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ISSN: 2320 – 7051
Int. J. Pure App. Biosci. **2 (2):** 181-186 (2014)

Research Article

INTERNATIONAL JOURNAL OF PURE & APPLIED BIOSCIENCE

An Efficient Callus Induction from *Phyllanthus debilis* Klein Ex Willd- A Wild Medicinal Plant of Eastern Ghats, India

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ABSTRACT

An efficient tissue culture protocol is developed for the medicinally persuasive plant Phyllanthus debilis Klein ex Willd. (Euphorbiaceae) using leaf and internodal explants on Murashige and Skoog's medium with different combination and concentrations of growth regulators. The leaf segments produced maximum callus induction on 45 days (82.5%) when MS medium was fortified with BAP (3.5 mg/l), NAA (2.5 mg/l), 2, 4-D (0.5 mg/l) and the best response was observed on internodal callusing (80%) when the MS medium contained 3.0 mg/l BAP, 2.0 mg/l NAA and 0.5 mg/l 2, 4-D. It was observed that the explants produced scanty callus when the concentration of the hormones are low.

Keywords: *Phyllanthus debilis, MS medium, Callus induction, Plant growth regulators.*

INTRODUCTION

Medicinal plants are of great interest to the researchers in the field of biotechnology as most of the drug industries depend, in part, on plants for the production of pharmaceutical compounds[1]. Among the world's 25 best selling pharmaceutical medicines, 12 are plant derived[2]. Plants are the main source of many modern medicines. It is estimated that approximately one quarter of prescribed drugs contain plant extracts or active ingredients obtained from or modeled on plant substances. Recent estimates suggest that over 9,000 plant species have known medicinal applications in various cultures and countries and this is without comprehensive research amongst several indigenous and other communities. In India, approximately 1700 plants species are used in Ayurveda, 500 for Siddha, 400 for Unani, 300 for Amchi systems of medicine with substantial overlaps of common plants among the system[3].

The genus *Phyllanthus* (Euphorbiaceae) has 750 species and several of them produce useful secondary metabolites which have been extracted from whole plants[4]. *Phyllanthus* species are traditionally used in the treatment of a variety of ailments including jaundice, asthma, ulcer, hepatitis, tuberculosis, malaria, dysentery, gonorrhea, flu, diabetes, dropsy, syphilis, cough, diarrhea, vaginitis and urinary diseases and other hepatic disorders[5, 6, 7]. *Phyllanthus debilis* Klein ex willd. belongs to the family Euphorbiaceae , a small herb 75cm long grown in hills 900-1200 m altitude, distributed in Tropical Africa, India, New Guinea[8]. *Phyllanthus amarus* and *Phyllanthus debilis* are closely related similar looking species commonly available in India. *Phyllanthus amarus* is widely distributed throughout India, while *Phyllanthus debilis* has its distribution restricted towards southern India[9].

Very few reports available on the tissue culture of *P. emblica, P. urinaria, P. amarus, P. abnormis, P. caroliniensis, P. tenellus, P. stipulatus* and *P. niruri*on transformed root cultures of *P. stipulatus, P. Niruri*[10, 11, 12, 13, 14, 15, 16]. Additional studies on callus and root extracts of these different species have shown the presence of phyllemblin[17].

India has a great wealth of traditional knowledge and wisdom, and the value of medicinal plants related trade in India is estimated at ₹5000 crores per annum. As the demand for the plant-derived pharmaceutical compounds is increasing, possibilities for mass production need to be explored. Plant tissue culture techniques offer the rare opportunity to tailor the chemical profile of a phytochemical product by manipulation of the chemical or physical microenvironment, to produce a compound of potentially more value for human use[19]. In recent year, there has been an increased in *in vitro culture* techniques which offer a viable tool for mass multiplication and germplasm conservation of rare, endangered and threatened medicinal plants[20, 21]. Tissue culturing of medicinal plants is widely used to produce active compounds for herbal and pharmaceutical industries. Advances in plant tissue culture will enable rapid multiplication and sustainable use of medicinal plants for future generations. Comparing with traditional methods of producing medicinal plant, *in vitro* micropropagation have many advantages such as the independent of seasonal variation, mass production, identification and production of clones with desired characteristics, conservation of threatened plant species, production of new and improved genetically engineered plant, preservation of genetic material by cryopreservation and production of secondary metabolites. Micropropagation of Several medicinal plants has been reported. Few studies reported the micropropagation of *P. Harmala*[22, 23]. Mass propagation of plant species through *in vitro* culture is one of the best and most successful examples of commercial application of plant tissue culture technology. Recently, there has been much progress in this technology for some medicinal plants. Tissue culture propagation and its importance in conservation of genetic resources and clonal improvement have been described[24]. There are no earlier reports on *invitro* studies of *P.debilis.* Therefore a standard protocol is developed for the efficient, medicinal plant.

MATERIALS AND METHODS

Phyllanthus debilis Klein ex Willd. was collected directly from the Yercaud hills, Eastern Ghats, Tamil Nadu. The plant was identified by The Rapinat Herbarium at Tiruchirappallai, Tamil Nadu. Leaf and internodes were excised from mother plant and used as the explants for further experiments. The explants were first washed with running tap water for half an hour to remove the soil particles and other extraneous tiny particles. Explants were treated with 2% Teepol for 5 minutes followed by repeated running tap water for 25 minutes after which they washed twice in sterilized distilled water. Further sterilization was done under aseptic condition in Laminar Airflow chamber. Explants were then surface sterilized by using 0.2% $HgCl_2$ for 2 minutes. They were rinsed thoroughly in sterilized distilled water three times and basal ends cut with a sterilized sharp blade. The leaf and internodes were cut into convenient size (0.5 to 1.0 cm). The glasswares were first soaked in sulphuric acid (H_2SO_4) for 4h and then washed well with a tap water, again soaked in detergent and washed under the running tap water to remove the traces of the detergent. They were finally rinsed using double distilled water and dried in hot air oven at high temperature of 90^o C for 30 minutes. MS medium[25] was used with different concentration and combination of BAP ranging from 1-3.5mg/l, NAA ranging from 1-2.5mg/l and 2, 4-D 0.5mg/l. The medium containing 3% sucrose and solidified with 0.8% agar was used. The *p*H of the media was adjusted to 5.6 with 1 N NaOH or 1 N HCl solutions prior to autoclaving. Media poured in culture vessels were steam sterilized by autoclaving at 121°C and 15 psi for 15-20 min. The cultures were incubated under controlled conditions of temperature (25±2°C), light (1000-2000 lux for 16/8 h provided by white fluorescent tubes).

RESULTS AND DISCUSSION

The leaf and internodal explants excised from the sterilized wild plants were inoculated on MS medium fortified with different concentrations and combination of BAP (1-3.5mg/l), NAA (0.5-2.5mg/l) and 2, 4-D (0.5mg/l). After 15 days of inoculation callus formation takes place at the cut end of leaf and internodes. The pale yellow colour callus proliferated from the leaf explants and light green colour from internodal explants. Higher concentration of BAP(3.5mgl) and NAA(2.5mg/l)gives best calli formation in leaf (61.5%) and intermodal (60%) explants. In leaf explants maximum percentage of callusing (82.5%)

was achieved with 3.5mg/l BAP, 2.5 mg/l NAA and 0.5 mg/l 2, 4-D followed by 72.5% of callusing was noticed in 3.0mg/l BAP, 2.0 mg/l NAA and 0.5 mg/l 2, 4-D. The minimum percentage of callusing (19.1%) was observed in MS medium supplemented with 1.5mg/l BAP, 0.5 mg/l NAA. In internodal explants maximum percentage of callusing (80%) was achieved with 3.0mg/l BAP, 2.0 mg/l NAA and 0.5 mg/l 2, 4-D followed that 75% of callusing was noticed in 3.5mg/l BAP, 2.5 mg/l NAA and 0.5 mg/l 2, 4-D. The minimum percentage of callusing (20%) was observed in MS medium supplemented with 1.5mg/l BAP, 0.5 mg/l NAA. To increase the frequency of callusing, plant growth regulators were supplied in different combination (Table: 1, Fig: 2). It was observed that the explants produce scanty callus when the concentrations of the hormones are low, where moderate and profuse callus was produced when the concentration was increased.

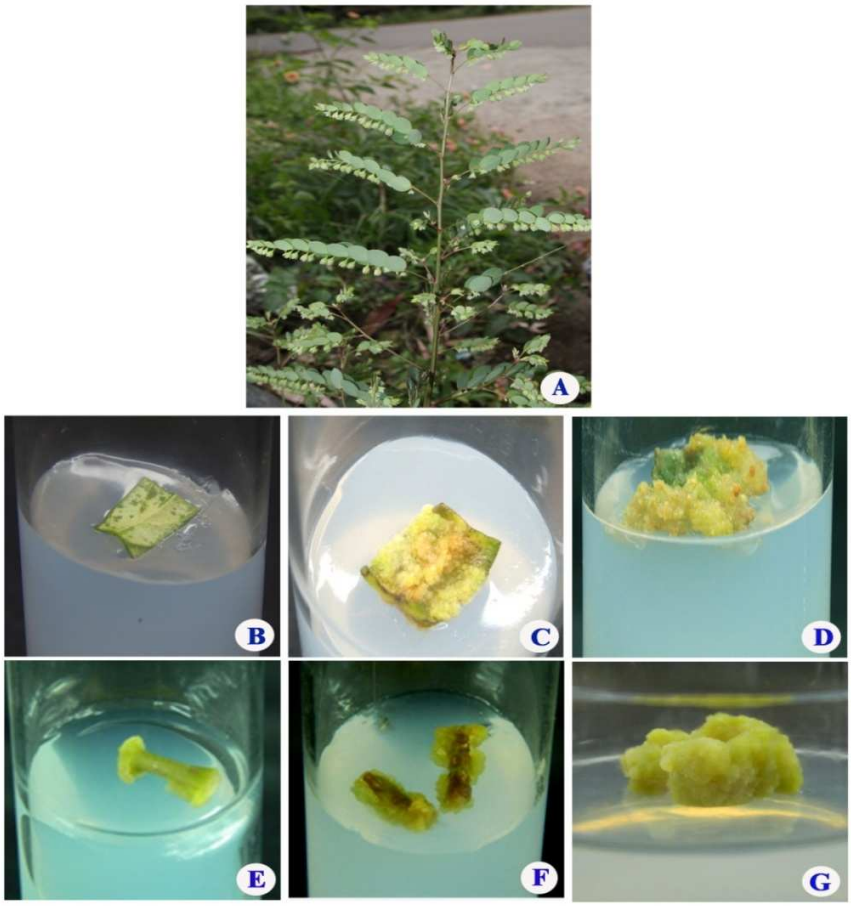
The observation was further supported by other works which reported that young leaves were used for callus induction in *Phyllanthus niruri* L. with the help of growth hormones Kinetin and 2, 4-D in different concentration[26], reported high frequency of callus initiation and growth was achieved by nodal segments, explants were inoculated in the vertical position on MS medium supplemented with 5 µM 2, 4-D[27]. The leaf petiole explants were used for the purpose of callus induction. Highest diameter of callus was observed on MS Medium fortified with 0.25 mg/l 2, 4-D+ 0.5 mg/l Kn callus diameter 4.6 cm and 0.25 mg/l 2,4-D+ 0.5mg/l BAP callus diameter 3.4 cm[28]. The combinations and concentrations of different growth regulators were shown to be critical factors for both the frequency and the type of callus formation as well as for the potential of callus differentiation. Of the four morphologically distinct types of calli that were induced, a friable, yellow callus induced on MS medium supplemented with 9.0 m*M* 2,4- dichloro phenoxy acetic acid[29]. From the perusal of data, it was obvious that low concentration of 2, 4-D plays a pivotal role in the augmentation of calli both in leaf and internodal explants of *P.debilis*. Our results are corroborated with earlier finding of other *Phyllanthus* species.

Table: 1 Percentage of callus induction from *Phyllanthus debilis* Klein ex Willd. in leaf and internodal explants at various combination of BAP, NAA and 2,4-D

Plant growth regulators mg/l			**Leaf**			**Internode**		
BAP	NAA	2,4-D	Degree of callus	% of callus induction Mean± Std. Error	Colour of callus	Degree of callus	% of callus induction Mean± Std. Error	Colour of Callus
1.0	--	--	No callus	--	--	No callus	--	--
1.5	0.5	--	+	19.16±0.33	PYL	+	20.00±0.05	LGR
2.0	1.0	--	++	33.00±0.05	PYL	++	32.00±0.05	LGR
2.5	1.5	--	++	38.00±0.47	PYL	++	36.53±0.33	LGR
3.0	2.0	--	+++	54.00±0.05	PYL	+++	52.00±0.05	LGR
3.5	2.5	--	+++	61.50±0.17	PYL	+++	60.00±0.17	LGR
1.0	--	--	No callus	--	PYL	No callus	--	LGR
1.5	0.5	0.5	+	27.33±0.33	PYL	+	26.00±0.25	LGR
2.0	1.0	0.5	++	47.53±0.27	PYL	++	48.00±0.11	LGR
2.5	1.5	0.5	++	62.50±0.30	PYL	++	60.06±0.08	LGR
3.0	2.0	0.5	+++	72.50±0.11	PYL	+++	80.06±0.12	LGR
3.5	2.5	0.5	+++	82.50±0.17	PYL	+++	75.06±0.20	LGR

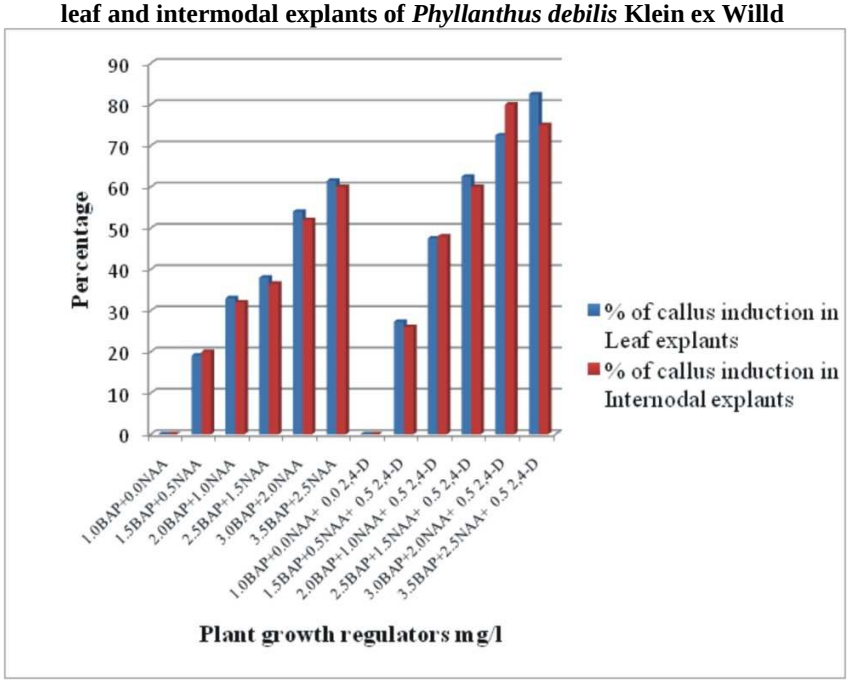
PYL = Pale Yellow, LGR=Light Green, - - = No callus, + =Scanty, ++ = Moderate and +++ = Profuse

Fig.1 Callus induction in *Phyllanthus debilis* Klein ex Willd from leaf and internode explants



A. Habit of *Phyllanthus debilis* Klein ex Willd., **B**. Leaf explants cultured on MS medium supplemented with 3.5 mg/l+2.5 mg/l+0.5 mg/l 2,4-D, **C**. Leaf callus initiation(15 days after inoculation), **D**. Well developed callus of leaf explants(45 days after inoculation), **E**. Internodal explants cultured on MS medium supplemented with 3.0mg/l+2.0 mg/l+0.5 mg/l 2,4-D, **F**. Internodal callus initiation(15 days after inoculation), **D**. Well developed internodal callus(45 days after inoculation).

Fig. 2 Effect of different combination and concentrations of growth regulators on induction of callus from leaf and intermodal explants of *Phyllanthus debilis* Klein ex Willd



CONCLUSIONS

In the present investigation a standard protocol was developed for the callus induction in *Phyllanthus debilis* Klein ex Willd. The present study reveals that the combinations of plant growth regulators produced best results for callus proliferation. The MS medium supplemented with 3.5mg/l BAP, 2.5 mg/l NAA and 0.5 mg/l 2, 4-D produced the highest amount of callus in leaf explants(82.5%). The best response was observed on Internodal callusing (80%) when the MS medium contained 3.0 mg/l BAP, 2.0 mg/l NAA and 0.5 mg/l 2, 4-D. The callus culture is an important preliminary stage to the regeneration of whole plants. The growth regulators requirement for callus initiation has been modified in the nutritional status of medium. In the present investigation, a standard protocol was developed for the callus induction, which is a prime stage for micropropagation. BAP, NAA and 2, 4-D was found to play an important role for callus production from the leaf and internodal explants of *Phyllanthus debilis.*

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