UK Journal of Pharmaceutical and Biosciences
Available at www.ukjpb.com
ISSN: 2347-9442

Standardization of Protocol for *in vitro* Micropropagation of *Phyllanthus niruri*: An Important Medicinal Plant

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Article Information
Received 22 June 2018
Received in revised form 29 Aug 2018
Accepted 30 Aug 2018

Keywords:
Phyllanthus niruri,
MS medium,
Regeneration,
Multiplication

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Abstract

An efficient regeneration protocol for *Phyllanthus niruri* was developed using nodal explants on MS medium augmented with different concentrations and combinations of plant growth regulators. The highest frequency (85.5%) of bud break and shoot induction was observed on medium supplemented with 1.0 mg/l BAP along with 0.5 mg/l IBA with the formation of 4.2±0.5 number of shoots having length of 3.0±0.1 centimeters within ten days. MS medium fortified with 1.0 mg/l BAP and 1.0 mg/l Kinetin in combination with NAA (0.5 mg/L) showed the highest percentage (80.0%) shoot multiplication with an average of 9.0±0.7 adventitious shoots, directly from the explants, without any callus formation. Shoots formed were remarkable healthy and achieved the length of 10.0±0.5 centimeters in 25 days. Elongated shoots were rooted best on MS medium with 1.0 mg/l IBA producing maximum number of roots with 85% response with average length 8.0±0.3 cm within 15 days. The plantlets were gradually acclimatized and successfully transferred to field condition with 98% survival rate after rooting. The standardized protocol reported in this study may help in large scale propagation of this plant species which is currently exploited from the nature.

1 Introduction

Plants are the traditional source for many chemicals used as pharmaceuticals, biochemicals, fragrances, food colours and flavours. Near about 1,000 plant varieties out of 17.000 plants were commonly used for traditional herbal medicines in Ayurveda, Unani, siddha and Amchi. From many years the traditional medicines was made by wildly grown local plants. Phytochemicals extracted from different parts of plant is used for the treatment of various diseases, for supplementing nutrition in foods and cosmetic industries has a great potential in this century. Most valuable phytochemicals are products of secondary metabolism and possess sufficient chemical or structural complexity, so that artificial synthesis is difficult[1]. Plant tissue culture techniques now play an important role in the micropropagation and quantitative improvement of the medicinally important plant. Though the conventional breeding techniques have considerably increased the productivity of modern crops, the application of biotechnology could speed up further crop improvement. It overcomes the barriers in conventional vegetative propagation and fulfils the demand for large scale cultivation in a short period by rapid multiplication. We can speed up the production rate of the average plant by approximately 10,000 times and a large number of productive plants can be multiplied routinely through tissue culture[2].

Phyllanthus niruri L. is an important medicinal plant belongs to family Euphorbiacese (Fig 1.1). Genus *Phyllanthus* having species more than 600 plants were known for the presence of phytochemicals and their pharmacological importance. It is commonly found in the region of tropical and subtropical and the countries like India, China, Brazil, Peninsula, Egypt and Argentina. It has been widely used as a medicine for many diseases like diabetes, jaundice, dropsy flu genitourinary infections, skin diseases etc. It is reported that plant shows anti-tumor and anti-carcinogenic activities and has a great potential to the treatment for hepatitis B viral infection by which this plant develop more interest among pharmaceuticals. The plant of

Phyllanthus niruri developed Phenolic compounds which is more prominent in leaves compare to the stem. The main active component of the plant reported was phyllanthin and hypophyllanthin in 1891, and about fifty rich sources includes lignans, tannins, saponins, coumarins, terpenes from root, stem, and leaves. Important Phytochemicals like lignansniranthin, nirtetralin and Phyltetralin have recently been reported from the leaves of plant[3-5].

Because of very small embryo due to which most of the seeds are abortive, germination is difficult. Due to less availability, low viability seed germination and the seeds germinates only when fresh under specific environmental condition and lost viability with the storage period of a year, Conventional propagation of this species is limited to seasonal means, which is difficult and slow in meeting the commercial quantities required[6, 7].

Thus, traditional propagation methods through seeds and vegetative method is not sufficient and reliable hence, plant production through tissue culture method is required. In view of above the present research work has designed for standardization of an efficient protocol for *in vitro* micro propagation of the *Phyllanthus niruri* plant.

2 Materials and Methods

2.1 Plant material and explants

The Plant *Phyllanthus niruri* were collected from Sanjeevini medicinal park Bhopal in the month of July and were identified by Technical Officer Sanjeevini Bhopal. Nodal segments, for axillary and apical meristems of 5-10 centimeter length were excised from the mother plants were selected as explants for inoculation.

The explants were washed under running tap water for 30 minutes and then washed thoroughly in sterile double distilled water (DDW). These explants were then kept in 1.0% Bavistin (BVN) (Carbendazim Powder BASF India Limited) a broad spectrum fungicide for 10 minutes followed by 5.0% (v/v) Teepol, a liquid detergent for 5 minutes by continuous shaking method. The treated explants were washed in sterile double distilled water (DDW) for 4 to 5 times to remove the chemical inhibitors. Further surface sterilization treatment was conducted in a laminar air flow chamber.

2.2 Effect of growth Hormones on shoot induction

For shoot initiation surface sterilized axillary and apical meristem explants of *P. niruri* were inoculated on Murashige and Skoog's (MS) media supplemented with different concentrations of 0.1-2.0 mg/l BAP (6-benzylaminopurine) either alone or with different combinations of 1.0 mg/l IBA (Indole-3-butyric acid), 0.5 mg/l NAA (α-naphthalene acetic acid). The cultures were incubated in a culture room at 25±2^{o} C under 16 hours photoperiod provided by cool white fluorescent tubes**.**

2.3 Effect of different growth hormones on shoot multiplication

To standardize the media for shoot multiplication protocol, initiated and regenerated shoots from different explants from nodal segments were separated, isolated and transferred on fresh medium for rapid multiple shoot formation.

Experiments were carried out to check the effect of different concentration of higher cytokinin like 0.5- 2.0 mg/l BAP (6 benzylaminopurine) and 0.5-1.0 mg/l KN (kinetin) either alone or with combination of auxins 1.0 mg/l of NAA (Naphthalene acetic acid). Subsequent sub culture on fresh medium maximizes the multiplication of shoot. The growth response of cultures were studied at weekly interval. The parameters were taken as the average number of shoots initiated and multiplied, and the length of regenerated and multiplied shoots were recorded**.**

2.4 Root Induction

After continuous shoot multiplication the shoots were separated and transferred from multiplication cytokinins containing medium to the root induction medium.

A low salt medium supplemented with auxin was found satisfactory for rooting of shoots in large number of plant species. Hence after multiplication phase the regenerated multiplied shoots with apex were separated, isolated and transferred for root induction medium containing different of Auxins like 1.0-2.0 mg/l IAA (indole-3-aceticacid), and 1.0 mg/l IBA (Indole-3-butyric acid). The growth parameters were observed as per percentage of root initiation, length in centimeters (cm) and number of roots formed.

2.5 Hardening and acclimatization

For the hardening of *in vitro* raised plants of *P. niruri* the well developed rooted shoots /plantlets were taken out from the from culture medium flasks and washes thoroughly with running tap water to remove all traces of medium attached to the roots. Finally the plants were planted in pots containing mixture of soil sand and farmyard in the ratio of (1:2:1) for acclimatization and were maintained in green house.

3 Results and Discussions

The present experiment was investigated to standardize the protocols for the initiation of shoot for shoot multiplication/ production, *in vitro* root induction, and hardening of tissue culture raised plantlets.

Selection and sterilization of plant material

For the standardization of efficient protocol of micropropagation young shoots of *Phyllanthes niruri* were collected from healthy plants, nodal segments with dormant bud for axillary and apical meristems from the field in the month of July to October were proved the best explants.

For the sterilization, it was observed that 0.1% $HgCl_2$ solution for 5-9 minutes shows 80% sterile cultures more than 5 minutes treatment explants dehydration is observed may be due to the soft texture of young shoots. It was found that this treatment was standardized to sterilize the explants without a prior treatment of ethanol and antifungal supplements.

Shoot induction of Phyllanthes niruri

Preliminary initiation experiments were conducted to examine the effect of BAP (6-benzylaminopurine) along with NAA (α-naphthalene acetic acid) and IBA (Indole-3-butyric acid) with Murashige and Skoog (MS) medium for shoot bud induction.

Effect of Cytokinins:

To standardize the growth hormones on bud break and shoot induction was stimulated on the Murashige and Skoog (MS) medium fortified with the cytokinin BAP (6-benzylaminopurine) in the concentrations ranging from (0.1-2.0 mg/l). Among different concentrations of BAP (6-benzylaminopurine) tested, maximum bud break and shoot induction (70.8%) was achieved on media supplemented with 1.0 mg/l BAP (6-benzylaminopurine) about 2.5± 0.1 numbers of shoots having length of 2.1±0.1 centimeters were initiated within 15 days culture. The frequency of shoot initiation from explants increased with the increase of culture period.

Effect of cytokinin with auxins

The shoot induction through nodal explants on MS medium supplemented with BAP (6-benzylaminopurine) along with auxins, IBA (Indole-3-butyric acid), and NAA (α-naphthalene acetic acid) also showed a significant variation in terms of bud break, number of shoots induced per explant (Table 1). Maximum (85.5%) bud break and shoot induction was observed on medium supplemented with 1.0 mg/l BAP (6-benzylaminopurine) along with 0.5 mg/l IBA (Indole-3-butyric acid) with the formation of 4.2±0.5 number of shoots having length of 3.0±0.1 centimeters within ten days. While minimum bud break and shoot induction (50.5% - 40.3%) was observed on the medium supplemented with 0.5 mg/l BAP (6-benzylaminopurine) and 0.5 mg/l IBA (indole-3- butyric acid) 1.0 mg/l BAP (6-benzylaminopurine) and 0.5 mg/l NAA (Fig 1.2 & 1.3).

Table 1: Effect of MS with growth regulators on Shoot induction of *Phyllanthus niruri*

MS+Auxin+cytokinin (mg/l).	%age of bud break.	No. of days required.	Mean No. of shoots initiated ± SE.	Mean shoot length in cm±SE.
0.1 BAP.	50	25	1.0±0.2	1.0±0.5
0.5 BAP.	55.0	20	3.8± 0.1	4.3±0.1
1.0 BAP.	70.0	10	2.5± 0.1	3.0±1.3
2.0 BAP.	55.0	15	1.1±0.5	2.5±0.9
0.5 BAP + 0.5 IBA.	60.5	15	6.2±0.8	8.0±0.1
0.5 BAP + 0.5 NAA.	50.5	10-15	3.0±0.4	2.0±0.5
1.0 BAP + 0.5 IBA.	85.5	10-15	4.2±0.5	3.0±0.1
2.0 BAP + 0.5 NAA.	70.0	25	3.2.±0.5	3.2.±0.5

The shoots induced on this enriched medium were found to retain their vigour and health. It is observed that addition of auxin like 0.5 mg/l NAA (α-naphthalene acetic acid) and IBA (indole-3- butyric acid) in combination with BAP (6-benzylaminopurine) shows good response in shoots induction. The initiated shoots were further transferred to fresh medium for multiplication experiment.

Our results thus conclusively showed that low concentration of growth hormones auxin and cytokinin levels (0.5-1.0 mg/l) shows best shoot induction from nodal cultures. The stimulating effect of BAP (6-benzylaminopurine) on bud break and multiple shoot formation has been earlier reported for several species of phyllanthus medicinal plant. Bhattacharya and Bhattacharya (2001)[8] and Ghanti et al., (2004)[9] reported similar results in *P. amarus* and Karthikeyan *et al.,* (2008)[10,11] have reported in *P. niruri* by using shoot tip nodal segments on MS medium supplemented with different growth hormones.

Multiplication of shoots on different growth hormones of Phyllanthus niruri

To standardize a suitable medium for mass multiplication of shoots from single initiated nodal meristems, the effect of various media were assessed. Highest number of shoot formation were observed on MS medium containing high concentration of BAP (6-benzylaminopurine), with the addition of KN (kinetin) along with NAA (α-naphthalene acetic acid) and IBA (Indole-3-butyric acid). These media shows rapid

production of about 10 to 15 auxiliary's shoots per culture and shows mass shoot production when sub cultured on the same fresh medium within the duration 15 days. Among the cytokinins tested, BAP (6-benzylaminopurine) showed the most optimal effect on multiples elongation (Table 2).

Effect of BAP (6-benzylaminopurine) and KN (kinetin)

The effect of BAP and KN in combination at concentration of 0.5 mg/l was studied on multiplication and growth response. Since, good cluster of shoot formation and growth was obtained after the first 3 to 4 sub-cultures without or very little intervening callus phase. Interestingly, in this medium, not only elongation of shoot buds occurred but there was also a further proliferation of additional shoot buds. The best response was recorded on MS with 0.5 mg/l of BAP and KN 50.0 % of shoot multiplication within 25 days. About 4.6±0.8 numbers of shoots were initiated having 6.3±0.9 centimeter in length. Whereas 40.0% of shoot multiplication was achieved on medium fortified with 0.5 mg/l BAP (6-Benzyl aminopurine) in which only 3.4±0.05 number of shoots of 4.5±0.9 centimeters in length were observed (Table. 2).

Table 2: Effect of MS media and growth regulators on shoot multiplication of *Phyllanthus niruri*

MS+Auxin/cytokinin (mg/l)	%age of shoot multiplication	No. of days	Mean No. of shoots	Shoot length in cm.±SE
0.5 BAP	40.0	20	3.4.±0.5	4.5±0.9
0.5 BAP+ 0.5 KN	50.0	25	4.6±0.08	6.3±0.9
0.5 BAP + 1.0 IBA	60.0	15	7.2±0.4	6.0.0±0.2
0.5 BAP + 0.5 NAA	50.5	20	5.3±0.1	6.3±0.9
1.0 BAP+1.0 KN + 0.5 NAA	80.0	25	9.0±0.7	10.0±0.5
1.0 BAP + 0.5 NAA	75.4	30	6.5.±0.5	5.0.±0.1
2.0 BAP + I.0 NAA	70.0	30	7.0±0.9	8.7±0.6
2.0 BAP+1.0 KN + 0.5 NAA	75.0	30	8.8±0.5	4.5±0.0
2.0 BAP + 0.5 NAA	60.0	30	6.5±0.9	7.5±0.4

In the present experiment the shoot multiplication has achieved in the medium supplemented with 1.0 mg/l BAP with 0.5 mg/l IBA shows the most responding and desirable medium both in terms of multiplication fold and cluster elongation, Thus, as usual BAP was found to be the most effective indicating, the cytokinin specificity of any nodal explants of *Phyllanthus niruri* for shoot formation.

Effect of cytokinins with auxins

Auxins, like IBA (Indole-3-butyric acid) and NAA (α-naphthalene acetic acid) were added and tried along with varied concentration of BAP (6-benzylaminopurine) and KN (kinetin) to study their effect on shoot regeneration and to maximize elongated shoot multiplication, have a positive effect on multiplication fold and shoot elongation. A prolonged incubation of 3-4 weeks culture period results significant increase in shoot formation with good elongation.

Cultures showed highest percentage (80.0%) of shoot formation with an average of 9.0±0.7 adventitious shoots, directly from the explants, without any callus formation on medium supplemented with 1.0 mg/l BAP and 1.0 mg/l Kinetin with NAA. Shoots formed were remarkable healthy and achieved the length of 10.0±0.5 centimeters in 25 days. (Table. 2, Fig 1.4 & 1.5).

It was further evaluated by the addition of 0.5-1.0 mg/l NAA supplemented with 2.0 mg/l BAP. The frequency of shoot proliferation was obtained in the average of 75.0% of about 8.0±0.7 of 8.7±0.5 centimeters within 25-30 days.

In contrast to the synergistic effect of BAP in combination with another cytokinin KN and an auxins IBA/NAA has improved the multiplication rate has been previously reported in *P. tenellus* important parameter for the large production of plant. For shoot formation from nodal explants on the medium having 1.0 mg/l Kinetin in *P. urinaria* in MS containing high (5.0 µM) BA, with (1.25 to 5.0 µM) in plant species *P. amarus* reported by Cristiane et al., (2010)[12] and Rajasubramaniam and Saradhi (1997)[13]. An average number of 21-23 shoots have induced on medium MS containing 5.0 µM BA, 1.25 to 5.0 µM Kinetin or 2.5 to 5.0 µM 2iP, on *P. caroliniensis*, *P. fraternus* about 14-16 shoot from shoot tips explants on B5 medium having 10_5 M BAP[14].

The developed of an efficient multiplication protocol for this important medicinal plant *P. niruri by* using nodal segment for axillary shoot proliferation is directly contrast with the results obtained in *P. urinaria* Linn, reported *in vitro* micropropagation was achieved on the medium having BAP (1.5 mg/l) with KN (2.0 mg/l)[15].

Effect of growth regulators for Root induction

Various experiments were designed for root induction on MS medium with and without (control) growth regulators. Maximum (85.0%) root induction has achieved directly from the base of the shoots on medium supplemented with 1.0 mg/l IBA of average length 8.0±0.3 cm within 15 days. Comparatively 1.0 mg/l IAA was not more responding to develop an efficient root system from in *vitro* raised shoots. The roots formed were very slender and thin in the medium containing NAA. Among the various concentration and combinations full strength of Murashige and Skoog (MS) basal medium supplemented with 1.0 mg/l IBA shows best root induction protocol for healthy roots within minimum time period (Table 3, Fig 1.6 & 1.7).

Table 3: Effect of MS media and growth regulators on root induction in *Phyllanthus niruri*

Media composition (mg/l)	% age of root formation	No. of days	Mean root length in cm ± SE
1.0 IAA.	60.0	20	4.0±0.4
1.0 IBA.	85.0	15	8.0±0.3
1.5 IAA	68.2	20	5.3±0.8
2.0 IAA.	50.0	25	3.5±0.0
1.5 IBA.	75.0	20	6.5±0.6
2.0 IBA	55.0	25	3.8±0.2

Similar results of rooting were reported in other species of *Phyllanthus* by *Cristiane et al*., 2010[12] in *P. tenellsus* due to auxin type and its concentration since replace higher IAA to IBA (0.2 and 0.4 mg L-1) improved rooting.

The effectiveness of IBA acid over other auxins for root induction has been already reported in several other species such as in *Phyllanthus niruri*[10,11], *P. amarus*[8] and in *P. urinaria*[14].

Hardening of Tissue culture raised plant

Many times an *in vitro* grown plant does not survive when they are taken out from optimized physical culture conditions. The transfer of plants from the culture flasks to the soil requires a careful, stepwise procedure.

The plantlets were successfully hardened in sterilized soil sand and vermicompost in the ratio of (1:2:1) mixture gave the maximum (98%) survival percentage with better plant growth resulting as a suitable medium for hardening (Figure 1.8). Similarly in *P. amarus*, acclimatized in *vitro* raised rooted plantlets were effectively transferred to soil sand and farmyard in the ratio of (1:2:1) mixture and the plants were successfully transferred in the field and grown up to flowering stage[8, 10,11].

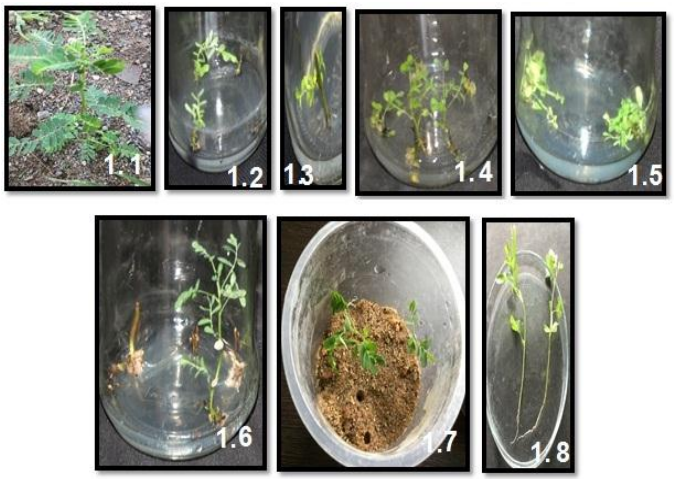

Fig 1: Details of Micropropagation of *Phyllanthus niruri*

1.1: Plant in field, 1.2 &1.3: Shoot initiation in MS + 0.5 mg/l BAP +0.5 mg/l IBA, 1.4 & 1.5: Shoot multiplication in 2. 0 mg/l +0.5 mg/l NAA, 1.6 & 1.7: Root induction MS + 1.0 mg/l IBA, 1.8: Hardened plant of *Phyllanthus niruri*

4 Conclusion

The results achieved from the present investigation are quite encouraging and is concluded that the standardize protocols for culture establishment multiplication and plantlets hardening of *Phyllanthes niruri* an important medicinal plant. The significance of developed efficient *in vitro* protocol would be to obtain maximum number of plantlets in minimum period of time with proper rooting along with acclimatization in the field. In conclusion, the protocol developed through this investigation will be useful for large scale multiplication and providing of quality planting material of the important medicinal plant possibly other related species used for cultivation. Increased awareness about potential of this group of interesting and useful plants has encouraged many innovative and progressive growers and entrepreneurs to take up their cultivation as a commercially enterprise.

5 Conflict of interest

We declared that we have no conflict of interest.

6 Author contributions

AP, PS and SK have carried out the research work in the laboratory. SK compiled and analyzed the data of present work. All authors approved the final manuscript.

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