International Journal of Multidisciplinary Research and Development
Online ISSN: 2349-4182, Print ISSN: 2349-5979
Impact Factor: RJIF 5.72
Received: 02-03-2018; Accepted: 05-04-2018
www.allsubjectjournal.com
Volume 5; Issue 4; April 2018; Page No. 01-04

In vitro micropropagation of nodal segments of *Phyla nodiflora* (L.) Verbenaceae

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Abstract
Phyla nodiflora (L.) is one of the highly demanded medicinal crops. Nodal explants of *Phyla nodiflora* (L.) were cultured on Murashigi and Skoog (MS) medium supplemented with different combination and concentration of cytokinins for multiple shoot regeneration. Maximum number of shoot regeneration occurred in1.0 mg/l BAP Compared to kinetin. Among twenty five different combinations of BAP & NAA, BAP was found to be the best for producing creamish green fast growing and friable callus. This protocol can be used for continuous and rapid multiplication of *Phyla nodiflora*. Moreover, plant secondary products are often produced only in small quantities in most of the plant species. It is not always feasible to isolate secondary compounds from intact plants. Therefore, plant cell and tissue culture techniques can be an alternative approach to maintain sustainability supply of plant materials for producing bioactive compounds continuously under artificially controlled condition.

Keywords: *Phyla nodiflora* (L.), nodal esplants, cytokinins, kinetin

Introduction
Phyla nodiflora (L.) Greene (= *Lippia nodiflora* (L.) Mihex) belongs to Verbenaceae family, which is widely distributed in South Africa and Central America (Terblanche and Kornelius, 1996) [22]. It is a runner plant with scanty roots possessing various ethanobotanical and medical applications in adenopathy, chronic indolent ulcers, etc. (Kirtikar and Basu, 1975) [13]. It has been used as a traditional and Unani system of medicine to treat diarrhea, pain in knee joints, lithiasis, cold, and fever, which also has been used for inducing bowel moments in the stomachic patients (Nadkarni and Nadkami 1954) [16]. *P. nodiflora* contains flavonoids, sugars, sterols, essential oils, resins, tannins and other medicinally valuable constituents. In addition, the compounds halleridone and hallerone serve as anti-cancer, antitumor, anti-malarial, anti-fungal and other cytotoxic activities (Ravikanth *et al.,* 2000) [24]. The aerial parts of this plant are used as anodyne, antibacterial, diuretic, emmenagogue, parasiticide, refrigerant and febrifuge agents (Agarwal, 1997) [23]. Several researchers have reported various pharmacological properties including antispasmodic, hypotensive, anti-inflammatory, analgesic, antipyretic (Forestieri *et al.,* 1996) [25], antibacterial, antinociceptive, antifungal, antioxidant and free radical scavenging activities (Shukla *et al.,* 2009) [17]. *P. nodiflora* extracts have been used to cure multiple skin diseases and hair afflictions (Abbasi *et al.,* 2010) [26]. This plant is over-exploited due to its high medicinal value and hence, propagation of this plant by tissue culture may be mandatory, which offers a greater potential to deliver large quantities of disease-free, true-type healthy stock within a short span of time. While, the systematic manipulation of media, phytohormone concentrations and selection of suitable explants are among several key factors that control the process of shoot regeneration from callus; the callus-mediated regeneration protocol is a critical requirement as it allows exploitation of *in vitro* selection, somaclonal variation and genetic engineering techniques, which aims at the genetic improvement of plants (Georges *et al.,* 1993). Since the last decade, very few *in vitro* studies have been done in this genus; the latest studies have been performed on the propagation of *Lippia junelliana* and *Lippia alba* (Juliani *et al.,* 1999; Gupta *et al.,* 2001) [12, 11]. Direct shoot propagation using axillary node explants has been implemented in our previous studies and somatic embryogenesis was observed to be very successful in this medicinal plant (Ahmed *et al.,* 2005) [4].

Moreover, plant secondary products are often produced only in small quantities in most of the plant species. It is not always feasible to isolate secondary compounds from intact plants. Besides, plants are endangered by a combination of factors such as over-collecting, unsustainable agriculture practices, urbanization, pollution and climate change, no proper regulation on management and conservation. Therefore, plant cell and tissue culture techniques can be an alternative approach to maintain sustainability supply of plant materials for producing bioactive compounds continuously under artificially controlled conditions (Thorpe 1994) [18].

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Garden of Mother Theresa Post Graduate and Research Institute of Health Sciences, Pondicherry.

Materials and Methods

Maintenance of aseptic environment

All culture vessels, media and instruments used in handling tissues as well as the explants must be sterilized. The importance is to keep the air surface and floor free of dust. All operations are carried out in laminar air-flow, a sterile cabinet.

1. Preparation of sterile media, culture vessels and instruments (sterilization is done in autoclave).
2. Preparation of sterile plant growth regulators stocks (by filter sterilization)
3. Aseptic working condition
4. Explants (isolated tissues) are sterilized using chemical sterilents, e.g. $HgCl_2$ and NaOCl.

Media Preparation

Plant growth regulators

The plant growth regulators BAP and NAA were procured from sigma-Aldrich, USA.6-Benzylaminopurine (BAP): 50 mg was dissolved in a few drops of 1 N NaOH and the volume was made to 50 ml with distilled water to give 1mg/ml stock.

Alpha naphthalene acetic acid (NAA): 50 mg of NAA was dissolved in a few drops of 1 N NaOH and the volume was made to 50 ml with distilled water to give 1mg/ml stock.

Preparation of MS basal medium

Murashige and Skoog (MS) medium was prepared by adding required volumes of Macronutrient (10X), Micronutrient (200X), Iron Sodium (100 X) and vitamin (200X) Stock solutions to a known volume of distilled water, Myo-inositol (100 mg/l) and Sucrose (3%) were then added with gentle mixing. Subsequently the P^H was adjusted to 5.8 using 1 N NaOH or 1N HCl before making the final volume. Agar (0.8 %) was added to the medium and the media was melted in the microwave oven and allowed to cool. Then pour 20 ml each in 25x 150 mm glass rimless culture tubes. Then the tubes were closed with cotton plugs. Autoclave, the tubes containing media at 121°C for 15 minutes. After autoclaving the tubes were kept in a slanted position and allowed to solidify.

Establishment of aseptic plants

Plant Material

The present study protocol is to develop the in-vitro multiplication of *Phyla nodiflora*. For this purpose the plant materials (*Phyla nodiflora*) are collected in the month of October 2011 from the Medicinal Garden of Mother Theresa Institute of Post Graduate and Research Institute of Health Sciences, Indira nagar Puducherry. The plant was authenticated from the French Institute, Puducherry and a Voucher specimen is deposited in the French Institute with the specimen number 25892.

Preparation of explants and Surface sterilization

Nodal explants were excised from 2 years old plant of *Phyla nodiflora* and washed thoroughly in running tap water for 15 minutes and then washed thoroughly with autoclaved sterile distilled water for once for 2 minutes. Then washed with 0.5% v/v savlon for 5 minutes and washed thoroughly in running tap water for 15 minutes and then followed by sterile distilled water for 5 minutes. The surface sterilization was done with 70% (v/v) alcohol for 3 minutes and washed with sterile distilled water for three times, followed by 0.5 % (v/v) sodium hypochlorite for 10 minutes and thorough wash with sterile distilled water for 2 minutes thrice and followed by aqueous mercuric chloride solution 0.1% (w/v) for 10 minutes. Then washed thoroughly with sterile distilled water in sterile condition or under laminar flow. The explants were sterilized with different sterilizing agents and observations were recorded in accordance with growth parameters illustrated in Table.4

In-vitro micro propagation from nodal explants

1. Excise nodal sections from stem with a scalpel. Stem should be 1-2 cm long and the leaf should be detached.
2. Nodes were inoculated (nodal segments) vertically one node per culture slants containing basal MS medium supplemented with different concentration of BAP (0.1mg/l, 0.5 mg/l, 1.0 mg/l BAP), Kinetin (0.0, 0.1, 0.5, 1.0 mg/l). γ, γ, diphenyl dimethyl amino purine (2 ip) (0.0, 0.1, 0.5, 1.0 mg/l).
3. The cultures were maintained at 26± 2ºC with 16 hr photoperiod.

Results and Discussion

Generation of aseptic shoot culture for used as source of explant

Explants from the field grown plants are infested with a large number of contamination of microbes. A sterilizing protocol was developed in order to raise aseptic plants from nodal explants from the field grown plants. The effect of two different concentrations of sodium hypochlorite and magnesium chloride tested is given in Table 1. The procedure consisting of treatment with 0.5% (v/v) NaOCl followed by 1.0% (v/v) $HgCl_2$ gave the best percentage of aseptic cultures and therefore considered optimal for raising aseptic shoot cultures in *Phyla nodiflora*. Following these procedure several aseptic cultures were raised from the field grown plants were raised and cultured (Fig. 1).

Table 1: Effect of sterilization treatment on percent survival and shoot regeneration of *Phyla nodiflora*

1	No of explants inoculated	% of No. of explants producing shoots±SD
0.5% (v/v) Naocl	24	25±0.44
1.0% (v/v) Naocl	24	37±0.49
0.5% (v/v) Naocl followed by 0.05% (v/v) $Hgcl_2$	24	79±0.50
0.5% (v/v) Naocl followed by 1.0% (v/v) $Hgcl_2$	24	97±0.66

Excised nodal explants are treated with various sterilizing agents cultured on MS basal medium. Data recorded after one month old culture and the values expressed in mean ± SD

MS supplemented with either BAP, Kninetin and γ, γ diphenyl dimethyl allyl amino purine (2 ip) at different concentration were tested for their effect on induction of shoot multiplication from nodal cuttings. Three millimeter to five millimeter sized nodal cuttings were obtained from 20 days-old in vitro grown aseptic plantlets raised as above. The nodes were then cultured on MS supplemented with BAP (0.0, 0.1, 0.5 and 1.0 mg/1) / kinetin (0.0, 0.1, 0.5 and 1.0 mg/1) / 2 iP (0.0, 0.1, 0.5 and 1.0 mg/1) to assess their ability to give rise to multiple shoots. Within a week‘s time the nodal cuttings sprouted to produce new shoot (Fig.1). The frequency of shoot formation increased with increase in concentrations of the cytokinin (Fig 2). The intensity of shoot regeneration form nodes is presented in Table 6. From the table it can be deduced that maximum of four shoots /explants could be obtained on 0.5 mg/l BAP and six number of shoots / explants on 1.0 mg/l BAP. The explants cultured in 1.0 mg/l kinetin showed that only three number of shoots / explants and showed a better shoot length. MS media supplemented with 2 ip showed formation of less number of shoots/explants. Therefore it may be concluded that among the three cytokinin tested, 1.0 mg/l BAP is optimal for shoot multiplication through nodal cuttings.

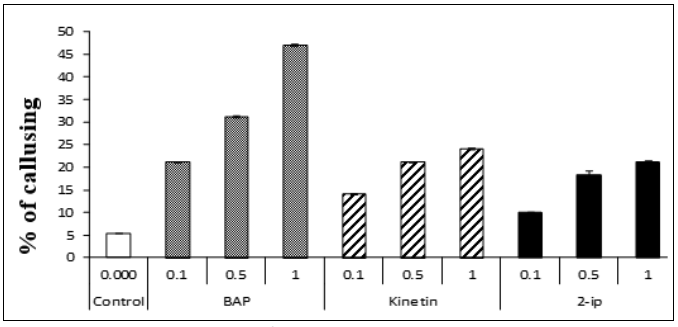

Fig.1: Effect of cytokinin on shoot multiplication

Fig 2: Multiple shoot formation in nodal explants of *Phyla nodiflora*. A four week old culture of showing multiple shoots formed from node cultured on MS supplemented with (A) 0.5mg/l BAP and (B) 1.0 mg/l BAP (C) 5mg/l kinetin and (D) 1.0 mg/l 2 ip(γ,γ dimethylallylamino- purine)Table 1.

Induction and proliferation of callus from leaf explants

From the two month old *in vitro* grown plants the leaves are excised and are used as explants for the callus induction. Leaf segments were inoculated (0.5-1.0 cm^2) on MS medium fortified with different concentrations of cytokinin (0,0.1,0.2,0.5,1.0 mg/l BAA) and auxin (0,0.1,0.2,0.5,1.0 mg/l NAA)(Table 1,Fig: 2A).The explants failed to produce callus on MS medium lacking growth regulators but the swelling of the explant is observed. Callus produced from the leaf segments were dark green, light green, and brown and nodular to fragile in nature. In comniation of NAA (1.0 mg/l) and BAP (0.1 mg/l) a profuse greenish coloured callus was formed. In lower concentration of NAA and BAP less amount of callus was formed in comparison to higher concentration. Among the growth regulators tested in combination of (BAP -0.5 mg/l and NAA 0.1 mg/l) induced maximum frequency of shoot regeneration.(Fig 2).The minimum frequency regeneration of shoot was noted in (BAP -0.1 mg/l and NAA 0.1 mg/l) (Table-2).

Effect of BAP and NAA for shoot regeneration on intact petiole end leaf explants

Young leaf explants were derived from *in vitro* nodal culture of 2 month old plants. Approximately 1cm^2 leaf segments were inoculated on MS medium supplemented with BAP and NAA at the rate of around eight segments per petridish with their adaxial surface facing down. The presence of cytokinin along with auxin is necessary for indirect adventitious shoot induction. The induction of callus and subsequent differentiation and organogenesis is accomplished by the differential application of growth regulators such as BAP and NAA in a culture medium. Leaf segments were inoculated (0.5-1.0 cm^2) on MS medium fortified with different concentrations of cytokinin (0, 1.5, 2.0, 2.5 mg/l BAA) and auxin (0,0.1,0.2,0.5,1.0 mg/l NAA)(Fig: 2).The explants failed to produce shoots on MS medium lacking growth regulators but the swelling of the explants is observed. Among the growth regulators tested in combination with (BAP -2.5 mg/l and NAA 0.2 mg/l) induced maximum frequency of shoot regeneration (Fig 3).

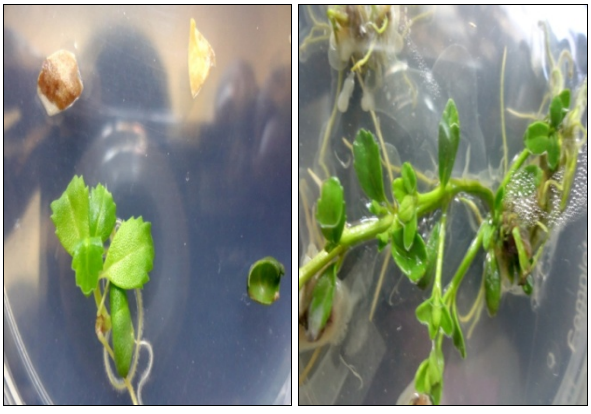

Fig 3: Effect of BAP and NAA for shoot regeneration on intact petiole end leaf explants

Summary and Conclusion

Phyla nodiflora (family-Verbinaceae) is an endangered medicinal plant traditionally used as antidiuretic, antiseptic for women after delivery and also it has antifungal activity. *Phyla nodiflora* provides a standardized protocol for the efficient propagation. The described protocol could be worked as a useful tool for adapting *in vitro* culture strategies to increase the bio mass and yield of active principles or other secondary metabolites of pharmaceutical importance. In the present investigation an efficient micro propagation protocol for *Phyla nodiflora* is described for shoot multiplication from nodal explants and shoot organogenesis from leaf explants. Shoot multiplication occurred when nodal explants from *in vitro* grown plant were cultured on media containing different concentration and combination of cytokinin. Successful shoot multiplication was observed in BAP 1.0 mg/l compared to kinetin. Regeneration of shoots occurred, when intact petiole leaf end cultured on MS media containing different concentration and combination of auxin and cytokinin. The maximum percentage of shoot organogenesis occurred in 1.5 mg/l BAP and 0.1 mg/l NAA. These *in vitro* raised plants did not show any morphological abnormality when compared to original plants.

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