In vitro micropropagation of *Phyla Nodiflora* (Linn.) Greene a native medicinal plant

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**ABSTRACT**

*Phyla nodiflora* L. is a well known native medicinal plant in Indian systems of medicine. Explants of shoots with nodal region with and without the axillary bud were cultured on MS medium supplemented with PGRs. Segments without the axillary buds produced callus at the cut end in light. Roots were also initiated at this region. In explants with axillary bud only one of the two buds was released. This grew into normal branch in the test tube. In the presence of charcoal the growth of the axillary bud is more rapid although the internodes were abnormally elongated. At low concentration of 2,4,5-T the axillary buds and cut surfaces produced callus. Explants of leaf produced roots from the basal cut end of the major veins. Such roots elongated excessively. This study will help in the biotechnological manipulations of *P. nodiflora* to enhance and isolate useful medicinal substances.

**Keywords:** in vitro tissue culture, *Phyla nodiflora*, micropropagation, medicinal plants, Verbenaceae.

**Abbreviations:** MS-Murashige-Skoog Medium; NAA – Naphthalene acetic acid, BAP-6-Benzyl amino purine; 2,4,5-T – 2,4,5-trichloroacetic acid, PGRs-Plant growth regulators.

1. **INTRODUCTION**

From time immemorial plants and plant products have a great source to human kind. Some of the useful products include food, fibres, wood, paper, drugs, dyes, resins, spices and condiments. Based on the utility plants have been categorized as food plants, plants of industrial value, fibre yielding plants and medicinal plants (Sambamurthy and Subrahmanyam, 1989). Medicinal plants are the most essential component of the health care systems of human kind. The use of plant parts in the treatment of physical injuries and metabolic disorders must have attracted the attention of people at an early stage of their evolution. Most ancient civilization such as the Indian, Egyptian and Chinese has left us records of use of medicinal plants. Medicinal plants are found growing wild in all parts of the world, especially in the tropical countries. In India, China and the Middle Eastern countries, collection and preservation of crude drugs from medicinal plants have been in practice from the beginning of history (Sambamurthy and Subrahmanyam, 1989).

The use of herbal medicines is growing in developed countries, presently 25% of the UK population use herbal medicine (Vines, 2004; Zhou and Wu, 2006). About 40% of compounds used in pharmaceutical industry are directly or indirectly derived from plants because the chemical synthesis of such compounds is either not possible and/or economically not viable. Therefore a large number plant species (especially medicinal) are under threat of extinction because of their over exploitation (Vines, 2004; Rout et al., 2000; Edwards, 2004). The success of plant biotechnology relies on the fundamental techniques of plant tissue culture. Understanding basic biology of plants is a prerequisite for proper utilization of the plant system or parts thereof. Plant tissue culture helps in providing a basic understanding of physical and chemical requirements of cell, tissue, organ culture, their growth and development. Establishment of cell, tissue and organ culture and regeneration of plantlets under in vitro conditions has opened up new avenues in the area of plant biotechnology (Dagla, 2012). Micropropagation is the process of vegetative growth and multiplication from plants tissues or seeds. It is carried out in aseptic and favourable conditions on growth media, using various plant tissue culture techniques (Zhoud and Wu, 2006; Leifert et al., 1989; Bhojwani and Razdan, 1996). Tissue culture is based on concept of totipotency; the ability of plant cells and tissues to develop into whole new plant (Fowler et al., 1993). Gottlieb Haberlandt (1854-1945), a German botanist is considered as the father of plant tissue culture, was the first to separate and culture plant cells on Knop’s salt solution in 1898 (Krkorian and Berquam, 1969). In conventional cultivation many plants do not germinate, flower and produce seed under certain climatic conditions or have long periods of growth and multiplication. Micropropagation insures a good regular supply of medicinal plants, using minimum space and time (Prakash and Van Staden, 2007). The advantages of *In vitro* micro propagation of medicinal plant are listed below:

1. Higher rate of multiplication.
2. Environment can be controlled or altered to meet specific needs of the plant.
3. Plant available all year round (independent of regional or seasonal variation).
4. Identification and production of clones with desired characteristics.
5. Production of secondary metabolites.
6. New and improved genetically engineered plant can be produced.
8. Preservation of genetic material by cryopreservation.  

1.2. Tissue culture as a means of propagation  

Tissue culture techniques are used for rapid propagation of plant in vitro conditions. Tissue culture has been in practice for several decades for laboratory and commercial cultivation of a large number of economically important plants (George and Sherrington, 1984; Narayanaswamy, 1994). Somatic embryogenesis provides an effective method for rapid propagation of a large number of plants. Plant tissue culture techniques also have great potential as a means of vegetatively propagating economically important crops and crops of future potential on a commercial basis (Brown and Thrope, 1984). Using micropropagation, multiplication of economically important plants, elite genotypes and rare and endangered species is possible in a relatively short period of time (George and Sherrington, 1984). In the traditional methods of vegetative propagation, a rooted cutting can produce a single plant from which several years later further cuttings are available whereas in in vitro culture system, even the resting buds can produce several axillary as well as adventitious shoots. These in turn can be induced to produce axillary or adventitious shoots. Plant tissues also show an enormous rate of multiplication in suitable in vitro conditions. Large scale production of plants with selected qualities like disease resistance, drought and frost tolerance is achieved through tissue culture (Rao, 1981).  

1.3. Plant Growth Regulators in Tissue Culture and Vegetative Propagation  

The growth and development of higher plant tissues in vitro conditions is controlled by gradients of endogenous plant growth substances. These compounds which are generally active at very low concentrations can be either plant hormones (naturally occurring) or synthetic plant growth regulators (PGRs). There are five known classes of plant growth regulators Auxins, Cytokinins, Gibberellins, Ethylene, Absciscic acid and other regulators. The role of PGRs in plant tissue culture, the mode of actions, and the application of PGRs are extensively described by George and Sherrington (1984), Bhogwani and Razon (1985). Growth and morphogenesis in the culture are regulated by the interaction and balance between the growth substances produced endogenously by cultured cells (George and Sherrington, 1984). Auxins and Cytokinins are the two important classes of PGRs used for regulating growth and morphogenesis in plant tissue and organ cultures. Auxins promote the growth of callus, cells in suspension, isolated protoplast and organs such as meristems, shoot or root tips. The auxins commonly used for callus induction for different explants of monocots are 2,4-D; 2,4,5-T and NAA (Gamborg et al., 1975). The use of 2,4-D as a PGR at high concentration is not encouraged as it induced genetic instability and suppresses organogenesis. Somatic embryogenesis is often initiated in media containing high levels of auxin, especially 2,4-D; but embryo are not formed until the auxin concentration is reduced (Ammirato, 1983). Induction of caulogenesis or rhizogenesis in culture requires adjustment of the level of auxin/cytokinin (George and Sherrington, 1984). Cytokinin in tissue culture induces shoot morphogenesis. Cell division in callus tissue without the addition of cytokinin is believed to occur due to natural endogenous growth substances (Skoog and Miller, 1957). Callus which was induced to form shoots by the addition of cytokinin, was more compact than non-shoot forming callus. Shoot forming in tobacco callus could be induced by using relatively low levels of auxin and high levels of cytokinin (Skoog and Miller, 1957).  

1.4. Botany of Phyla nodiflora  

This genus is distributed throughout India, common in wet places along bunds. In tamil it is commonly known as “poduthalai”. A creeping branched perennial herb, rooting at the nodes, and with sub-quadrangular stems. Leaves simple opposite, sub-sessile, cuneate-spathular sharply serrate towards the apex. Flowers white or pale pink, sessile, densely packed in axillary spikes. Plant is more or less clothed with appressed modified white hairs, glabrous. Fruits globular, oblong dry splitting into one seeded pyrenes. Flowers and fruits throughout the year.  

1.6. Compounds reported from Phyla nodiflora  

Phyla nodiflora “poduthalai” is well known in Siddha and local systems of medicine. The plant is used to treat diseases of heart, blood, eye, asthma, bronchitis, stomach ache, for body cooling and for many ailments. Alkaloids reported from phyla nodiflora showed significant analgesic, anti-inflammatory, and anti pyretic activities (Costa et al., 1989; Forestieri et al.,1996). Halliderne and Hallerone compounds isolated from P. nodiflora (Ravikanth et al., 2000) are used for anti-inflammatory, insecticidal, antimalarial and cytoxic activities (Nishino et al., 1998). Micropropagation of Lippia junelliana (Mold.) Tronc (Juliani et al., 1999) and Lippia alba (Gupta et al., 2001) was reported. It is used for loss of consciousness in Ayurveda medicinal system. In Unani medicine it is used for treatment of fever and in Urinary concretions, it is called, (1991). The tender stalks and leaves are slightly bitter and prescribed in the form of infusion to children suffering from indigestion and women after delivery. Chutney made from the leaves and fruits are used to relieve the irritation of internal piles. Fumigation with entire Phyla plant between two red hot bricks is said to give relief of inflamed and bleeding piles.  

2. STATEMENT OF THE PROBLEM  

The present investigation was undertaken to determine a suitable in vitro culture technique for rapid multiplication and regeneration of Phyla nodiflora. Study was also carried out to determine suitable explants and elucidate the effects of different PGRs like 2,4-D; 2,4,5-T, NAA and BAP under in vitro conditions.  

2.1. Scope of the Study  

Phyla nodiflora L. is a well known native medicinal plant in Indian systems of medicine. This study will help in the biotechnological manipulations of P. nodiflora to enhance and isolate useful medicinal substances.  

2.2. Limitations of the Study  

- It is a preliminary investigation of medicinally important plant species (Phyla nodiflora (L.) Greene)  
- Different explants of single plant species used in this study.  
- In this study we have used only MS medium with different PGRs.  

2.3. Data Collection  

For this particular study, research papers from various journals, books, reviews, abstracts were collected and used. The research papers published by web of science in the field of Science and Technology covered and index database were taken as the prime source for the present study.  

3. MATERIALS AND METHODS  

3.1. Source of the Plant material  

Fresh and healthy twigs of Phyla nodiflora (Kurikkar) Greene (syn. Lippia nodiflora (L.) Mihex) (Fig 1) were collected around chennai city. Plants readily establish in pot cultures. Expalnts for tissue culture were collected from both field and pots. Disease free portions were selected and used in this investigation.
3.2. Sterilization Methods
Sterilization of all glassware, instruments, inoculating hood and explants is very essential in order to obtain axenic cultures. Borosil beakers, conical flasks, Borosil test tubes (50ml), and culture bottles (100ml) were used for media preparation during this investigation. Borosil test tubes and culture bottles were initially soaked in dilute sulphuric acid for 24 hours followed by a wash in detergent followed by running tap water. The washed bottles were transferred to hot air oven for drying at 140°C for 2 hours. The equipments used for inoculation such as forceps, cotton, blade, cutting board, aluminium foil, beaker, tissue paper, conical flask and distilled water were subjected to wet sterilization using autoclave at 15 lbs pressure for 15 minutes.

The incubation room and inoculation hood (Laminar air flow chamber) were fumigated once in 15 days using formaldehydrin and ethanolin 2:1 ratio. Ethanol was used to enhance the spreading of formaldehyde vapours. Fumigation was carried out for about 24 hours. The excess formaldehyde vapours were removed by introducing ammonia in Petri dish in the area of fumigation.

3.3. Medium Preparation
MS Medium (1962), supplemented with PGRs like 2,4-D; 2,4,5-T, NAA and BAP were used for investigation. Required amount of stock solutions were transferred in a sterile conical flask using sterilized syringes. PGRs of desired concentration were also added. The PGRs were used independently as well as in combination with other PGRs. The medium was made up to the required volume by adding double distilled water. 2% sucrose and 0.3% activated charcoal was added and pH of the medium was adjusted to 5.8 before addition of 0.8% agar. The medium was boiled to melt the agar. The dispensing of the medium was confined to the proximal region and no callus was produced on the distal cut end (Fig.6) is a microtome section of the proximal end showing the occurrence of the meristematic zone and enlarged cells of the callus adjacent zone. In dark, in addition to callus production, shoot initiation also occurs (Figs. 5, 7-10). The shoot develops from the residual axillary bud tissue. The shoot is thin, elongated and darketiolated. At the nodal regions a pair of leaves occur but remain unexpanded (Fig. 9, 10). On NAA supplemented medium the shoot becomes multiple (Fig 10), and callus production is reduced. Wang et al. (2006) reported that the NAA (0.2 mg/L) and 2,4-D (0.2 mg/L) induced the embryogenic callus in Chorispora benthamiana; similar results were observed in Phoenix dactylifera (Fki et al., 2003, Lin et al., 2004). While in Abdul Bakrudeen Ali Ahmed et al., 2005 reported that when the concentration of BA was raised to 2.5 mg/l and combined with KN 0.5 mg/l, a higher number of multiple shoots were formed. MS medium supplemented with BA and IBA was most effective for shoot regeneration. Similar results were reported in Holostemma annulare (Sudha et al., 1998), Hemidesmus indicus (Sreekumar et al., 2000) and Holostemma adakolien (Martin, 2002).

As in light treated cultures, roots are also initiated in the dark. In addition, in some of the dark treatments as in BS5N6 medium, the distal end also produces some callus (Fig. 9). Abdul Bakrudeen Ali Ahmed et al., 2005 reported that full-strength MS medium supplemented with IAA or IBA were developed higher basal callus. Explants with the axillary bud were cultured in light. In all treatments only one of the two axillary buds was released and developed into a small plantlet (Figs. 11-13). Ragava Swamy et al., (1992) reported that IBA was the most efficient cytokinin for the axillary bud initiation and subsequent proliferation of axillary buds. A similar result was observed by Baskaran and Jayabalai (2005). The plantlet had about 7 nodes within a month after inoculation. While in Abdul Bakrudeen Ali Ahmed et al., 2005 axillary nodal explants cultured on MS basal medium without growth regulators induced of two shoots as well as 6.0 cm shoot length were observed with a slow media without charcoal. However the plants were healthy with normal morphology. On media containing charcoal the growth of the axillary bud was rapid and within 20 days 8 to 8 nodes were produced. Abdul Bakrudeen Ali Ahmed et al., 2005 reported that BA and KN in combination of polyvinylpyrrolidone and malt extracts, however significantly reduced the somatic embryos quality. The stem was elongated and slender. In all treatments roots were initiated at later stage (Figs. 12, 13). Explants of the main shoot apex also elongated and produced the single leading shoot.
without much callus production (Fig. 14). On MS medium supplemented with lesser amount of 2,4,5-T callus was produced at the nodal cut end. The leaf also swelled and started differentiating callus (Fig. 15). Explants of small segments of leaf on MS medium supplemented with 2,4-D produced small amount of callus and well grown root (Fig. 16). Callus initiation and proliferation was better in 2,4-

Figure 2
Callus formation from the node with root production after 10 days of incubation in light on MS supplemented with 2,4-Dx 10^{-5}M.

Figure 3
Callus initiation from the node with root production after 14 days of incubation in light supplemented with 2,4-Dx 10^{-5}M (1.5ml).

Figure 4
Callus initiation and root production from the nodal region after 12 days of incubation in light in light supplemented with 2,4,5-Tx 10^{-5}M (1.5ml).

Figure 5
Axillary bud explants producing shoots with root initiation in dark after 14 days of incubation supplemented with 2,4,5-Tx 10^{-5}M.

Figure 6
Microtome section of nodal callus stained with tolutine blue shows meristematic region.

Figure 7
Callus with shoot initiation in dark condition after 18 days of incubation supplemented with 2,4,5-Tx 10^{-5}M.

Figure 8
Callus proliferation with root initiation after 14 days of dark incubation on MS medium supplemented with 2,4-Dx 10^{-6}M.

Figure 9
Callus proliferation from the explants with shoot proliferation after 17 days of incubation in dark condition supplemented with B5 N6 x 10^{-5}M.

Figure 10
Multiple shoot production with root formation after 20 days of incubation in dark supplemented with NAA x 10^{-5}M.

Abbreviations: C- Callus; SH-Shoot; ME-Meristematic zone; S-Stem.

Figure 11
Shoot initiation from the nodal region after 35 days of light incubation on MS medium supplemented with BAPx 10^{-5}M (1.5ml).

Figure 12
Nodal explants producing shoot after 30 days of light incubation on MS medium supplemented with 2,4-Tx 10^{-5}M (1.5ml).

Figure 13
Nodal response forming shoot after 25 days of light incubation on MS medium supplemented with B5 N6 x 10^{-6}M (2+1.5ml).

Figure 14
Shoot formation from shoot apex portion after 12 days of light incubation in light on MS medium supplemented with B5 N6 x 10^{-6}M (1.5 +1ml).

Figure 15
Callus formation from the axillary bud region after 30 days of incubation in light on MS medium supplemented with 2,4,5-Tx 10^{-5}M (1ml).

Figure 16
Root production from the callus produced of the base of the midrib after 15 days of incubation in the MS medium supplemented with 2,4-Dx 10^{-6}M.

Figure 17
Plant regeneration from the axillary bud after 20 days of light incubation in the MS medium supplemented with 2,4-Tx 10^{-6}M (1.5ml).

Abbreviations: N- Node; Ax-Axillary bud; LE-Leaf; C-Callus; RT-Root.

Ahmed et al., 2011)

In this 15 days old culture the root length was more than 15 cm. The root elongation has continued even to date. However, highest root induction was observed on half-strength MS basal medium supplemented with IBA1.0 mg/l -1 resulted in 72.7% root initiation by Abdul Bakrudeen Ali Ahmed et al., (2005). Interestingly root initiation occurs only when a cut vein such as the midrib is in contact with the medium. Roots were initiated from the basal region of the

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explants and not from the apical region. More roots were initiated from segments from the basal portion of the leaf. No roots were initiated from leaf segments obtained from the leaf margins. This investigation on an important native medicinal plant was carried out with an aim to understand the physiology of propagation in response to PGRs in isolated segments in simple cultures and in well defined tissue culture procedures. Tissue culture studies have established the response of various explants and media and light-dark conditions. One of the most interesting observations is the response of leaf explants. The profuse elongation of root initiation at the basal cut ends of vein is worthy of further investigation. Similarly Bhatt et al., 2002 reported that the adventitious roots were generated using leaf explants of P. nodiflora cultured on Murashige and Skoog (MS) medium supplemented with naphthylacetic acid. Full-strength MS medium supplemented with IAA or IBA were developed higher basal callus. Leaf explants under proper hormonal protocol may be ideally suited for producing plantlets with shoots and roots. Propagation may not be difficult in P. nodiflora. The plant grows vigorously through vegetative propagation and can be easily established through division (Fig. 17). However tissue culture experiments can establish procedures for future biotechnology with this plant. Such future investigation could include in vitro extraction of important P. nodiflora chemicals and genetic engineering to alter the growth and biochemistry of the plant.

SUMMARY OF RESEARCH
1. The response of explants on tissue culture media was investigated using different kinds of explants namely shoot with axillary bud, shoot without axillary bud, leaf segments and shoot apex.
2. The explants were inoculated on MS media with or without adding PGR. The PGRs added were 2,4-D, 2,4,5-T, NAA and BAP. Segments without the axillary buds produced callus at the cut end in light. Roots were also initiated at this region.
3. In all treatments only one of the two axillary buds was released and developed into a small plantlet.

FUTURE ISSUES
The most interesting observations are the response of leaf explants. The profuse elongation of root initiation at the basal cut ends of vein is worthy of further investigation. Future investigation could include in vitro extraction of important P. nodiflora chemicals and genetic engineering to alter the growth and biochemistry of the plant.

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