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ORIGINAL ARTICLE

Improved protocol for micropropagation of genetically uniform plants of pharmacological importance - *Phyllanthus niruri* using a single-step nodal cutting technique

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ABSTRACT

Phyllanthus niruri, a plant with high medicinal importance, is used as natural remedies for Bronchitis, Anaemia, Leprosy, Asthma, Urinary disorders neurotonic, immuno modulator in India due to presence of different classes of organic compounds. The study was attempted in order to shorten the protocol for in vitro propagation. Multiple shoots were obtained by culturing the nodal explants on MS medium supplemented with various concentrations of cytokinins viz., BAP alone or in combination with auxins auxin viz IBA and 2-4 D (1mg/l BAP, 0.5mg/l IBA and 1mg/l 2-4-D). Further, to multiplication and simultaneous rooting was obtained with lowered concentration of hormones (1mg/l BAP and 0.5 mg/l 2-4-D). The micropropagated plantlets were successfully acclimatized and transferred to soil supplemented with vermicompost and farm yard mixture.

Keywords: *Phyllanthus niruri, nodal explants, tissue culture, vermicompost.*

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INTRODUCTION

COVID-19 caused the enormous loss of human lives, it has also put extraordinary stress on agricultural food systems, bringing to forefront the role of nutrition as an equally important need for humankind [1,2,3]. In present context, issues like environment sustainability, loss of biodiversity, global warming and climate change, population explosion and ever continuing covid pandemic, it's even more pertinent to keep our Plant genetic resources (PGR) timely available for maintaining our food/nutrition/health security [4]. This would imply enhancing our agricultural production systems to not only meet the current demands but also be ready for any unforeseen future challenges [5]. National Medicinal Plant Board has estimated that India has 17,000–18,000 species of flowering plants growing in 15 agroclimatic zone, out of which 6000–7000 have reported medicinal applications since ancient times in traditional medicine including ayurveda, siddha, unani, and homeopathy [6]. Out of the vast repository of medicinal plants, more than 960 medicinal plants are commercially exploited and within these 178 are known to have annual consumption levels of more than 100 MT (metric tons). The Indian market for medicinal plants was valued at Rs. 4.2 billion (US$ 56.6 million) in 2019 and since then has been growing at a rate of 38.5 % to reach valuation of up to Rs. 14 billion (US$ 188.6 million) by the end of the year 2026 [7,8]. At global level, the market is estimated to be around US$ 120 billion. India happens to be one of the largest contributors to traditional and medicinal herbs and has been exporting the same, contributing significantly to the Indian economy. India exports have also been consistently rising for these products by as high as 27% translating to 104,511 tonnes of Ayush medicines exports during the period of Apr 21- Jan

22 [9]. There also exists a big demand and supply gap for these products which was evidenced as increase in demand by as high as 50% and decrease in availability by 26%. Additionally, the extraction of these is done primarily from the wild and natural plants using excessive farming techniques that cause a great stress on the resource availability as well as biodiversity in terms of overexploitation by pharma and drug industry. The loss of biodiversity has been at such high proportions that caused 65 species (i.e., 10% of the total species) falling into the critically endangered, endangered, vulnerable, and nearly threatened categories [10].

The scale and pace of technology revolution has begun to impact in so many ways that it appears the civilization is moving into the age of biotechnology. Plant cell biotechnology has evolved as a new era in the 21st century merging the area of genetic engineering and molecular biology with conventional methods of breeding. It has been globally accepted as one of the important tools for crop improvement and has allows the appearance of new agricultural products with improved quality and management of plant diversity. Plant tissue culture has been extensively exploited to meet the growing demands for elite planting material in the current century. In these processes, tissues or cells, either as suspensions or as solids are maintained under conditions conducive for their growth and multiplication. Advances in plant biotechnology, especially those associated to *in vitro culture* and molecular biology, have also provided powerful tools for the optimization of micropropagation, callus culture, and root culture protocols and it also offered the possibility to use cell/root culture techniques for vegetative propagation and secondary metabolite production in medicinal plants of high value [11,12,13 14]. Global recognition of the science of Ayurveda has been exemplified with increased availability of medicinal plants-based products. This has been further advocated through Indian government impetus to integrate the science of allopathy and ayurveda at all clinical levels including secondary and tertiary health care facilities. This has led to an ever-increasing demand for the raw materials, adding pressure on the limited available resources. Further compounding the limitations, the very fact that the production and cultivation of these plants is unorganised and over exploitation of these resources can cause these species to become endangered if not extinct. This needs to be counteracted through sustainable cultivation and harvesting methods to prevent the loss of biodiversity. Micropropagation, especially for traditional Indian medicinal plants has emerged as an important tool to address these concerns. Extensive work and demonstration of scalable technologies in applied plant biotechnology, particularly in micropropagation have made them an important mode for large scale propagation of medicinally important plants through all seasons, round the year. This has led to an exponential increase in commercial value and now represents an efficient way to produce several valuable natural products [15]. Many of these protocols are already established for medicinally important plants like *Artemisia annua* (L) *Catharanthus roseus* (L.) *Rauwolfia serpentina* (L.) *Bacopa monnnieri* , *Stevia rebaudiana* and *Moringa oleifera* [16]. As more and more population move towards the practise of wholistic healing through traditional systems of medicine, the cultivation of these plants will acquire much larger significance and relevance. The side effects associated with western medicinal practises have further mobilised Indian population to utilise the traditional knowledge for general wellness as well as therapeutics. Hence, this requires an urgent intervention to adopt an alternate means of production and conservation, which could ensure large-scale and high-quality plant materials as well as conserve them to fulfill the growing demand [17].

Recently, *Phyllanthus niruri*, a small herb belonging to the family Euphorbiaceae family has been recognised for its pharmacological potential and has been put on second place in priority list of most significant Indian medicinal plants assessed based on its therapeutic significance, business worth and potential for further innovative work [18,19]. The plant is cultivated in the coastal regions and finds its mention in the ancient text Chraka Samhita for the treatment of asthma liver and urinary disorders [20]. The usage is not just restricted to Ayurveda but similar mentions are found in Siddha and Unani medicine also. Animal studies have validated the use of this extract for medicinal purposes [21,22]. The extracts of whole plant, roots, leaves have been reported to have excellent antioxidant and hepato- protective properties as validated through cell line animal based experiments through marked decrease in biomarker enzymes [23]. This is currently being explored towards treatment of Hepatis B infections. Other pharmacological activities associated with *P. niruri* include antidiabetic, anti-inflammatory, antinociceptive and analgesic, cardioprotective, hypolipidaemic, antibacterial, antiparasitic, antiviral, analgesic and anti-urolithiatic and wound healing activities [24,25]. Variety of secondary metabolites including find lignans, tannins, polyphenols, alkaloids, flavonoids, terpenoids and steroids have been identified as the bioactive ingredients [26]. Some major bioactive constituents isolated from this plant include alkaloids like 4-methoxy-nor-securinine, nirurine and ent-norsecurinine, gallic acid, coumarins like ellagic acid, ethyl brevifolin carboxylate and methyl brevifolin carboxylate and flavonoids including quercetin, rutin, astragalin, isoquercitrin and kaempferol-4′-rhamnopyranoside [27,28]. Secondary

metabolites are reported to be responsible for many of the biological activities exhibited by medicinal plants and hence *P. niruri* seems to be an ideal candidate for usage in traditional medicine system and needs to be investigated further for its commercial production [29,30] Table 1. One of the major concerns with its commercialisation has been to ensuring a regular and consistent supply and quality of the planting material throughout the year. Increased demands for these materials are threatening to disrupt sustainable production to provide the basic raw material, thus disrupting the supply chain. To overcome these issues, tissue culture-based solutions are being explored for ensuring the supply chain and also result in overproduction of secondary metabolites. Additionally, these processes are also quite time effective. Although, these techniques have been developed for variety of species, however, till now the micropropagation of pharmacologically important *P. niruri* has not yet been explored in detail as evidenced from very few studies conducted on this species. This gap in information is being covered in the present study where we have assessed the effects of multiple plant growth regulators (single and in combination in varying concentrations) to evaluate their effect on effective multiplication, rooting and acclimatization to establish protocol for large-scale propagation of aseptic cultures of *P. niruri.*

S. No.	Class	Compounds	References
1	Alkaloid	4-Methoxy-nor-securinine, nirurin, ent-norsecurin	Anjaneyulu *et.al*., O'Neil *et.al*., Singh *et.al*.
2	Benzenoid	Gallic acid, Corilagin	Shimizu *et.al*.
3	Coumarin	Ellagic acid, ethyl brevifolin carboxylate	Shimizu *et.al*., Toru Lizuka *et.al.*
4	Flavonoid	Quercetin, rutin, astragalin, quercitrin, isoquercitrin, kaempferol-4- rhamnopyranoside,eridictyol-7-rhamnopyranoside, fisetin-4-O-glucoside, nirurin	Nara *et.al.,* Singh *et.al*.
5	Lignin	Phyllanthin, hypophyllanthin, niranthin, nirtetralin, phyltetralin, hinokinin, isolintetralin	Row *et.al.,* Anjaneyulu *et.al.* Calixto *et. al.*
6	Lipid	Ricinoleic acid	O'Neil *et.al*.
7	Phytallate	Phyllester	Foo *et. al.*
8	Sterol	Estradiol, ß-sitosterol, isopropyl-24-cholesterol	Chauhan *et.al.,* O'Neil *et.al*.
9	Tannin	Geranin	Ueno *et. al.*
10	Triterpene	Lupeol acetate, lupeol, 3,7,11,15,19,23-hexamethyl-2Z,6Z,10Z,14E,18E, 22E-tetracoshenen-1- ol, phyllanthenol, phyllanthenone, phyllantheol	Hossain *et. al.,* K. Narendra *et al.*

Table 1 - List of compounds and their class present in *P. niruri*

MATERIAL AND METHODS

Micropropagation by node culture has been reported to be an effective method for micropropagation and the same was utilised for *P. niruri*. Growing healthy plants were collected from Botanic Garden of Indian Republic, Noida. Uttar Pradesh, India (coordinates 26°28′ to 29°30′ N latitudes and 91°30′ to 97°30′East longitude) along the bank of river Yamuna growing on predominantly sandy soil. The nodal segments were processed through standard protocols that involved washing running tap water for thirty minutes followed by subsequent rinsing with 0.5% mild detergent tween 20. Detergent removal was ensured through triple rinsing with distilled water. Subsequent steps were carried out under aseptic conditions under laminar flow. For surface sterilization, the explants were dipped in 0.2% aqueous solution(w/v) of $HgCl_2$ for 3 minutes then they were washed in sterilized distilled water for 3-4 times, to ensure complete removal of $HgCl_2$. To evaluate the effect of 6-benzylaminopurine (6-BAP), indole-3-butyric acid (IBA) 2- 4 D on callus formation and shoot initiation basal MS media with 3% sucrose was prepared and further supplemented with varying (0.0 – 1.5 mg/L) of 6-benzylaminopurine (6-BAP), indole-3-butyric acid (IBA) 2- 4 D. Medium pH was adjusted to 5.8 and 0.8% agar was added for facilitating solidification. After autoclaving at 121°C and 15 lb pressure, the media was poured in 25×100 mm glass culture tubes (Borosil, India), cooled and then inoculated with sterile explants. The cultures were incubated at 26±2°C, 16/8 hr photoperiod, relative humidity (55±5%), provided in culture shelves by cool fluorescent tubes with 4000 lux light intensity. Data was recorded after 3-5 weeks. Each culture treatment consisted of 15 replicates and each experiment was repeated thrice. The parameters that were assessed were growth and multiplication in respect of shoot induction, their number and length, callus induction, number of shoots from callus, number of leaves, root induction and root length. The most sturdy plantlets were hardened in soil and survival % was measured.

RESULTS AND DISCUSSION

In vitro establishment of *P. niruri* in aseptic cultures condition was carried from plants grown in the natural environment condition. Induction of multiple shoots from nodal explants has been reported in a couple of earlier studies. In the present study about 1 – 1.5 cm of nodal portion were inoculated onto the different concentrations of BAP, IBA and 2-4-D supplemented in MS medium and their growth and multiplication in respect of shoot induction, their number and length, callus induction, number of shoots from callus, number of leaves, root induction and their length was compared. The maximum number of shoots 12 per explants was obtained on the MS medium supplemented with a concentration of BAP ranging from 1.0 – 1.5 mg/l along with the concentration of 2-4-D ranging from 0.5 – 1.0 mg/l. (Table 2). Multiple shoot initiation was obtained within 2-3 weeks of culturing. A similar trend was also seen for shoot length (5.16 cm) and root length (3.24 cm) at the same combination. There was a decrease in the number of shoots as well as shoot length as the concentration of cytokinin - BAP was increased to 1.5 mg/l. It was also observed that addition of other auxin – IBA decreased the number of shoots and their length. A concentration of BAP at 1.0 mg/l supplemented with1.0 mg/l IBA and 1.5 mg/l 2-4 D in the medium gradually reduced the number of shoot production and yellowish callus was observed after 5 weeks of culture, highest efficiency (90%) was noted after 4 weeks of inoculation. However, it was noticed that the other satisfactory results for in vitro shoot multiplication with sizable development were obtained on MS- BAP (1 mg/l) alone and 1.0 mg/l BAP and 1.0 mg/l 2-4-D in combination. Formation of 1-2 shoots from callus started, subcultured on fresh MS media supplemented with equal concentration of BAP and IBA (1.0 mg/l) in combination with 2-4-D (1.5 mg/l). All these media show simultaneous root initiation, and it inhibit further shoot multiplication. The effect of different concentrations of hormones on the number of shoots, length of the shoot (cm), and the number of leaves are represented in Table 3. Our study clearly indicates the requirement of only BAP and no other hormone for shoot proliferation with additional benefits including stem thickening, elongation, and leaf size increase. Sub culturing of *in vitro* induced plant propagates was done after every four weeks to provide fresh medium and nutrients. After 5-6 weeks of culture, the number of roots per shoot cluster and rooting percentage were recorded before hardening (Fig. 1(a-i). Maximum induction of roots (64.28 %) and length (03.24) cm shoots was achieved from nodal explants inoculated on MS medium supplemented with BAP (1.0 mg/) and 2-4-D (0.5 mg/l). More importantly, within a period of 7 -8 weeks, almost 30 plantlets were generated, which has not yet been previously reported. Additional benefits of our protocol include usage of a single hormone (BAP) at low dosage unlike previous protocols which required multiple hormones at high concentrations. It is also a single step protocol to obtain rooted plantlets. All of the above features of this protocol, make it ideal and economic for the micropropagation of *P. niruri*.

To further move towards hardening, the plantlets with well-developed root systems were acclimatized. This was done by taking plantlets with sufficiently developed roots from the culture tubes, washing roots with running tap water, followed by treatment with fungicide - Bavistin 5 % w/v. These were then transferred to small earthen cups containing the farm yard mixture (FYM), sand and vermicompost (VC) compost in 1:1:1 ratio. The plants along with pots were covered with transparent polythene bags to prevent sudden desiccation. The inner side of the polythene bags was sprayed with water supplemented with 20% NPK at every 8 hours to maintain high humidity and supplement the essential elements of the plantlets. The polythene bags were gradually perforated to expose the plantlets to the outer normal environment and subsequently removed after 3 weeks. This ensured the establishment of plantlets in soil. The plantlet showed over 80% of survival rate. *In vitro* regenerated plantlets adapted well to acclimatization as plantlet leaves grew more prominent and thickened with new leaves emergence from 2 week onwards (Fig.). Plantlets of *P. niruri* were successfully induced and multiplied via *in vitro* culture by using nodal explants, which could be a step closer to its commercialization and availability of plants throughout the year and makes it a short and economic protocol for the *in vitro* propagation of plants. Plants produced can be also used in pharmacological and eco physiological research, avoiding the need to collect specimens in a natural environment ensuring reduced genetic variability, thus ensuring more standardised product.

MS medium + Plant Growth Regulators Concentration (mg/l)			Morphogenic Responses						
BAP	IBA	2-4-D	Shoot induction (%)	Average Number of shoot per explants	Average Shoot length (cm)	Callus Induction (%)	Average Number of shoots from	Root Induction (%)	Average Root Length (cm)
0.5	0.5	0.5	45.27	02.67	1.53	54.33	02.64	36.62	01.23
0.5	0.0	0.5	74.57	04.84	1.72	58.67	03.27	43.86	01.24
1.0	0.0	1.0	81.35	07.46	2.64	64.89	04.26	56.27	01.26
1.0	0.0	0.5	95.35	12.07	5.16	54.34	06.24	64.28	03.24
1.0	0.5	1.0	85.54	10.12	4.56	56.76	04.15	48.56	02.89
1.0	1.0	1.0	76.67	6.91	3.46	62.54	03.45	43.34	02.54
1.0	1.0	1.5	64.37	5.76	3.25	94.35	03.28	39.58	02.16
1.0	1.5	1.0	47.79	4.37	2.54	74.24	02.57	37.67	02.17
1.5	1.0	1.0	39.56	2.74	2.36	61.05	02.29	29.25	02.87
1.5	1.0	1.5	36.26	2.64	2.22	46.84	02.17	28.39	01.65
1.5	1.5	1.5	34.78	2.82	2.14	38.57	01.87	27.58	01.12

Table 2- Effect of various combination of plant growth regulators supplemented with MS media on organogenesis in *P. niruri*.

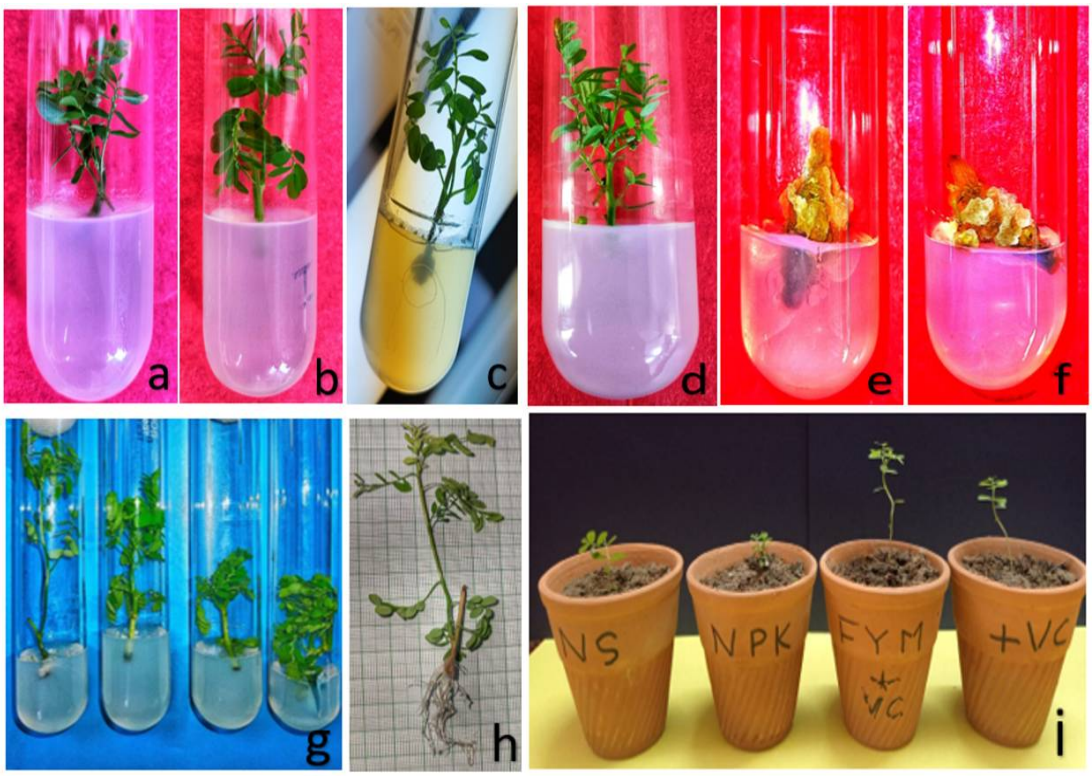

Figure 1- a, b and c - Multiple shoot initiation from nodal segment at MS medium supplemented with 1mg/l BAP, 0.5mg/l IBA and 1mg/l 2-4-D, further shoot multiplication and simultaneous rooting. **d –** Proliferation axillary bud on MS medium supplemented with 1 mg BAP + 0.5 2-4 D mg/l. **e and f -** Callus induction from internode on MS medium supplemented with 1mg/l BAP, 1mg/l IBA and 1.5 mg/l 2-4-D. and 1mg/l BAP, 1.5 mg/l IBA and 1 mg/l 2-4-D. **g –** Early and late multiplication stage after consecutive 2 weeks of inoculation on MS medium supplanted with 1mg/l BAP, 1 mg/l IBA and 1 mg/l 2-4-D. **h** – Regenerated plant with multiple root and shoot before hardening. **i** – Survived plantlets in mixture of cocopeat + peat moss (1:1), supplanted with NPK, FYM and VC and VC only after 4 weeks of acclimatization.

CONCLUSION

Micropropagation is currently being advocated as an important strategy to reinforce the supply and quality of crops used for medicinal purposes. In conclusion, the present study describes an improved protocol for the mass propagation of medicinal plant species, *P. niruri*, from stem nodal segments. MS medium supplemented with combination of 1mg/l BAP, 1 mg/l IBA and 1 mg/l 2-4-D was most successful for multiple shoot proliferation. This method avoids overuse of multiple plant hormones and provides a rapid and quick system for whole plant regeneration which could be used for the large scale micropropagation of the plant. The *in vitro* propagated plants acclimatized well with more than 80 % survival rate. Furthermore, there was no detectable variation seen among the acclimatised plants with regard to morphological growth characteristics, growth features noticeable abnormalities. The study would provide provision for large scale cultivation of *P. niruri* to meet the increasing demand for it in the herbal medicinal industry. However, we recommend that additional studies may be carried out as an extension work in future to enhance the production of bioactive metabolites in the in vitro culture through metabolic engineering strategies.

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