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Germplasm conservation of *Phyllanthus virgatus* G. Forst. by encapsulation of *In vitro* derived leaf segments

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ABSTRACT

The present study describes an efficient plant regeneration system for *in vitro* propagation of *Phyllanthus virgatus* through leaf explant. The leaf explants were cultured on Murashige & Skoog (MS) medium supplemented with BAP (1.5 mg L^{-1}) and NAA (0.9 mg L^{-1} for efficient callus induction (82.26%). The maximum percentage of shoots (77.85%) was developed from BAP (2.0 mg L^{-1}) and GA3 (0.5 mg L^{-1}). The efficient rooting response (76.28%) was noted on a nutrient medium containing NAA (1.5 mg L^{-1}) and IBA (0.2 mg L^{-1}). Regenerated plantlets were successfully acclimatized and hardened off inside the culture room and then transferred to green house with a 72% survival rate.

KEYWORDS: *Phyllanthus virgatus*, Micropropagation, Callus culture, Organogenesis

INTRODUCTION

The modern biotechnological tool like micropropagation offers a tremendous potential for conservation and multiplication in large scale levels of tropical useful medicinal plant species and subsequent exploitation. *In vitro* culture techniques are required to develop genetic transformation systems, and to establish cell suspension for the production of secondary metabolites. In this regard, the special attention to vegetative propagation particularly tissue culture, is worth and also a possible alternative to overcome the limited success of more conventional techniques (Jones *et al*., 1990; Galiana *et al*., 1991). Among the important medicinal plants, *Phyllanthus virgatus* (Euphorbiaceae) is one of the species. All parts are used as medicine for infantile malnutrition due to intestinal parasites; fresh plant ground and the paste applied on bleeding piles. Leaf juices are used in eye diseases and dysentery, itch and gonorrhea. Roots are used in mammary abscess. Plant used as antiseptic. Veterinary medicine, whole plant and root preparations externally applied to abscesses, mammary abscesses. Further, the whole plant extracts have been used since ancient times, for treating hypertension, diabetes, hepatic, urinary, sexual disorders, and other common ailments.

The present study describes the optimization of culture conditions and plant growth regulators required for callus induction, shoot regeneration and rooting of plantlets from an immature leaflets of *Phyllanthus virgatus*.

MATERIALS AND METHODS

The disease free and tender leaf segments of *Phyllanthus virgatus* were collected from three month old plants grown under shade house conditions and they were used as explant materials. The collected explant materials were washed under normal tap water followed by treatment with a surface sterilant, tween 80 (5% w/v) for 5 minutes. The explant materials were washed in double distilled water and then treated with fungicide such as carbendazim (50% w/v) fungicide (10%) also for 15 minutes to eliminate the fungal contamination followed by repeated washes in double distilled water 2 or 3 times. After the treatment with fungicide, explant materials were treated with antibiotics such as 5% antibiotics (Ampicillin & Rifampicin) for 30 minutes to eliminate bacterial contamination and followed by three rinses in sterile double distilled water. The explants were also treated with furthermore; surface sterilization was carried out by dipping the explants in 0.1% $HgCl_2$ for 3 minutes followed by 3-4 rinses in sterilized double distilled water.

Media and Culture Condition

Murashige and Skoog (MS) (1962), medium containing 3 % sucrose solidified with 1 % agar (tissue culture grade, Himedia, India) was used. The following conditions are required for the medium preparation. The pH 5.6-5.8 was adjusted prior to the addition of agar and also before autoclaving at 121° C for 15 min. The temperature condition should be maintained for all the culture bottles which were kept in culture chamber at 25± 2°C under 16/8 hr (light/dark) photoperiod with a light intensity of 2000 lux supplied by cool white fluorescent tubes and with 60-65% relative humidity.

Callus Induction Medium

The well sterilized explants were inoculated in 25 ml MS medium containing culture bottles which supplemented with different concentrations and combinations of BAP and NAA for callus induction.

Shoot Induction Medium

The shoot induction medium containing different combinations and concentrations of PGR like BAP (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg L^{-1}) and GA_3 at 0.5 mg $L^{-1}$was used for shooting characters.

Rooting of Elongated Shoots and Acclimatization

The well-developed shoots containing plantlets were carefully removed from the shooting culture medium and properly washed in double distilled water to avoid any trace of the medium on roots. The minimum length of 5-6 cm long *in vitro* regenerated shoots were excised and transferred to the rooting induction media which contain the NAA and IBA for rooting. After the well development of roots, these rooted plantlets were transferred to a hardening medium composed of garden soil, sand and vermicompost in different proportions and maintained in greenhouse conditions to know the survivability rate.

Statistical Analysis

All the experiment was done at least twice using triplicate. The data were statistically processed and means were compared using Duncan's Multiple Range Test ($P<0.05$).

RESULTS AND DISCUSSION

Optimization of micropropagation protocol depends upon various factors *viz*., media, explants, growth factors and cultural conditions. Hence, the different factors responsible for the standardization of present various stages of micropropagation. The effect of different combinations and concentrations of growth regulators on callus initiation from leaf explants of *P. virgatus* is shown in Table 1. An effective callus formation (82%) by leaf explants was observed on the MS medium fortified with the growth hormone, BAP (1.5 mg L^{-1}) and NAA (0.9 mg L^{-1}) (Figure 1). Senthilkumar *et al.* (2009b) also reported that the

Table 1: Effect of growth regulators on callus induction from leaf explants of the species, *Phyllanthus virgatus*

Growth regulators (mg/L^{-1})				Days required for callus formation after inoculation	Callus formation (%)
BAP	2, 4-D	NAA	Kn	Leaf Explant	Leaf Explant
0.5	0.0	0.0	0.0	16	$31.46^{a}\pm0.78$
1.0	0.0	0.0	0.0	19	$39.15^{c}\pm1.21$
1.5	0.0	0.0	0.0	17	$43.86^{e}\pm0.47$
2.0	0.0	0.0	0.0	20	$59.05^{g}\pm1.54$
2.5	0.0	0.0	0.0	21	$66.34^{i}\pm2.16$
3.0	0.0	0.0	0.0	19	$72.48^{f}\pm1.28$
0.0	0.5	0.4	0.0	16	$64.75^{c}\pm0.36$
0.0	1.0	0.4	0.0	15	$58.63^{e}\pm1.24$
0.0	1.5	0.4	0.0	20	$63.00^{f}\pm0.73$
0.0	2.0	0.4	0.0	23	$69.85^{i}\pm1.21$
0.0	2.5	0.4	0.0	16	$75.79^{a}\pm0.85$
0.5	0.0	0.3	0.0	15	$46.80^{cd}\pm1.39$
1.0	0.0	0.6	0.0	16	$53.88^{e}\pm1.98$
1.5	0.0	0.9	0.0	24	$82.26^{i}\pm0.61$
2.0	0.0	1.2	0.0	24	$80.13^{j}\pm1.53$
2.5	0.0	1.5	0.0	21	$66.52^{h}\pm1.36$
3.0	0.0	1.8	0.0	18	$48.28^{b}\pm0.17$
0.0	0.3	0.0	0.2	15	$39.84^{c}\pm1.72$
0.0	0.6	0.0	0.4	17	$44.95^{d}\pm1.24$
0.0	0.9	0.0	0.6	16	$49.23^{e}\pm0.84$
0.0	1.2	0.0	0.8	18	$53.12^{f}\pm1.93$
0.0	1.5	0.0	1.0	13	$57.76^{cd}\pm0.69$

Means in columns followed by different letter (s) are significant to each other at 5% level according to DMRT

growth hormone cytokinin which has an important role in callus induction. Gaspar *et al*., (1996 & 2003) reported that cytokinin has the ability to stimulate cell division and then develop into an un-organized tissue (callus). Uddin *et al.* (2006) also suggested that high degree of totipotency and meristematic activity in the cells of leaves margins of many species resulted in the formation of more effective prominent calli than any other explants.

Shoot initiation occurred from immature leaf explants derived callus. The combination treatment of BAP (2.0 mg L^{-1}) along with GA3 (0.5 mg L^{-1}) was found to exhibit the highest frequency of shoot multiplication (77%), the maximum number of shoots (14.11 shoots/callus) and shoot length (7.9 cm) (Table 2 and Figure 1) . Arya and Shekhawat (1987), Cobman and Ernst (1990) and Dewan *et al* (1992) reported cytokinins as an obligatory part of the media for shoot differentiation. These results are in agreement with the present study. For the induction of root organs from the *in vitro* grown shoots by using MS medium supplemented with different concentration of growth regulators. The maximum rooting (76 %) maximum number of roots (10.47 roots/shoot) and root length (7.8 cm) was obtained by using the growth regulator viz., NAA and IBA combination and the concentration of growth regulators at 1.5 and 0.2 mg L^{-1} respectively (Table 3 and Figure 1). A similar kind of observation based on the requirement of lower doses of auxins for better rooting was already reported in the species of *Phyllanthus amarus* by Chitra *et al.* (2009). The presence of auxin at higher concentrations facilitated better rhizogenesis. Similar findings have been reported in several medicinal plants like *Catharanthus roseus* (Faheem *et al*., 2011), *Leptadenia reticulate* (Sudipta *et al*., 2011).

Table 2: Effect of shooting attributes from different growth regulators on leaf derived callus of the species, *Phyllanthus virgatus*

Growth regulators (mg/L^{-1})				Culture response (%)	No. of shoots/ callus	Shoot length (cm)
BAP	NAA	GA_3	IAA			
0.3	0.0	0.0	0.0	$46.63^{d}\pm1.27$	$3.15^{ab}\pm1.28$	$3.6^{ab}\pm1.62$
0.3	0.0	0.0	0.0	$54.22^{hi}\pm1.36$	$6.47^{bcd}\pm0.35$	$4.3^{abc}\pm0.36$
0.3	0.0	0.0	0.0	$62.47^{j}\pm0.74$	$8.00^{def}\pm1.43$	$5.0^{abc}\pm1.36$
0.3	0.0	0.0	0.0	$57.63^{fg}\pm1.04$	$7.05^{abc}\pm0.66$	$4.6^{bc}\pm1.28$
0.3	0.0	0.0	0.0	$47.71^{gh}\pm0.46$	$6.83^{fgh}\pm1.53$	$5.2^{abc}\pm0.45$
0.3	0.0	0.0	0.0	$39.87^{kl}\pm1.62$	$8.64^{fgh}\pm0.91$	$5.9^{c}\pm0.56$
0.5	0.0	0.5	0.0	$59.09^{k}\pm1.85$	$12.35^{hi}\pm1.61$	$6.2^{abc}\pm1.33$
1.0	0.0	0.5	0.0	$69.72^{m}\pm1.52$	$9.53^{ghi}\pm1.27$	$4.7^{abc}\pm1.18$
1.5	0.0	0.5	0.0	$71.21^{n}\pm1.56$	$10.73^{i}\pm0.77$	$6.4^{c}\pm0.47$
2.0	0.0	0.5	0.0	$77.85^{n}\pm1.09$	$14.11^{i}\pm1.31$	$7.9^{c}\pm1.88$
2.5	0.0	0.5	0.0	$73.69^{b}\pm0.36$	$11.89^{efg}\pm0.73$	$6.3^{bc}\pm1.57$
3.0	0.0	0.5	0.0	$48.26^{a}\pm1.82$	$9.53^{bcd}\pm1.23$	$3.8^{ab}\pm0.40$
0.0	0.5	0.0	0.0	$43.07^{de}\pm1.22$	$8.14^{ghi}\pm1.46$	$4.1^{abc}\pm0.27$
0.0	0.5	0.0	0.0	$39.72^{a}\pm1.44$	$7.76^{fgh}\pm0.44$	$5.6^{bc}\pm1.73$
0.0	0.5	0.0	0.0	$47.61^{c}\pm1.11$	$7.25^{cde}\pm1.28$	$5.3^{bc}\pm0.82$
0.0	0.5	0.0	0.0	$50.48^{fg}\pm0.81$	$6.83^{ab}\pm1.36$	$4.8^{abc}\pm1.55$
0.0	0.5	0.0	0.0	$52.62^{ij}\pm1.34$	$4.18^{bcd}\pm1.72$	$4.6^{abc}\pm1.63$
0.0	0.5	0.0	0.0	$53.78^{l}\pm1.59$	$6.20^{efg}\pm0.44$	$3.7^{ab}\pm1.49$
0.5	0.0	0.0	0.2	$23.94^{d}\pm1.76$	$1.97^{def}\pm1.73$	$1.9^{abc}\pm0.32$
1.0	0.0	0.0	0.2	$46.00^{k}\pm0.38$	$2.06^{ghi}\pm1.31$	$2.4^{a}\pm0.21$
1.5	0.0	0.0	0.2	$57.49^{ef}\pm1.09$	$5.51^{abc}\pm0.26$	$4.7^{abc}\pm1.63$
2.0	0.0	0.0	0.2	$61.24^{hi}\pm1.86$	$3.74^{a}\pm1.17$	$3.0^{abc}\pm0.88$
2.5	0.0	0.0	0.2	$64.06^{fg}\pm1.56$	$4.47^{ab}\pm0.63$	$3.7^{a}\pm1.82$
3.0	0.0	0.0	0.2	$66.82^{ij}\pm1.93$	$7.30^{fgh}\pm0.43$	$3.1^{ab}\pm1.62$

Means in column followed by different letter (s) are significant to each other at 5% level according to DMRT

Table 3: Effect of rooting attributes from different concentrations of growth regulators on leaf callus derived *in vitro* produced shoots of the species, *Phyllanthus virgatus*

Growth regulators (mg/L^{-1})			Shoots rooted (%)	No. of roots/shoot	Root length (cm)
NAA	IAA	IBA			
0.5	0.0	0.2	$58.16^{j}\pm0.22$	$5.27^{abc}\pm0.74$	$4.2^{a-d}\pm1.43$
1.0	0.0	0.2	$63.47^{k}\pm1.34$	$8.16^{def}\pm0.33$	$3.6^{ef}\pm1.62$
1.5	0.0	0.2	$76.28^{m}\pm1.82$	$10.47^{f}\pm0.88$	$7.8^{f}\pm1.09$
2.0	0.0	0.2	$72.34^{l}\pm0.28$	$9.31^{ef}\pm1.14$	$5.4^{ef}\pm0.28$
2.5	0.0	0.2	$61.76^{h}\pm1.36$	$6.72^{abc}\pm1.39$	6.2de±1.19
3.0	0.0	0.2	$54.83^{g}\pm1.07$	$4.68^{ab}\pm0.21$	$4.8^{a-d}\pm1.73$
0.5	0.0	0.3	$51.77^{e}\pm1.15$	$5.33^{bcd}\pm0.93$	$2.5^{bcd}\pm1.27$
1.0	0.0	0.3	$44.62^{d}\pm1.72$	$4.72^{ab}\pm0.46$	$1.3^{a-d}\pm1.88$
1.5	0.0	0.3	$40.32^{c}\pm0.68$	$6.53^{abc}\pm1.27$	$3.6^{abc}\pm0.79$
2.0	0.0	0.3	$39.63^{b}\pm1.75$	$5.77^{abc}\pm1.58$	$5.3^{a-d}\pm1.26$
2.5	0.0	0.3	$34.71^{a}\pm1.61$	$3.92^{a}\pm1.36$	$3.4^{ab}\pm1.83$
3.0	0.0	0.3	$41.28^{a}\pm1.74$	$4.49^{ab}\pm1.27$	$1.8^{a}\pm0.67$
0.0	0.2	0.1	$32.09^{c}\pm1.23$	$5.83^{ab}\pm0.82$	$4.1^{a-d}\pm1.96$
0.0	0.4	0.2	$43.84^{f}\pm0.63$	$6.41^{bcd}\pm1.94$	$3.9^{abc}\pm1.78$
0.0	0.6	0.3	$54.28^{h}\pm0.46$	$7.29^{cde}\pm0.52$	$2.4^{bcd}\pm1.35$
0.0	0.8	0.4	$37.47^{h}\pm1.18$	$6.36^{bcd}\pm1.38$	$3.7^{cde}\pm1.51$
0.0	1.0	0.5	$28.09^{g}\pm1.85$	$3.19^{abc}\pm0.73$	$4.8^{a-d}\pm1.44$
0.0	1.2	0.6	$21.72^{i}\pm1.98$	$2.06^{bcd}\pm1.32$	$2.2^{de}\pm1.61$

Means in columns followed by different letter (s) are significant to each other at 5% level according to DMRT

Table 4: Effect of plantlet survivability from leaf callus derived *in vitro* rooted plantlets of the species, *Phyllanthusvirgatus*

Hardening medium composition (V/V)	No. of plantlets under hardening	No. of plantlets survived	Survivability (%)
Red soil + sand (1:1)	50	27	$43^{a}\pm0.68$
Garden soil + sand + vermicompost (1:1:1)	50	48	$72^{d}\pm0.59$
Decomposed coir waste + perlite + compost (1:1:1)	50	40	$63^{c}\pm1.37$
Vermicompost + soil (1:1)	50	36	$53^{b}\pm0.31$
Red soil + sand + vermicompost (1:1:1)	50	20	$50^{a}\pm1.26$

Means in column followed by different letter (s) are significant to each other at 5% level according to DMRT

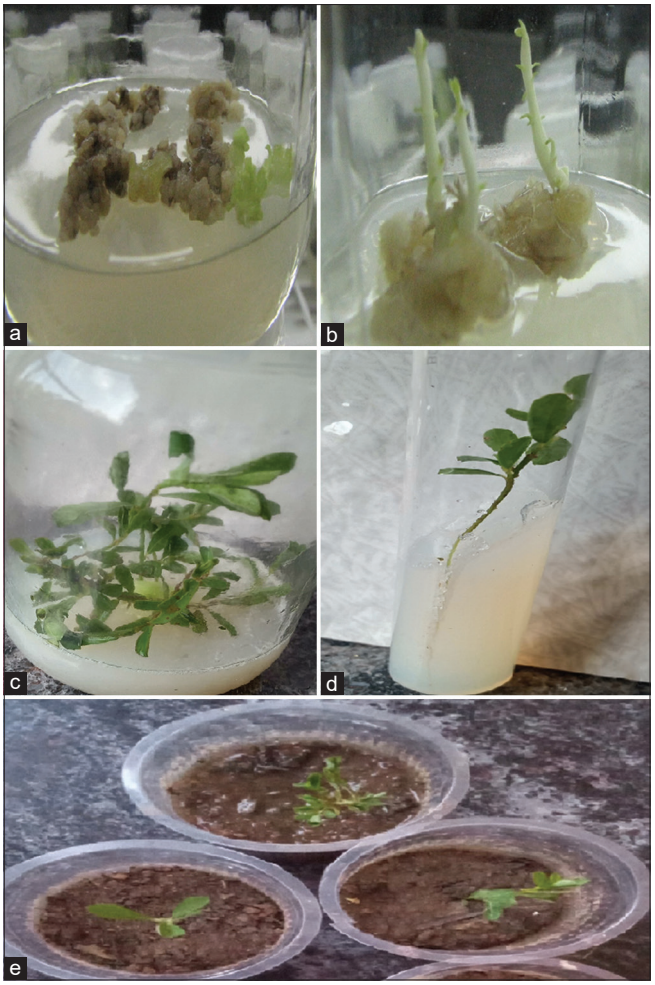

Figure 1: *In vitro* regeneration through leaf explant *Phyllanthus virgatus*. a-b: Effective callusing in MS medium supplemented with BAP and NAA at 1.5 and 0.9mg/L^{-1} respectively. c: Successful shooting by subculturing of leaf derived callus in the MS medium with BAP and GA_3 at 2.0 and 0.5mg/L^{-1} respectively. d: High amount of rooting during the subculturing of shoots in the MS medium fortified with NAA and IBA at 1.5 and 0.2mg/L^{-1} respectively. e: Under hardening in the mist chamber

After the development of roots, the well sprouted plantlets were taken out from the culture bottles and washed with double distilled water to remove adhering agar medium. It facilitates that the chance of contamination could be stopped. Then these juvenile plantlets were transferred to garden soil, sand and vermicompost (1:1:1 ratio by volume) composed hardening medium. The young leaf callus derived plantlets have the

maximum rate of survivability was higher than 72% (Table 4 and Figure 1). Admixture of all these three compositions may provide suitable environmental conditions by providing optimum nutrients, adequate aeration and essential minerals respectively to the plantlets. The present paper describes a prime and easy proliferation protocol for large scale production of *Phyllanthus virgatus*. This useful method is considered not only as an aspect of micropropagation but also useful to breeding and gene transfer purposes.

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