Journal of Academia and Industrial Research (JAIR)
Volume 5, Issue 4 September 2016

ISSN: 2278-5213

RESEARCH ARTICLE

Optimization of *In Vitro* Regeneration of *Phyllanthus amarus* and its Antibacterial Potential

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Abstract

An efficient tissue culture protocol was developed for the medicinally important plant *Phyllanthus amarus* using leaf, node and internode explants on Murashige and Skoog's basal medium with different combination and concentrations of growth regulators. The nodal explant produced maximum callus induction on 45 d (80%) when MS medium was fortified with 2,4-D (0.5 mg/L), the internodal segment produced maximum shoot formation (80%), at the 2.0 mg/L BAP concentration and the internode explants produced maximum percentage of root (60%) when the MS medium contained 3.0 mg/L IAA. The tissue cultured *Phyllanthus amarus* crude extracts at different concentrations were evaluated for antimicrobial activity against human bacterial pathogen *Pseudomonas aeruginosa*. Among the different concentrations of crude extracts, 150 µL (17.0%) recorded the maximum percentage of inhibition when compared to the standard antibiotic (3.3%).

Keywords: Tissue culture, *Phyllanthus amarus*, shoot formation, antimicrobial activity.

Introduction

Many plants are used as traditional medicines for the treatment of various infective diseases (Ozgen *et al*., 2012). 'O'Neill and Lewis (1993) have reported that the medicinal plants are of great interest to the researchers in the field of biotechnology as most of the drug industries depend on plants for the production of pharmaceutical compounds. Plants are the main source of many modern medicines. Among the world's 25 best selling pharmaceutical 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 to possess medicinal applications in various countries and this is without comprehensive research amongst several indigenous and other communities. In India, Gayathri *et al*. (2006) have approximately studied 1700 plants species which are used in Ayurveda, Siddha and Unani. *Phyllanthus* (Euphorbiaceae) has 750 species and several of them produce useful secondary metabolites which have been extracted from whole plants (Unander, (1996). *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 (Unander, 1991; Bharatiya, 1992). 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 by various researchers (Jusekutty *et al*., 1993; Ehsanpour and Sa-Adat, 2002). Hence, the present investigation was undertaken to standardize a suitable protocol for the mass multiplication of this medicinally important plant *Phyllanthus amarus* and also screen them for antibacterial activity against human bacterial pathogen *Pseudomonas aeruginosa.*

Materials and methods

Collection of plant materials: As per information given by the Siddha medical practitioner, the plant material was collected at Golden Jubilee Biotech Park herbal garden at Chennai in the month of July 2014. The plant parts were used and the local names were recorded. The identification was carried out with the help of Flora of the Presidency of Madras.

Plant material and explant preparation: Phyllanthus amarus explants (leaves, nodes and internodes) were collected and washed thoroughly under running tap water for 10 min and washed with double distilled water and added 3 drops of Tween-20 (liquid detergent) and add sodium hypochlorite (bleach) and swirled it for 8½ min.

Table 1. Effect of 2,4-D on callus induction from explants of *Phyllanthus amarus*.

Explants	No. of explants	Response (%)
Leaves	100	60
Nodes	100	80
Internodes	100	70

Table 2. Effect of BAP on shoot induction from explants of *Phyllanthus amarus*.

Explants	No. of explants	Response (%)
Leaves	30	20
Nodes	50	60
Internodes	50	80

Table 3. Effect of IAA on root formation from explants of *Phyllanthus amarus*.

Explants	No. of explants	Response (%)
Leaves	20	10
Nodes	40	40
Internodes	40	60

Afterwards it was washed thoroughly with distilled water and 0.1% $HgCl_2$ was added and swirled it for 2-3 min followed by 70% alcohol. Then it was washed 3-4 times with distilled water and used for further process.

Culture medium and condition: The culture vial containing Murashige and Skoog's (1962) medium with 3% sucrose and 0.7% agar, supplemented with different hormone (2,4-D, BAP and IAA) concentrations was used for *in vitro* regeneration, pH was adjusted to 5.7 prior to autoclaving. The explants were inoculated and incubated at 25°C with 16 h photoperiod.

Plant crude extract preparation for antibacterial screening: The plant materials were washed with water to remove the adhering dust particles and were shade dried at room temperature. The dried plant materials were ground into a fine powder in an electric blender and subsequently sieved using a sieve for obtaining fine powder. Thereafter, 5 g each of fine powdered sample was weighed and soaked separately in 15 mL of different solvents (Methanol: chloroform (1:1)) in the ratio of 1:3 weights for volume (W/V) and allowed to stand for 24 h at ambient room temperature. The soaked plant powder was filtered through filter paper (Whatman No.1) and the filtrate was dried and used as crude extract. The lyophilized *Pseudomonas aeruginosa* culture (MTCC 1035) was purchased from the IMTECH, Pune and maintained in the nutrient medium. Antimicrobial activity was performed according to Perez *et al.* (1990). About 0.1 mL of the diluted *Pseudomonas aeruginosa* microbial culture was spread on sterile nutrient agar (NA) plate. Using a sterile cork borer (9 mm dia), the well was cut from agar in the centre of the plate. The crude extract was diluted in DMSO (mg/mL) and poured at different concentrations (25, 50, 75, 100, 125 and 150 µL) of the crude extracts in well. After suitable incubation period the inhibition zone was measured in mm. Streptomycin (25 µg/µL) antibiotic was used as standard.

Results and discussion

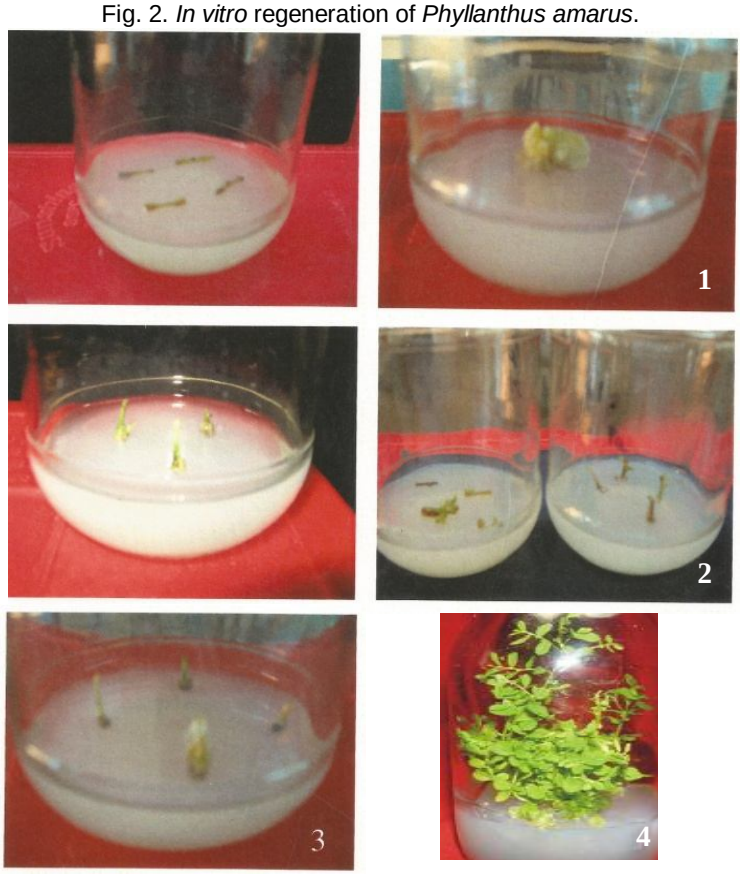
In vitro regeneration: The *Phyllanthus amarus* leaves, nodes and internodes explants were used for callus induction. The nodal explant produced maximum callus induction on 45th d (80%) when MS medium was fortified with 2,4-D (0.5 mg/L) and internodal segment produced maximum shoot formation (80%), at 2.0 mg/L BAP concentration and the internode explants produced maximum percentage of root (60%) when the MS medium contained 3.0 mg/L IAA (Tables 1-3, Figs. 1 and 2). *Phyllanthus debilis* using leaf and internodal explants on Murashige and Skoog's medium with different combination and concentrations of growth regulators produced maximum callus induction on 45 d (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 (Malayaman *et al.,* 2014).

Ugandhar (2014) reported the different concentrations of BAP, Kin and 2iP tested, individual treatment of 1.0 mg/L of BAP showed the best response and produced average of 7.2 shoots per shoot tip explants with 76% of response. In nodal explants also 1.0 mg/L BAP showed the best response (82%) and produced 8.5 shoots per explant. Among the different concentrations tested, 0.5 mg/L Kin showed the best response and produced 8.5 shoots per shoot tip explants. Root induction was achieved within 7 d of culture. IAA alone was effective for induction of roots. Usually root induction in *Phyllanthus* was difficult when compared to other medicinal plants. The combinations of BAP and IAA produced high percentage (85%) of response. In the present study, media comprising of MS salts, B5 vitamins, BAP + IAA (0. 5+1. 5 mg/L) was effective for induction of roots (9.5 roots/explant).

Fig. 1. *Phyllanthus amarus* wild plant.

1-Wild plant; 2-Flowers on branchlet; 3-Flowers; 4-Fruits.

Fig. 2. *In vitro* regeneration of *Phyllanthus amarus*.



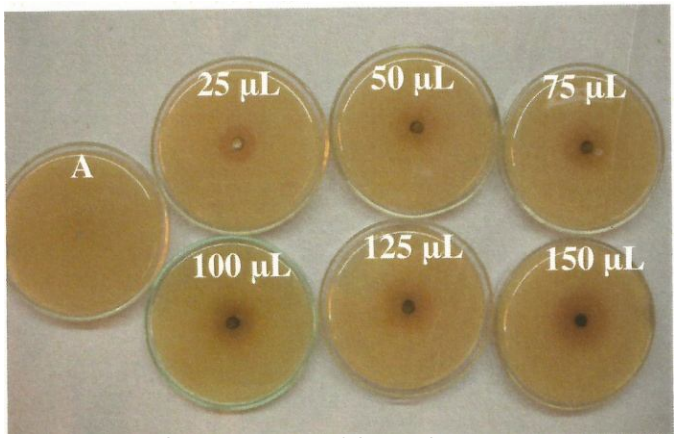
1-Callus; 2-Shoot; 3-Root induction; 4-*In vitro* plant.

Table 4. Effect of *P. amarus* crude extracts on *Pseudomonas aeruginosa*.

Antibiotic (25 µL)		Different concentrations of *P. amarus* crude extract											
		25 µL		50 µL		75 µL		100 µL		125 µL		150 µL	
ZOI	I %	ZOI	I %	ZOI	I %	ZOI	I %	ZOI	I %	ZOI	I %	ZOI	I %
3.0	3.3	8.0	9.0	9.0	10.0	10.0	11.1	12.0	13.3	14.0	16.0	15.0	17.0

Zone of inhibition (ZOI) in dia (mm); I% is inhibition percentage.

Fig. 3. Antibacterial activity of *P. amarus* crude extracts on *Pseudomonas aeruginosa*.



A-Streptomycin-25 µL; Conc. of the crude extracts-25-150 µL.

Antibacterial activity: Plant crude extract of *Phyllanthus amarus* was tested against human pathogenic bacteria *Pseudomonas aeruginosa*. Among the different concentration of crude extracts tested, 150 µL (17.0%) recorded the maximum percentage of inhibition when compared to standard antibiotic (3.3%) (Table 4, Fig. 3). Kanthimathi and Soranam (2013) in their study recorded zone of inhibition of 16 mm at 90 µL volume with 500 mg/mL concentration of the aqueous extract of *P. niruri* against the test bacteria *Lactobacillus* sp. There was no inhibition zone formation against the other test bacterial isolates namely *Bacillus* sp., *Pseudomonas* sp., *Proteus* sp., and *Streptococcus* sp.

Conclusion

Phyllanthus amarus nodal explant produced maximum callus induction on 45 d (80%) when MS medium was fortified with 2,4-D (0.5 mg/L), the internodal segment produced maximum shoot formation (80%), at the 2.0 mg/L BAP concentration and the internode explants produced maximum percentage of root (60%) when the MS medium contained 3.0 mg/L IAA. Among the different concentrations of *P. amarus* crude extracts, 150 µL (17.0%) recorded the maximum percentage of inhibition when compared to the standard antibiotic (3.3%). The present findings may be further explored to find alternative techniques for *in vitro* regeneration of various indigenous medicinal plants.

Acknowledgements

Authors are greatly acknowledged to Prof. Dr. P. Gajivaradhan, Principal, Pachaiyappa's College for providing laboratory facilities.

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