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# CRUCIAL ROLE OF NITROGEN IN *IN- VITRO* REGENERATION OF *PHYLLANTHUS AMARUS* SCHUM. AND THONN.

Antara Sen* and Amla Batra

Plant Biotechnology Laboratory No.- 5, Department of Botany, University of Rajasthan, Jaipur, Rajasthan, India

**Keywords:**

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**Abbreviations:**

AdS -Adenine sulphate,

BAP -6-Benzyladenino purine,

IAA -Indole-3-acetic acid,

IBA -Indole-3-butyric acid,

MS -Murashige and Skoog medium

**Correspondence to Author:**

**Miss. Antara Sen**

Plant Biotechnology Laboratory Number 5, Department of Botany, University of Rajasthan, Jaipur, Rajasthan, India

## ABSTRACT

Nitrogen plays a pivotal role in growth and differentiation of cells of *Phyllanthus amarus* Schum. and Thonn. Both form and amount of nitrogen in nutrient medium have significant effects on the rate of cell growth and differentiation. In this study, effects of different inorganic nutrients ($KNO_3$ and $NH_4NO_3$) and organic nutrients (glycine) on regeneration of *P. amarus* were evaluated. Murashige and Skoog's medium (MS) was used as basal medium along with plant growth hormones and different concentrations of nitrogen. Among the various growth hormones tried, BAP (0.5 mg/l) gave maximum number of shoots (17.33±0.86) after 3-4 weeks of inoculation. However, various strength of $KNO_3$, $NH_4NO_3$ and glycine were optimized in the medium in order to get the maximum number of shoots, nodes per shoot, shoot length as well as the size of leaves respectively. Amongst all modifications, less or more, MS medium with standard amount of nitrogen source was found to be most effective. The shoots regenerated on MS medium along with BAP (0.5 mg/l) were subjected to their elongation on the same medium and their root induction was done on half strength MS medium along with IBA (0.5 mg/l). These complete plantlets were hardened and acclimatized in natural conditions. 80% survival rate was observed under field conditions.

**INTRODUCTION:** Tissue culture technique is an alterative method to obtain rapid clonal multiplication. Growth and morphogenesis of plant tissues under *in vitro* conditions are largely influenced by the composition of the culture media. Mineral nutrients are one of the most important and basic components of plant tissue culture. Nitrogen assimilation and its role in plant growth and development play a pivotal role in establishing and understanding of cell differentiation in plants. Both the form and amount of nitrogen in the *in vitro* medium have significant effects on rate of cell growth, differentiation and cell totipotency [1].

In plant nutrient medium, nitrate ions, ammonium salt, amino acids and complex organic compounds supply nitrogen. Nitrate ions and ammonium salts are the good sources of nitrogen because they can be absorbed and metabolized by the cells easily, which further lead to root branching, breaking of seed and bud dormancy and stop apical dominance. Hence, nitrogen in the form of $NH_4^+$ and $NO_3^-$ is the dominant mineral nutrient in most plant tissue culture formulations, in both proportion and the concentration of $NH_4^+$ and $NO_3^-$. Optimization of such compounds stimulates regeneration in recalcitrant cultivars also [2].

The potential benefits of optimizing the nutrient components of culture media for a particular response are well documented across a wide range of plant species and applications. For example, the concentration of $NH_4^+$ and $NO_3^-$ affects numerous *in vitro* responses including the development of somatic embryos [3, 4, 5] the efficiency of plant recovery after ovule culture [6], shoot regeneration [7, 8], regulation of growth and biomass of bioreactor-grown plantlets [9], and also controls the rate of root initiation in shoot cultures [10].

*Phyllanthus amarus* Schum. and Thonn. commonly known as Bhui amla , is a herbaceous medicinal plant belonging to family Euphorbiaceace. It has been traditionally used in the treatment of a variety of ailments such as jaundice, asthma, ulcer, hepatitis, tuberculosis, malaria, dysentery, gonorrhea, syphilis, cough, diarrhoea, vaginitis and urinary diseases and other hepatic disorders [11, 12, 13, 14]. It has immense medicinal properties like antidote against liver diseases, antiviral properties**,** antioxidant, hepatoprotective, anti inflammatory and strong inhibitory effects against neurogenic diseases [15, 16, 17].

The present research endeavor emphasizes on the role of nitrogen source in tissue culture of *P. amarus,* which would reveal the understanding of its plant physiology regarding their growth, differentiation and development for researchers and plant growers in laboratory as well as field conditions.

**MATERIALS AND METHODS:** Tender twigs were collected from field grown juvenile plants of *P. amarus* and sectioned into 0.5-1.5 cm long nodal segments. They were washed under running tap water for 30 minutes and then treated with a solution of the teepol (1% v/v) for 10 minutes. Then they were rinsed with sterile double distilled water at least thrice to get rid of teepol and finally surface sterilized with $HgCl_2$ (0.1% w/v) for 2 min. Lastly, the material was washed 3 -5 times with autoclaved distilled water to remove any trace of $HgCl_2$. For shoot culture initiation, full strength MS [18] was taken as basal medium containing 3% sucrose, 0.8 % agar and supplemented with various cytokinins like BAP and Kinetin. The media were adjusted to pH 5.8±0.2 and autoclaved at 1.1 $kg/cm^2$ for 20 minutes at 121°C.

Cultures were incubated at 25±1°C with a photoperiod of 16 hours at 2000- 2500 lux of cool white fluorescent light. Shoots were initiated after 5-7 days of inoculation and then subcultured regularly on fresh medium at four week interval.

**Experimental Design:** After the media were optimized with hormones they were then allowed to test in 7-modified MS media (A, B, C, D, E, F, G), which differed in their nitrogen sources i.e., $NH_4NO_3$, $KNO_3$ and glycine formulation (**Table 2**). After 3-4 weeks of inoculation, the regenerated shoots were then studied morphologically and effects of nitrogen on growth and differentiation were evaluated.

**Establishment of optimized culture condition, root culture and hardening:** After the concentrations of all the nitrogen sources were optimized, the shoots were further subcultured regularly on the same optimized nutrient medium for development and shoot multiplication. These *in vitro* elongated shoots were taken out carefully from the culture vials and transferred to half strength MS medium with different concentrations (0.05- 2.5 mg/l) of auxins such as IBA, IAA and 2,4-D respectively, for root induction.

The plantlets were taken out from culture vessel without damaging the delicate root system and rinsed with distilled water to remove the adhering agar and then transferred to polycups containing vermicompost and autoclaved soil (1:3). Polycups were covered with inverted glass beakers to maintain high humidity and kept in growth chamber. They were gradually exposed from artificial environmental conditions to natural conditions for their acclimatization.

**RESULTS AND DISCUSSION:** During the present research endeavor, maximum number of shoots was obtained on MS medium supplemented with BAP (0.5 mg/l) after 3-4 weeks of inoculation [**Table 1, Figure A (1)**]. Similar observations were reported in many other plant species such as *Phyllanthus urinaria* [19] and *Codiaeum variegatum* [20]. However, in contrast to this, Captan *et al*. [21] observed that Kn proved to be the best for maximum shoot proliferation in *Phyllanthus urinaria.*

**TABLE 1: EFFECT OF CYTOKININS (BA AND KN) ON SHOOT INITIATION FROM NODAL STEM SEGMENTS IN *PHYLLANTHUS AMARUS* SCHUM. AND THONN.**

| Growth regulators (mg/l) | % response of shoot formation | Mean number of shoots produced/explants (*Mean ± $t_{0.05}$S.E.) | Av. Length of shoots (cm.) (*Mean ± $t_{0.05}$S.E.) |
|---|---|---|---|
| **BAP** | | | |
| Control (0) | 0 | - | - |
| 0.05 | 66 | 8.560±0.02 | 3. 04 ± 0.07 |
| 0.10 | 70 | 10.640±1.78 | 4.39 ± 0.18 |
| 0.5 | 92 | 17.330±0.86 | 8.92 ± 1.04 |
| 1.0 | 67 | 8.775±0.60 | 3.87 ± 0.14 |
| 1.5 | 56 | 7.460±0.28 | 3.70 ± 0.12 |
| 2.0 | 58 | 5.34±0.15 | 2.02 ± 0.26 |
| 2.5 | 42 | 4.30±0.27 | |
| **Kn** | | | - |
| 0.05 | 47 | 4.025±0.17 | 3.06 ± 0.45 |
| 0.10 | 54 | 7.075±1.88 | 4.67 ± 0.35 |
| 0.5 | 60 | 8.016±1.88 | 5.34 ± 0.28 |
| 1.0 | 52 | 6.325±0.31 | 4.40 ± 0.17 |
| 1.5 | 46 | 5.026±0.19 | 2.80 ± 0.42 |
| 2.0 | 30 | 3.30±1.06 | 2.03±0.08 |
| 2.5 | 20 | 2.460±1.02 | |

*Values are 95% confidence limits for Mean and mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.05% probability level.

The mineral nutrient needed in abundance by plants is nitrogen [22]. Nitrogen is an essential element in modern mineral salt formulations and is present in the form of both nitrate ($NO_3^-$) and ammonium ($NH_4^+$) ions. Murashige and Skoog medium [18] contains two nitrogen salts, ammonium nitrate (~1650 mg/l) and potassium nitrate (~1900 mg/l), giving a total of ~3550 mg/l for both nitrogen forms. This is considered to be a fairly high nitrogen concentration, which is supra-optimal for many plant species. The lack of nitrogen results in decreased and malformed development of plants.

In the present study, extensive observations were made for nitrogen effects, when nitrogen source as $NH_4NO_3$, $KNO_3$ and glycine were added to the MS medium in different concentrations [Table 2]. It was observed that nitrogen plays a crucial role in growth and differentiation. However, explants were not able to grow, neither they showed any morphological changes, when they were put on MS medium devoid of nitrogen source.

**TABLE 2: NITROGEN SOURCE OF MS AND MODIFIED MS MEDIUM (CITED AMOUNTS ARE IN mg/l)**

| N-source | MS Media+ BAP (0.5 mg/l) | Modified MS Media | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | A | B | C | D | E | F | G |
| NH4NO3 | 1650 | 0 | 0 | 0 | 1650 | 825 | 825 | 3300 |
| KNO3 | 1900 | 0 | 1900 | 1900 | 0 | 950 | 950 | 3800 |
| Glycine | 2 | 0 | 0 | 2 | 0 | 2 | 0 | 4 |

To study the effects of nitrogen on *in vitro* plant regeneration, various other reports are also available, like Rustaei et al. [23], that compared 2 different media (MS and N6) for *in vitro* regeneration of *Mallus domestica* and observed that MS medium with higher amount of nitrogen was good as compared to N6 medium (lower nitrogen content), hence proving that MS medium was optimum for *in vitro* plant regeneration.

Vinterhalter *et al* [7]. also showed that MS medium was the best for *in vitro* growth of *Ceratonia siliqua* L. with supraoptimal nitrogen content. However, reduced nitrogen nutrition (nitrogen deficiency) in plants generally results in stunted growth. It affects biosynthesis of proteins and nucleic acids in cells of meristematic tissues. Nitrogen deficiency is often accompanied by leaf necrosis.

Nevertheless, in *Clematis pitcheri*, lower level of nitrogen is sufficient for better shoot formation [24]. The studies with the incorporation of nitrogen salts in various concentrations, showed the shoot proliferation in all treatments. However, morphological parameters were also examined accordingly [**Table 3, Figure A (2)**]. Fine-quality new shoots were produced on media supplemented with $NO_3^-$ alone as the N source. They produced dark green leaves. There are many reports available where, $NO_3^-$ was used as the single N source and successful regeneration was obtained in various tissue culture systems [25, 26, 27]. Furthermore, a very small change in multiplication and shoot morphology was observed, when glycine was added along with $NO_3^-$ ions.

**TABLE 3: RATE OF SHOOT PRODUCTION AND QUALITY OF SHOOT PRODUCED ON TESTED MEDIA (MS, A, B, C, D, E, F, G)**

| MS Media + BAP (0.5 mg/l) | Multiplication index ±SE | No. of nodes ±SE | Shoot length(cm) ±SE | Leaf size(cm)± SE |
|---|---|---|---|---|
| MS | 15.27±0.96 | 4.12±0.2 | 30. 46±1.0 | 2.06±0.84 |
| Modified MS medium | | | | |
| A | 0 | 0 | 0 | 0 |
| B | 6.72±0.2 | 1.66±0.47 | 13.70±0.46 | 0.97±0.02 |
| C | 6.90±0.1 | 1.74±0.52 | 13.02± 1.56 | 0.90±0.06 |
| D | 4.56± 0.4 | 1.02±0.1 | 6.68±0.45 | 0.45±0.01 |
| E | 5.50±0.12 | 2.70±0.34 | 8.03±1.68 | 1.40±0.05 |
| F | 4.78±0.46 | 2.08±0.28 | 7.34±0.77 | 0.88±0.06 |
| G | 5.22±0.44 | 2.89±0.44 | 9.06±0.46 | 1.06±1.08 |

*Values are 95% confidence limits for Mean and mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.05% probability level

When $NH_4^+$ was used as the sole source of N, the multiplication rate was very low. The quality of the new shoots was poor, with yellowish color and frailness being the common feature. Ammonium used as the sole source of N appeared to have a negative effect on regeneration and growth of new shoots of *A. polyphylla* [28]. Although N assimilation is associated with the reduction of $NO_3^-$ to $NH_4^+$, many plant species showed inhibition of morphogenesis and growth, when ammonium was used as a sole source of N, like *Nicotiana tabacum* [29, 30] *Oryza sativa* [31,] *Solanum tuberosum* [32] and *Eucalyptus marginata* [27].

The inhibition of morphogenesis and growth in response to application of $NH_4^+$ as the sole N source has been attributed mainly to changes in medium pH and toxic effects of free $NH_4^+$ ions. Ammonium nutrition is associated with acidification of the medium. Low pH affects the availability of mineral nutrients in the medium, with most of them becoming limited, when pH falls below 5 [33] and therefore, restricting explant growth. Free $NH_4^+$ ions can cause toxicity within plant tissues. The highest multiplication rate with good quality new shoots, green to dark green leaves was observed on media containing both $NH_4^+$ and $NO_3^-$, respectively along with glycine at the normal concentrations. However, the increase and decrease in the concentrations of these three parameters showed significant decrease in shoot proliferation.

Thus, it was revealed from the present study that in the MS medium, N mixture (both organic and inorganic) was superior to any single N source for regeneration and growth of the shoots. Other studies also found that in MS medium, N regime was more efficient than a single N source [32, 25, 26.]

The optimized concentration of nitrogen in MS medium with BAP (0.5 mg/l) was then used for further subculturing to get better morphological responses and was regularly maintained this way [Figure A(3)]. They were then further transferred to half strength MS medium fortified with IBA (0.5 mg/l) [**Table 4, Figure A (4)**]. Omran *et al*. [34] also noticed *in vitro* rooting on IBA in *Lins culinaris* Medik., which is in consonance to the present research work. In contrast to this, Captan *et al.* [35] reported that NAA and IAA enhanced root proliferation in *Phyllanthus caroliniensis*.

**TABLE 4: EFFECT OF DIFFERENT CONCENTRATION OF AUXINS ON ADVENTITIOUS ROOT FORMATION FROM THE *IN VITRO* GROWN SHOOT CULTURED ON ½ MS MEDIUM. DATA (MEAN ± S.D.).**

| Growth regulators (types auxin) (mg/l) | % response of rooting | Av. Length of root (cm.) (*Mean ± $t_{0.05}$S.E.) |
|---|---|---|
| **0.0** | - | - |
| **IBA** | | |
| 0.05 | 52 | 1.49 ± 0.17 |
| 0.1 | 60 | 2.97 ± 0.44 |
| 0.5 | 95 | 4.04 ± 1.31 |
| 1.0 | 65 | 3.08 ± 1.15 |
| 1.5 | 40 | 1.68 ± 0.12 |
| **2,4-D** | | |
| 0.05 | 30 | 1.92 ± 0.13 |
| 0.1 | 32 | 1.79 ± 0.17 |
| 0.5 | 40 | 1.36 ± 0.04 |
| 1.0 | 35 | 1.08 ± 0.07 |
| 1.5 | 25 | 1.09 ± 0.02 |
| **IAA** | | |
| 0.05-1.5 | - | - |

*Values are 95% confidence limits for Mean and mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.05% probability level

The *in vitro* raised plantlets were taken out from culture bottles for hardening and acclimitization which was done by the method described in "Materials and Methods". In conclusion, N is an essential nutrient for regeneration and development of *P. amarus* shoots, and no proliferation and growth occurred in the absence of N. The results presented here clearly indicate the diverse responses to the various forms of N supplied, reflecting the previously overlooked importance of this nutrient in determining plant morphology and morphogenesis *in vitro*.

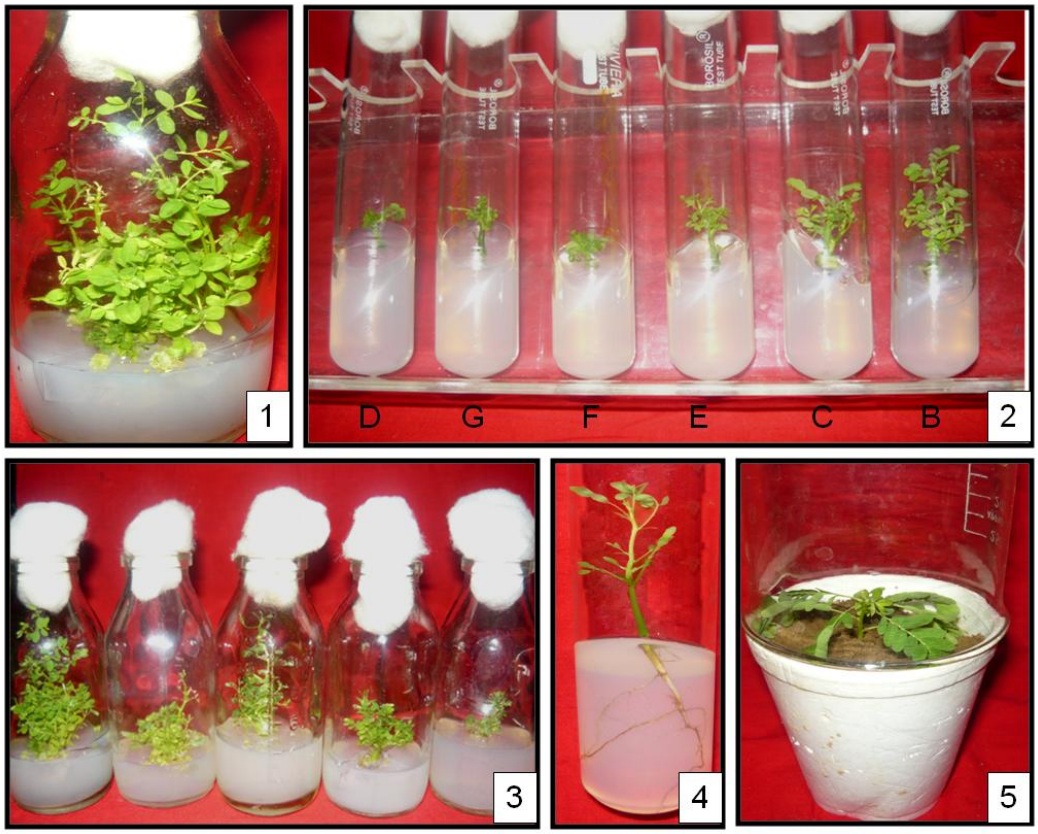


**FIG. A: 1:** Axillary shoots regeneration from nodal explants of *Phyllanthus amarus* cultured on MS medium +BAP (0.5 mg/l). **2:** Morphological variation and multiplication of shoots on Modified MS medium. **3:** Elongation and multiplication of shoots after 2 weeks of subculturing on MS + BAP (0.5 mg/l). **4:** *In vitro* rooting on ½ MS+ IBA (0.5 mg/l). **5:** *In Vitro* derived plantlets grown in pots.

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