Region 7 contained two bands, the first band (PRX7[1]-P17) was so specific to calli and calli mediated plants, whereas the second one was common to all (Table 3, Fig. 1G). In addition, in order to confirm the presence of phytochemical constituents, a comparative phytochemical study was carried out between the mother plant and calli derived from MS medium fortified with 1.0 mg/l of 2,4-D, and plantlets regenerated via indirect organogenesis from MS medium supplemented with 1.0 mg/l of BAP. The calli mediated tissues showed a higher percentage of metabolite constituents compared to the *in vivo* (mother plant) and nodal derived plants (data not shown). The phytochemical study revealed the presence of high quantities of saponins, alkaloids and phenolic compounds, etc., in calli and calli mediated tissues (Data are not shown).

DISCUSSION

The observations of the present study directly correlate with those of Rout *et al*. (1999), Manickam *et al*. (2000), Wilson *et al*. (2004) and Johnson *et al*. (2005). These investigators observed the maximum percentage of calli production on MS medium supplemented with 1.0 mg/l of 2,4-D. They also observed the maximum percentage of calli mediated shootlet formation on MS medium fortified with BAP (1.0 mg/l), in *Plumbago zeylanica, Withania somnifera* and *Plumbago rosea*. The hairy root formations coincided with that observed by Seeni (2004) in *Holostemma adakodian* callus cultures. Currently, scientists are using metabolic engineering and genetic transformation techniques to improve or modify the secondary metabolic content of medicinal and aromatic plants (Nisha *et al*., 2003).

Table 2. Effect of Plant growth regulators (PGRs) on Organogenesis of inter-nodal segment derived calli of *P. amarus.*

MS+growth regulators (mg/l)			Shooting response (%) ± S.D.	Rooting response (%) ± S.D.	Mean number of shootlets/ rootlets formation ± S.D.		Mean length of shootlets/ rootlets formation (cms ± S.D.)	
BAP	KIN	NAA			SHOOT	ROOT	SHOOT	ROOT
0.5	0.0	0.0	NIL	NIL	NIL	NIL	NIL	NIL
1.0	0.0	0.0	65.8±0.87	NIL	16.3±0.45	NIL	2.3±0.54	NIL
1.5	0.0	0.0	35.6±0.85	NIL	6.7±0.56	NIL	1.8±0.34	NIL
2.0	0.0	0.0	NIL	NIL	NIL	NIL	NIL	NIL
0.0	0.5	0.5	NIL	35.4±0.65	NIL	8.4±0.54	NIL	0.6±0.43
0.0	1.0	0.5	NIL	65.4±0.45	NIL	18.3±0.65	NIL	1.3±0.45
0.0	1.5	0.5	NIL	45.8±0.56	NIL	12.7±0.53	NIL	0.8±0.48

Table 3. Isoperoxidase analysis of *Phyllanthus amarus* Schum & Thonn.

ISOFORMS	Rf values	L_1	L_2	L_3	L_4
PRX1[1]	0.02	–	–	–	+
PRX1[2]	0.06	–	–	+	–
PRX1[3]	0.08	–	–	–	+
PRX2[1]	0.12	–	–	–	+
PRX2[2]	0.18	+	+	+	–
PRX2[3]	0.24	–	–	–	+
PRX3[1]	0.36	+	+	–	–
PRX3[2]	0.38	–	–	+	+
PRX4[1]	0.44	+	+	–	+
PRX4[2]	0.48	–	–	+	+
PRX5[1]	0.56	+	+	–	+
PRX5[2]	0.58	–	–	+	+
PRX6[1]	0.62	+	+	–	–
PRX6[2]	0.66	+	+	+	+
PRX6[3]	0.7	+	+	–	–
PRX7[1]	0.72	–	–	+	+
PRX7[2]	0.78	+	+	+	+

L_1 _ Mother plant. L_2 _ Nodal segment derived plantlets. L_3 _ Calli derived from the internodal segments. L_4 _ *In vitro* raised plant from *in vitro* derived calli. (+) – Sign indicates the isoform presence and (–) – Sign indicates the isoform absence.

For the purpose of metabolic engineering or plant breeding procedures, they have been totally dependent on the use of molecular markers for confirmation of their results. In the present study the isoenzyme system was used to reveal the morphological and biochemical variation. This study was further verified by the observations of Agarwal and Subhan, (2003) while studying *Centella asiatica* (Linn.), and those of Urban and Johnson *et al*. (2005) in the case of *Rhinacanthus nasutus*. The present study also revealed the somoclonal variation very clearly by the banding positions occupied by the calli mediated plants in the isoperoxidase gel system.Nair, 2000 observed the genetic uniformity between mother plant and in vitro raised plants of Calophyllum apetallum. The present study also supported the Nair analysis, by showing the equal appearance of bands between the mother plant and nodal derived plant (directly regenerated ones). The observations of the current phytochemical study were augmented by previous researches (Singh and Sudharsana 2003; Jain *et al*., 2004; Shariff *et al*., 2006). The present study has produced a valuable cell line protocol for the medicinally important plant *P. amarus*, which thus paves the way for the large scale production of this medicinally potential plant. Also, the isozyme marker will very much be useful in future research dealing with pharmaceuticals and molecular plant systematics.

References

Agarwal V, Subhan S (2003). *In vitro* plant regeneration and Protein profile analysis in *Centella asiatica* (Linn) urban, a medicinal plant. *Plant cell Biotechnol Molecular Bio*. 4: 83-90.

Anbalagan K (1999). *An introduction to electrophoresis*, Electrophoresis Institute Yercaud, Tamil Nadu, India, PP. 101.

Bratati DE, Datta PC, De B (1990). Pharmacognostic evaluation of *Phyllanthus amarus*. *Ind J Crud Drug Res*. 28: 81.

Castello M, Phatak A, Chandra N, Sharon M (2002). Antimicrobial activity of crude extracts from plant parts and corresponding calli of *Bixa orellana* L. *Ind J Exp Biol*. 40: 1378-1381.

Harborne JB (1964). *Biochemistry of Phenolic compounds*. Academic press, London. PP. 93-111.

Jain SC, Jain R, Vlietinck AJ (2004). *In vivo* and *in vitro* antimicrobial efficacy of *Mimosa hamata*. *Ind J Biotechnol*. 3: 271-273.

Johnson M (2003). *In vitro* studies on some rare and endangered ferns of Western Ghats, South India. PhD Thesis Submitted to Manonmanium Sundarnar University, Tmail Nadu, India

Johnson M, Berhanu Abraha, Mulugeta Kibret, Eyayu Molla, Manickam VS (2005). Regeneration from callus cultures of *Rhinacanthus nasutus* L. Kurtz. *Eth J Sci Tech*. 3: 17-24.

Johnson M (2006). *In vitro* multiplication of *Solanum anguivi* Lam, *Phyllanthus amarus* Schum & Thonn and *Jasminum calophyllum* wall-ethanomedicinal plants. *Eth J Sci Tech*. 3: 33-46.

Joy KL, Kuttan R (1998). Inhibition by *Phyllanthus amarus* of Hepatocarcinogenesis induced by N- Nitrosodiethylamine. *J Clin Biochem Nutr*. 24: 133.

Manickam VS, Madhavan ER, Antonisamy R (2000) Regeneration of Indian Ginseng plantlets from stem callus. *Plant Cell Tissue Organ Cult*. 62: 181-185.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture, *Physiol Plant*. 15: 473-497.

Nair LG (2000). Conservation through micropropagation restoration of selected woody medicinal plants. *Ph.D. Thesis*, Kerala University, Thiruvananthapuram, Kerala, India.

Nisha KK, Seetha K, Rajmohan K, Purushothaman MG (2003). Agrobacterium tumifaciens- mediated transformation of Brahmi (*Bacopa monniera* (L.)Wett st.), a popular medicinal herb of India. *Curr Sci*. 85: 85-89.

Raphel KR, Sabu MC, Kuttan R (2002). Hypoglycemic effect of Methanol extract of *Phyllanthus amarus* Schum and Thonn on Alloxan induced Diabetes mellitus in rats and its relation with antioxidant potential. *Ind J Expt Biol*. 40: 905-909.

Rout GR, Saxena C, Samantaray S, Das P (1999). Rapid plant regeneration from callus cultures of *Plumbago zeylanica*. *Plant Cell Tissue Organ Cult*. 56: 47-51.

Sabu KK, Padmesh P, Seeni S (2001). Estimation of active principle content and isozymes of *Andrographis paniculata* Nees (Kalmegh): An important medicinal plant of India. *J Med*

Arom Plant Sci. 23: 637-647.

Sadasivam S, Manickam A (1992). Biochemical methods for Agricultural Science. Wiley Eastern Ltd. and Tamil Nadu Agricultural University, Coimbatore, India. PP. 139.

Seeni S (2004). Bioprospecting of medicinal plants In: National Level First Biological Congress on Biome and Biosciences organized by Muthayammal College of Arts and Sciences, Rasipuram.

Shariff N, Sudharshana MS, Umesha S, Hariprasad P (2006). Antimicrobial activity of *Rauvolfia tetraphylla* and *Physalis minima* leaf and callus extracts. *Afr J Biotechnol*. 5: 946-950.

Singh K, Sudharshana MS (2003). Antimicrobial activity of *Baliospermum axillare* plant and callus extract. *Asian J Microbiol Biotecnol Environ Sci*. 5: 571-574.

Sivarajan VV, Balachandran I (1994). Ayurvedic drugs their plant sources. Oxford and IBH Publishers, New Delhi, India, PP. 466.

Smila H, Johnson M, Rajasekarapandian M (2007). Studies on varietal difference, tissue specificity and developmental variation of esterase and peroxidase isozymes in pearl millet (*Pennisetum glacum* (L.) R. Br.). *Ind J Biotechnol*. 6: 91-99.

Thyagarajan SP, Subramanian S, Thirunalsundari TPS, Blumberg BS (1988). Effect of Phyllanthus amarus on chronic carriers of hepatitis B virus. *Lancet* 2: 764.