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# IN VITRO PLANT REGENERATION AND CALLUS FORMATION FROM THE NODAL EXPLANTS OF PHYLLANTHUS NIRURI L. (EUPHORBIACEAE)-A MEDICINAL HERB

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**ABSTRACT**

**Aim**: *In* vitro propagation methods can be applied to conserve medicinal plant or to sustain the productivity of the biologically active compounds. An efficient *in vitro* plant regeneration protocol was developed for the medicinally potent *Phyllanthus niruri* L. (Euphorbiaceae) using nodal segment as explant.

**Methods**: In this study, two different media were used to test the individual effects of growth regulators in shoot and callus initiation and also to evaluate the ability of different basal media which supports the establishment of shoot culture.

**Results**:Maximum multiplication of shoots (15.28±0.96) was achieved on Murashige and Skoog's medium supplemented with BA (0.5 mg/L) after 3-4 weeks of inoculation. To induce callus culture, explants were oriented in both horizontal and vertical position on the surface of the medium. The results were comparatively promising when the explants oriented horizontally. The shoots were separated from cluster and subcultured for their elongation on the same medium. Individual in vitro shoot of *P. niruri* could produce normal root systems when they were cultured on basic MS medium without any plant growth regulator.

**Conclusion**:The results obtained reveals that the low cost system is now available for large scale production of selected clones and is necessary because of its medicinal potentiality is concerned.

**Key words**: Callus initiation, explant, growth regulators *Phyllanthus niruri* Linn, Shoot regeneration.

## INTRODUCTION

*Phyllanthus niruri* L. (Euphorbiaceae) is an herbaceous medicinal plant widely distributed in the tropics. It is a small erect annual herb growing upto 30-40 cm in height. [1] This plant wide spread through out the tropics and subtropics in sandy region as weed in cultivated and waste lands. [2] Leaves contain bitter substance Phyllanthin, Hypophyllanthin, Niranthin, Nirtetralin, Niruretin, Nirurin, Niruriside, Phyltetralin. Quercetin, kaempferol-4-rhamnopytanoside. [3], [4], [5] *P. niruri* was also found to have anti-oxidant and heptoprotective properties and anti-inflammatory potential. [6] Some of the flavonoids obtained from this plant had shown antinociceptive properties. [7] Latex with oil (Til) in Opthalmia, stem and leaves yields a black dye possesses antibacterial property. [8] This plant is a favorite choice of the rural people because of its immense medicinal properties like antidote, against liver diseases, antiviral properties**,** antioxidant, heptoprotective, anti inflammatory and strong inhibitory effect against neurogenic. [9], [6] ,[10]

Current medicinal knowledge of the activity of plant phenolics indicates that useful drugs may be developed from them in the future, or that they could be used as templates for further research and development. Due to these potentialities, the present study was taken up with a view to develop protocol for mass propagation of *Phyllanthus niruri* Linn., through *In* vitro techniques, so that enough quantity of genotypically superior quality plantlets can be produced.

## MATERIALS AND METHODS

### Explants preparation

Single nodal cuttings of *Phyllanthus niruri* of size 0.8 – 1.2 cm was collected from the 30 – 40 day old plants grow in the field from the nearby villages in Erode district. Plants are identified and confirmed with the authentic.

### Sterilization

The picked nodal segments were kept under running water for about 15-20 minutes to remove soil particles and then rinsed with liquid detergent (Teepol 1%) (V/v)) For 5-10 minutes. Then they were rinsed with sterile

double distilled water at least thrice to get rid of teepol. Prior to inoculation, these explants were subsequently surface sterilized in the laminar air flow chamber with 0.1% mercuric chloride (HgCl2; w/v) for 2 minutes followed by repeated rinsing with sterile double distilled water.

**Culture media**

The surfaced sterilized explants were then aseptically inoculated on sterile MS medium [11] comprising 3% sucrose as carbon source and 0.8% agar as solidifying agent. The medium was also supplemented with various growth regulators [auxins- IAA (indole-3-acetic acid), IBA (indole-3-butyric acid), 2,4-D (2, 4-dichlorophenoxyacetic acid)and cytokinins - BA (6-benzyl adenine), KN (6- kinetin)] and pH of the medium was adjusted to 5.8 before autoclaving at pressure 1.06 $Kg/cm^{-3}$. For multiple shoot induction different concentrations (0.5, , 1.0, 1.50, 2.0 mg/L) of BA, KN alone were used. For callus induction BA kinetin, 2, 4-D, IBA, IAA at different concentrations (0.5, 1.0, 1.50, 2.0 mg/L) were used. However, various auxins such as 2, 4-D, IBA, IAA at different concentrations (0.5 to 2.0 mg/L) were examined for *In vitro* root induction.

**Culture conditions**

The cultures were incubated at 25±2°C under cool, white and fluorescent light of 2000-2500 lux and relative humidity of about 55±5%. 16/8 hours photo and dark period were maintained in growth chamber, respectively. For each treatment, eight replicates were used and every experiment was repeated at least thrice. Data on multiple shoot induction; elongation and callus formation were taken statistically with two-way ANOVA followed by a comparison f measuring Tukey Test (HSD) at p=0.05 Observations were recorded periodically.

**Induction of Multiple shoots**

The aseptic nodal segments were cultured on MS medium supplemented with 0.5, 1.0, 1.50, 2.0 mg/L of BA and KN alone. Physiological conditions and number of shoots per explants were observed periodically.

**Medium for Shoot Culture Establishment**

For the establishment of shoot culture various salts such as MS (Full and half strength), B5 [12],AR [13],W [14]),SH [15],KM [16],WPM [17] were supplemented with 0.62 mg/L BA were used.

**Induction of Callus culture**

The aseptic nodal explants were cultured both in horizontal and vertical position for the induction of callus. The MS medium is supplemented with with 0.5, 1.0, 1.50, 2.0 mg/L 2, 4-D, IBA, IAA.

**RESULT AND DISCUSSION**

During the present research investigation, maximum number of shoots (15.3±1.0) was obtained when nodal explants inoculated on MS medium supplemented with BA (0.5 mg/L) after 3-4 weeks .Further, increase in the concentrations of BA decreased the number of multiple shoots [Table 1]. Similar observations were reported in other plant species such as *Phyllanthus urinaria,*[18] *Codiaeum variegatum,* [19] *Dictyospermum ovalifolium,* [20] *Cunila galioides,* [21] *Strawberry,* [22] *Gymemma sylvestre* [23] and *Vitex negundo.* [24] Whereas, in contrast to the present results, [25] observed that Kn was proved to be the best for maximum shoot proliferation in *Phyllanthus urinaria*. These multiple shoots were separated from the clump and sub cultured on the same medium for their elongation in I*n vitro* conditions. Elongation of shoots were also reported in other plant species like *Costus speciosus,* [26] and *Baliospermum montanum* [27] on the same medium in which they were initiated. However, in contrast to the multiplication medium, [28] reported elongation of shoots on MS medium having different growth regulators in *Jatropha curcas*. Most or all (97-100%) of the nodal segments produced roots on MS medium without BA or Kinetin.

**Table-1:** Shoot culture initiation of nodal explants of *Phyllanthus niruri* Linn., inoculated in MS medium containing Cytoxinins (BA and Kinetin).

| Growth Regulators(mg/L) | Concentration(mg/L) | Mean number of Shoots produced (*Mean ± $t_{0.05}$ S.E.) |
|---|---|---|
| Cytokinins | | |
| BA | 0.00 | 0 |
| | 0.50 | 15.3±1.0 |
| | 1.00 | 6.6±1.4 |
| | 1.50 | 4.5±1.9 |
| | 2.00 | 2.7±1.7 |
| Kinetin | 0.00 | 0.0 |
| | 0.50 | 3.5±1.2 |
| | 1.00 | 2.6±2.9 |
| | 1.50 | 1.3±0.9 |
| | 2.00 | 0.1±0.1 |

***Values are 95% confidence limits for Mean and mean values within the column followed by the same letter arenot significantly different by the Tukey's test at 0.05% probability level**

In this study, root initiation in the explants was totally inhibited at all concentrations of all growth regulators [Table 2, Figure 1] and similar results on the inhibition of root primordial differentiations by BA were reported earlier for sunflower. [29] Similarly, Auxins like IBA ad IAA did not have stimulatory effect in terms of promoting root proliferation and elongation as expected in the present study.

**Table 2:** Effect of MS medium supplemented with BA or Kinetin on Roots (%) from In vitro shoots obtained from the nodal explants of *Phyllanthus niruri* Linn.,

| Growth Regulators(mg/L) | | Concentration(mg/L) | *In Vitro* Shoots produce roots (%) |
|---|---|---|---|
| Cytokinins | BA | 0.00 | 100.0 |
| | | 0.50 | 0.0 |
| | | 1.00 | 0.0 |
| | | 1.50 | 0.0 |
| | | 2.00 | 0.0 |
| | Kinetin | 0.00 | 98.2 |
| | | 0.50 | 0.0 |
| | | 1.00 | 0.0 |
| | | 1.50 | 0.0 |
| | | 2.00 | 0.0 |

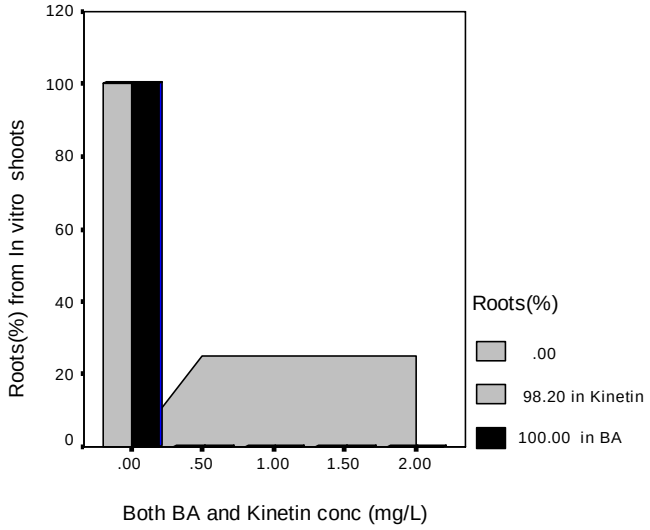


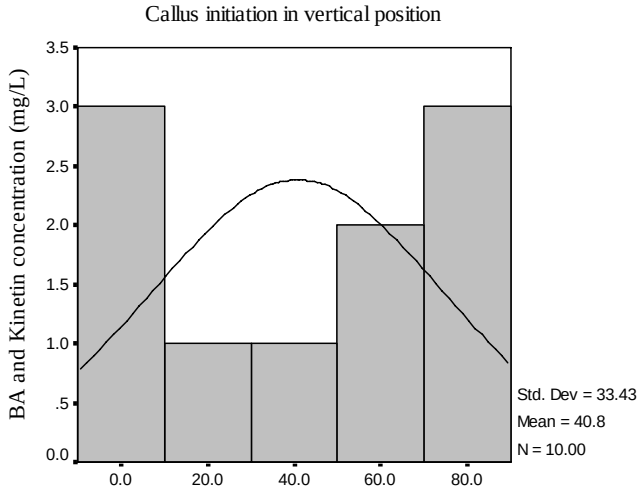

**Figure 1:** Rooting response with both BA and Kinetin at different concentrations(mg/L)

Callus initiation was stimulated (20-80%) in MS medium supplemented with diffferent concentrations (0, 0.5, 1.0, 1.5, 2.0 mg/L) of cytokinins (BA and kinetin) when explants oriented in vertical position [Table 3, Figure

2(a)]. The plant growth regulators BA and kinetin were equally effective in callus intiation (70 – 100%) from single nodal explants inoculated horizontally on the media [Table 3, Figure 2(b)]. When the explants oriented vertically in MS medium supplemented with different concentrations (0,0.5,1.0,1.5,2.0 mg/L) of auxins (2,4-D, IBA, IAA and BA), there was no callus inititation in all the concentrations IBA and IAA used whereas 76-82% callus induction was observed in 2,4-D supplemented medium [Table 4, Figure 3(a)]. Callus induction was observed (96 – 100%) in the explants oriented horizontally on the surface of the medium supplemented with auxins (2, 4-D, IBA and IAA) [Table 4, Figure 3(b)].

**Table 3:** The frequency (%) of callus fresh mass from nodal explats of *Phyllanthus niruri* Linn.,. inoculated in both verticaland horizontal position on MS medium supplemented with Cytokinins.

| Growth Regulators (mg/L) | | Concentration (mg/L) | Callus (%) explants oriented in Vertical position | Callus (%) explants oriented in horizontal position |
|---|---|---|---|---|
| Cytokinin | BA | 0.00 | 0.0 | 0.0 |
| | | 0.50 | 75.2 | 100.0 |
| | | 1.00 | 60.8 | 85.1 |
| | | 1.50 | 20.7 | 73.0 |
| | | 2.00 | 0 | 70.2 |
| | Kinetin | 0.00 | 0.0 | 0.0 |
| | | 0.50 | 80.1 | 84.0 |
| | | 1.00 | 78.0 | 72.1 |
| | | 1.50 | 40.2 | 52.0 |
| | | 2.00 | 52.9 | 66.3 |




Frequency (%) Callus initiation from the nodal explants

**Figure 2(a):** The frequency (%) of callus fresh mass initiation from explants oriented in vertical position on MS medium supplemented with Cytokinins.

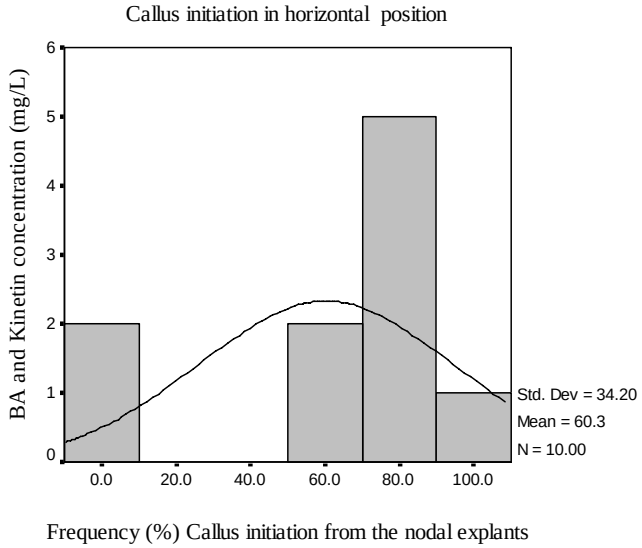



**Figure 2(b) :** The frequency (%) of callus fresh mass initiation from explants oriented in horizontal position on MS medium supplemented with Cytokinins.

**Table 4:** The frequency (%) of callus fresh mass from nodal explats of *Phyllanthus niruri* Linn.,. inoculated in both vertical and horizontal position on MS medium supplemented with Auxins.

| | Growth Regulators (mg/L) | Concentration (mg/L) | Callus (%) explants oriented in Vertical position | Callus (%) explants oriented in horizontal position |
|---|---|---|---|---|
| Auxins | 2,4-D | 0.00 | 0 | 0 |
| | | 0.50 | 82.4 | 100.0 |
| | | 1.00 | 40.5 | 99.0 |
| | | 1.50 | 65.8 | 99.0 |
| | | 2.00 | 76.0 | 98.0 |
| | 1BA | 0.00 | 0 | 0 |
| | | 0.50 | 0 | 100.0 |
| | | 1.00 | 0 | 98.0 |
| | | 1.50 | 0 | 97.0 |
| | | 2.00 | 0 | 96.0 |
| | 1AA | 0.00 | 0 | 0 |
| | | 0.50 | 0 | 100.0 |
| | | 1.00 | 0 | 100.0 |
| | | 1.50 | 0 | 99.0 |
| | | 2.00 | 0 | 99.0 |

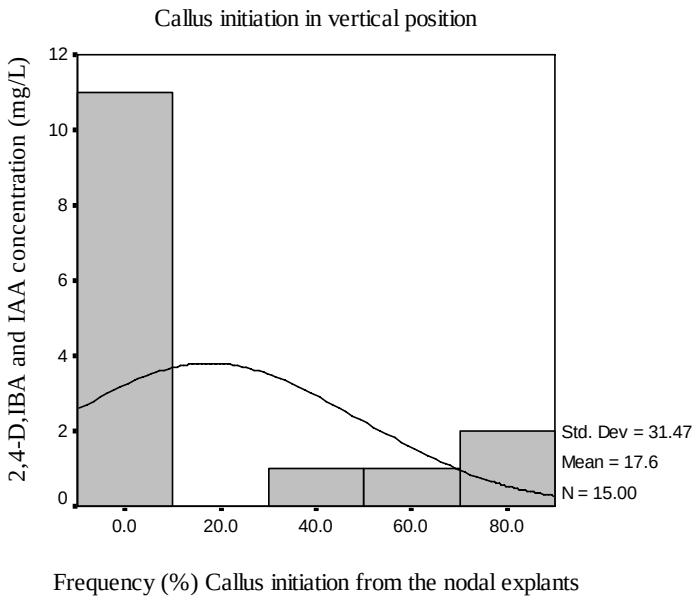



**Figure 3(a):** The frequency (%) of callus fresh mass initiation from explants oriented in vertical position on MS medium supplemented with auxins**.**

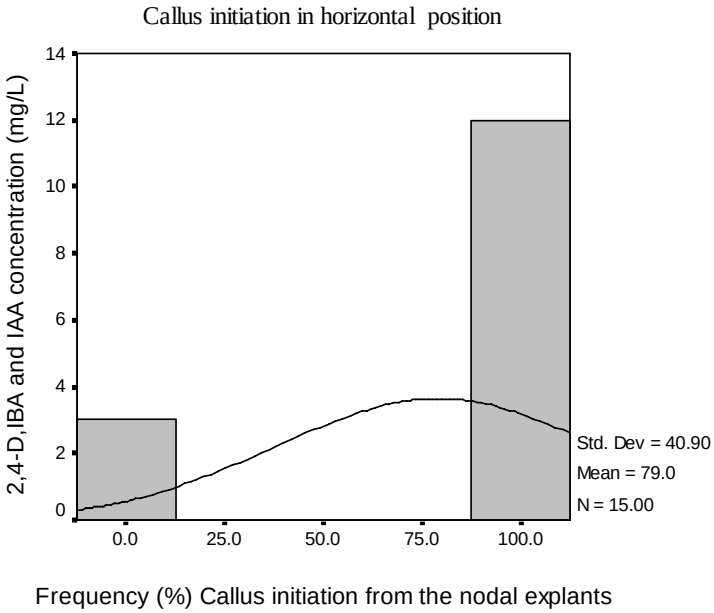



**Figure 3(b):** The frequency (%) of callus fresh mass initiation from explants oriented in horizontal position on MS medium supplemented with auxins.

In the present study, on comparison, the data obtained in both orientations was found to be contrast with the resul. They revealed that the inability of the auxins, (IBA and IAA) on callus induction of the explants in the vertical position and show the strong influence of explant orientation. Similarly, the rate of callus induction was comparatively increased (96 – 100%) in the explants oriented horizontally on the surface of the medium supplemented with auxins (2, 4-D, IBA and IAA). The fact that the cytokinins such as BA was effective as the auxins (2, 4-D, IBA and IAA) in promoting callus growth in this species is an indication that the level of uptake and metabolism of these plants growth regulators by the explants in the horizontal positions allows the appropriate endogenous levels of cytokinins to counter balance the levels of endogenous auxins and vice-versa. Preliminary experiments carried out with this species have also shown significant promotive effect of light on callus fresh mass in the explants oriented horizontally. [30]

Some of the tissue culture media supplemented with 0.62mg\L BA had variable effects on growth of uninodal explants [Table 5]. Shoot initiation was observed 60-100% of the cultures [Table 5, figure 4]. These differences and similarities might be due their basal salt formulation. The reduction in the salt strength of MS media to 50% did not reduce shoot culture growth as observed for *P.caroliniensis.* [30] The poorest shoot growth of the cultures in terms of number of shoots was observed on B5, SH, W and KM. This may be probably due to their low ammonium content compared to MS medium.

**Table 5:** Effect of basal media salt formulation supplemented with 0.62 mg/L BA on the frequency (%) of morphogenic responses of shoot initiation from nodal explants of *Phyllanthus niruri* Linn.

| Basal medium*(mg\L) | Shoots (%) |
|---|---|
| MS | 100 |
| B5 | 90 |
| SH | 60 |
| W | 90 |
| KM | 100 |
| WPM | 85 |
| AR | 100 |
| MS/2 | 100 |

*Reference cited MS[11] ,B5 [16],AR [17],W [18]),SH [19],KM [20],WPM [21]

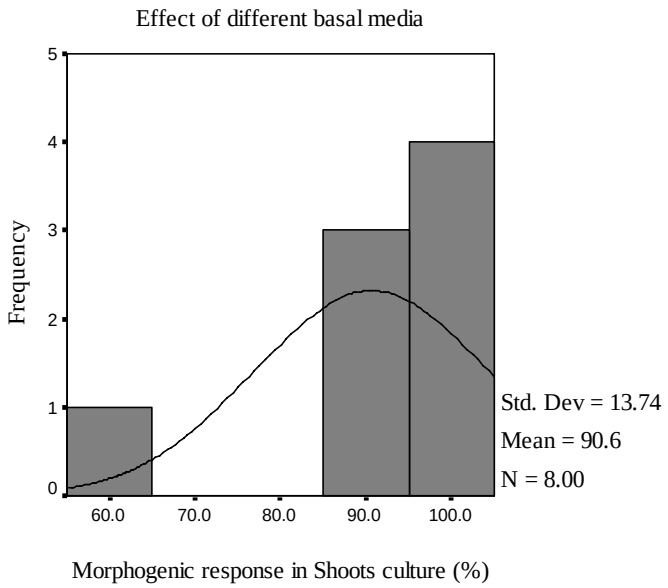



**Figure 4:** Effect of different basal media with 0.62mg/L of BA in shooting response.

## CONCLUSIONS

The results clearly indicate that a low cost system is now available through this study for large scale multiplication of selected clones of this species. The present protocol could be used for large scale clonal propagation without any seasonal constraint. The shoot cultures initiated in this study could prove to be a better source for getting antiviral compounds against hepatic damage and can also be used for genetic transformation studies using *Agrobacterium.* Therefore, further a through study of this plant is because of its potentiality concerned.

*In* vitro culture techniques could hence be used as alternatives for the mass production of the plant material to meet the market demand.

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