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In vitro Plant Regeneration, Flowering and Fruiting of *Phyllanthus niruri* L. (Euphorbiaceae)

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Abstract: A micropropagation protocol was developed for a rapid production of *Phyllanthus niruri* (Euphorbiaceae) plantlets using nodal segments of the mature plants as explants. The aseptic nodal segments cultured on MS medium supplemented with 1.0 mg L^{-1} benzyladenine (BA) produced an average of 6.6 shoots from each explant. Continuous subculturing of the nodal segments enabled the production of healthy shoots. The addition of kinetin (KN) (0.5, 1.0 mg L^{-1}) into the MS medium with the presence of BA (0.5, 1.0, 1.5, 2.0 and 2.5 mg L^{-1}) induced multiple shoot formation in all (100%) the nodal segments. None of the nodal segments produced multiple shoots when they were cultured on MS medium supplemented with 0.5 or 1.0 mg L^{-1} KN without BA. 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. *In vitro* flowering and fruiting also occurred in 97% of the micro-shoots on MS medium without plant growth regulators. The first *in vitro* flowering was observed 12 days after initial proliferation of nodal segments while fruiting occurred 20 days after culture. The addition of gibberellic acid (0.5 mg L^{-1}) into the MS medium was found to induce *in vitro* flowering within one week but inhibited *in vitro* fruiting. Basic MS medium supplemented with 30 g L^{-1} sucrose was found to promote healthy plantlet with 60 and 35% of the plantlets produced *in vitro* flowers and fruits, respectively within two weeks. All the *in vitro* plantlets produced flowers and 80% of them produced fruits after four weeks of culture on the same medium. The morphology of the *in vitro* flower and fruit were similar to that of the mother plant except they were smaller in size.

Key words: *In vitro* flowering and fruiting, nodal segments, shoot multiplication

INTRODUCTION

Phyllanthus niruri L. (Euphorbiaceae) is an herbaceous medicinal plant widely distributed in the tropics. It produces phyllanthoid branches with the presence of flowers and fruits at the base of each leaf, one of the identification characteristics of this plant. It is a popular and valuable traditional medicinal herb used for the treatment of various ailments such as flu, dropsy, diabetes and jaundice (Unander and Blumberg, 1991). Interest in this plant was further enhanced with reports of its anti-tumor and anti-carcinogenic activity and its potential as a remedy for hepatitis B viral infection (Rajeshkumar *et al*., 2002). *P. niruri* was also found to have anti-oxidant and hepatoprotective properties and anti-inflammatory potential (Kiemer *et al*., 2003). Some flavonoids obtained from this plant had shown antinociceptive properties (Santos *et al*., 2002). Several chemical compounds were identified from this plant such as amariin acid, amariin and phyllanthusiin D (Foo and Wong, 1992; Foo, 1993, 1995). Six phenolic compounds were also detected such as gallic acids, epicatechin, gallocatechin, epigallocatechin, epicatechin 3-0-gallate and epigallocatechin 3-0-gallate (Ishimaru *et al*., 1992). De Souza *et al*. (2002) reported that the leaves of *P. niruri* contained higher amount of the phenolic compounds than the branches. Although the anti-hepatotoxic potential of the plant was controversial, the major components that were responsible were phyllanthin and hypophyllanthin. Qian-Cutrone *et al*. (1996) successfully isolated niruriside, a new HIV REV/RRE binding inhibitor, using bioassay-guided fractionation technique.

The traditional practitioners usually obtained the whole plant materials including flowers and fruits of *P. niruri* for the preparation of traditional medicine via cultivation or collection from the wild. However, cultivation of this plant often encountered with low seed germination and seed heteromorphy (Unander *et al*., 1995). *In vitro* culture techniques could hence be used as alternatives for the mass production of the plant

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material to meet the market demand. *In vitro* flowering of *Phyllanthus caroliniensis* had been reported (Catapan *et al*., 2000). However, to date there was no report on *in vitro* flowering and fruiting in *P. niruri*. In the present study, we therefore report on rapid micropropagation protocol for *P. niruri* including the induction of *in vitro* flowering and fruiting of this plant.

MATERIALS AND METHODS

Establishment of aseptic explants: *Phyllanthus niruri* were collected from the herbal garden of Universiti Sains Malaysia, Penang, Malaysia. The 3-5 cm stem segments, with 3-5 nodes each segment, were used as explants. The stem explants were first washed with detergent several times and rinsed under running tap water for 30 min. They were then immersed in 70% (v/v) ethanol for 30 sec before surface-sterilized in 10% (v/v) Clorox® with the addition of three drops of Tween-20 (polyoxyethylene sorbitan monolaurate) for 10 min. They were subsequently rinsed three times with sterile distilled water and then surfaced-sterilized again with 5% (v/v) Clorox® with the addition of three drops of Tween-20 for 5 min. After three rinses with sterile distilled water, the stem explants were cut into single nodal segments before inoculated in the universal bottles containing MS medium (Murashige and Skoog, 1962) supplemented with 30g L^{-1} sucrose and 7.5g L^{-1} agar (Algas, Chile). The medium was adjusted to pH 5.7 before autoclaving at 121°C for 13 min under a pressure of 1.05 kg cm^{-1}. The cultures were placed in a culture room at 25±2°C under continuous lighting with cool white fluorescent tubes at an intensity of 44±9 μE m^{-2} s^{-1} for 10 days and the aseptic explants were used for the subsequent studies.

Induction of multiple shoots: The aseptic nodal segments were cultured vertically with the basal end placed into 250 mL jam bottles containing MS medium supplemented with 0.0, 2.0, 4.0, 6.0, 8.0, 10.0 mg L^{-1} benzyladenine (BA). Two explants were inoculated into each jam bottle and six bottles were used for each treatment. The experiment was repeated three times. After 4 weeks, the physical conditions of the plantlets and the number of shoots formed from each explant were recorded. To study the effect of kinetin on the induction of multiple shoots from the nodal segments of *P. niruri*, the same procedure was repeated using MS medium supplemented with 0.0, 2.0, 4.0, 6.0, 8.0 and 10.0 mg L^{-1} kinetin (KN).

Effect of combination and lower concentration of BA and KN on multiple shoot induction: Based on the results obtained from the previous section, the effect of combination of BA and kinetin (KN) at lower concentration on induction of multiple shoots was studied. The apical shoots of 4 weeks old *in vitro* plantlets were cultured on solid MS medium supplemented with BA (0.0, 0.5, 1.0, 1.5, 2.0 and 2.5 mg L^{-1}) combined with KN (0.0, 0.5 and 1.0 mg L^{-1}). The experiment was carried out using 6×3 factorial design. Two shoot explants were cultured in each culture vessel (250 mL jam jar) and eight jam jars were used for each combination medium. The experiment was repeated twice. The number of shoots formed from each explant was recorded after 4 weeks of culture. The cultures were maintained in a culture room at 25±2°C under continuous lighting with cool white fluorescent tubes at an intensity of 44±9 μE m^{-2} s^{-1}. The data was analyzed with Two-Way ANOVA followed by a comparison of means using Tukey Test (HSD) at $p = 0.05$.

Effect of subculture on multiple shoot formation: The multiple shoots obtained from the previous section were separated into individual shoots and subcultured on basic MS and proliferation medium (MS + 1.0 mg L^{-1} BA) respectively. Two individual shoots were cultured in each jam jar. Ten replicates were used for each subculture cycle. The number of shoots formed from each explant was recorded at each four week subculture cycle for seven cycles.

Effect of sucrose and GA_3 on *in vitro* flowering and fruiting: The stem nodal explants that remained aseptic were cultured vertically with the basal end placed into MS medium supplemented with 20, 30, 50 and 70 g L^{-1} of sucrose and MS supplemented with 0.0, 0.5, 1.0 and 1.5 mg L^{-1} of GA_3 respectively. Two individual shoots were cultured in each jam jar. Ten replicates were used for each sucrose and GA_3 concentration. The percentage of *in vitro* flowering and fruiting were recorded every week for four weeks.

Morphological study of flower and fruit using light microscope: The structures of the inflorescence and fruit from the *in vitro* plantlet and mother plant were observed immediately after collected fresh from the plants with the aid of light microscope fitted with colored video camera (Olympus BX 50 with JVC-K-F 55B Camera). Photographs were taken for each of the flower and fruit selected at random from the *in vitro* plantlet and mother plants.

RESULTS AND DISCUSSION

Establishment of *in vitro* plantlets and induction of multiple shoot formation of *Phyllanthus niruri*: The surface-sterilization protocol used for stem nodal segments of *P. niruri* enabled 90-100% of them remained

aseptic and survived on basic Murashige and Skoog (1962) medium (MS) without any plant growth regulators. When the aseptic nodal segments were cultured on MS medium supplemented with 2.0 mg L^{-1} BA, 91.7% of them produced multiple shoots. The percentage of nodal segments formed multiple shoots decreased when the amount of BA added into the MS medium was increased. When the nodal segments were cultured on MS medium added with 2-6 mg L^{-1} kinetin, only 22-33% of them formed multiple shoots. Multiple shoots were not induced from the nodal segments with the addition of 8 mg L^{-1} or higher concentration of kinetin into the culture medium. Most or all (97-100%) of the nodal segments produced roots on MS medium without BA or kinetin (Table 1).

The addition of KN (0.5, 1.0 mg L^{-1}) into MS medium with the presence of BA (0.5, 1.0, 1.5, 2.0 and 2.5 mg L^{-1}) induced multiple shoot formation in all (100%) the nodal segments. None of the nodal segments produced multiple shoots when they were cultured on MS medium supplemented with 0.5 or 1.0 mg L^{-1} KN without BA. But Catapan *et al.* (2002) reported that *P. urinaria* could produce 16-20 shoots per explant with the presence of kinetin in the culture medium. In this study, MS medium supplemented with BA (0.5-2.5 mg L^{-1}) without KN could still induce the nodal segments to produce multiple shoots. MS medium supplemented with 1.0 mg L^{-1} BA without kinetin induced the highest number of multiple shoot formation (6.6 shoots per explant) but the number of shoots induced was not significantly different with the presence of the 0.5 mg L^{-1} kinetin in MS medium plus 1.0 mg L^{-1} BA. The number of shoots induced from each nodal segment decreased as the concentration of BA and kinetin increased in the MS medium (Table 2). Hence, MS supplemented with 1.0 mg L^{-1} BA was chosen as the best medium for induction of multiple shoot from nodal segments of *P. niruri*. Rajasubramaniam and Saradhi (1997) had also proven that BA was effective for shoot multiplication of *Phyllanthus fraternus*. Catapan *et al.* (2000) reported that culture medium supplemented with BA, kinetin and 2iP induced a maximum of 4-5 shoots from each explant of *P. caroniensis*. In our study, it was found that 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.

Table 1: Effect of MS medium supplemented with BA or Kinetin (0-10 mg L^{-1}) on multiple shoot formation from nodal segments of *P. niruri*

Plant growth regulator	Amount of plant growth regulators (mg L^{-1})	Nodal segments formed multiple shoots (%)	*In vitro* shoots produce roots (%)
BA	0	0.0	100.0
	2	91.7	0.0
	4	41.7	0.0
	6	16.7	0.0
	8	4.2	0.0
	10	0.0	0.0
Kinetin	0	0.0	97.2
	2	22.2	0.0
	4	33.3	0.0
	6	22.2	0.0
	8	0.0	0.0
	10	0.0	0.0

Table 2: Effect of MS medium supplemented with BA (0-2.5 mg L^{-1}) or Kinetin (0-1.0 mg L^{-1}) on multiple shoot formation from nodal segments of *P. niruri*

MS + BA + KN		
BA (mg L^{-1})	KN (mg L^{-1})	No. of shoots produced from each nodal segment±SD
0.0	0.0	1.0±0.1hi
0.0	0.5	1.0±0.2i
0.0	1.0	1.2±0.2g-i
0.5	0.0	3.9±1.5c-f
0.5	0.5	4.5±1.6b-d
0.5	1.0	5.3±1.0ab
1.0	0.0	6.6±1.4a
1.0	0.5	6.3±0.8a
1.0	1.0	5.0±1.5ab
1.5	0.0	4.5±1.9b-d
1.5	0.5	3.7±1.1c-f
1.5	1.0	4.3±2.0c-e
2.0	0.0	2.7±1.7d-i
2.0	0.5	2.6±1.2e-i
2.0	1.0	2.8±1.2d-h
2.5	0.0	2.0±1.2f-i
2.5	0.5	2.9±1.7d-g
2.5	1.0	2.1±1.0f-i

Mean values with different letter (s) are not significantly different at p = 0.05

Effect of subculture on multiple shoot formation and *in vitro* flowering and fruiting: Subculturing was carried out to maintain the *P. niruri* cultures and to increase the number of plantlets. The nodal segments were subcultured on the proliferation medium, MS medium supplemented with 1.0 mg L^{-1} BA. A high number of multiple shoots (11.8 shoots per explant) was recorded at the first subculture cycle when they were cultured on the proliferation medium. MS medium without plant growth regulator could only induce the formation of 3.3 shoots from each explant at the first subculture cycle. The number of shoots formed from each explant on proliferation medium decreased in the subsequent subculture cycles (Fig. 1). Most of the multiple shoots that remained on proliferation medium became vitrified after seven subculture cycles. Vitrification of shoots could be due to the long exposure of the plantlets to the presence of BA in the culture medium. This was because the addition of BA, a cytokinin, in a culture medium could increase the cell water potential and resulted in vitrification of a plantlet (George and Sherrington, 1984). The same phenomenon was observed in *Spilanthes acmella* L.

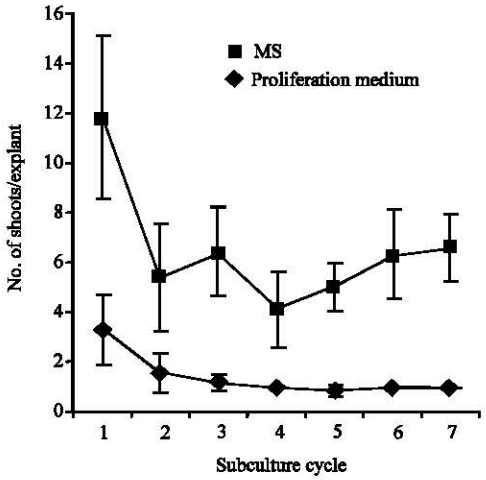

Fig. 1: Effect of subculture on multiple shoots formation of *Phyllanthus niruri* on MS and proliferation medium at 4 weeks culture interval

Roots were produced on 25% of the shoot microcuttings even after the first subculture cycle on MS medium without plant growth regulators. The percentage of microcuttings produced roots increased at each subculture cycle and almost all the microcuttings (97%) produced roots at the third subculture cycles and all of them produced roots on the 5th subculture cycle. *In vitro* flowering and fruiting were observed on 33% of the *in vitro* plantlets cultured on MS medium without any growth regulator on the first subculture cycle. Almost all the plantlets produced flowers and fruits after the second subculture cycle and continued to produce flowers and fruits even at the seventh subculture cycles (Fig. 2).

Effect of sucrose and GA3 on *in vitro* flowering and fruiting: MS medium supplemented with 30 g L^{-1} sucrose was found to promote healthy plantlet with 60% of the plantlets produced *in vitro* flowers after 2 weeks of culture and all the *in vitro* plantlets produced flowers on the 4th week. Thirty five percent of the *in vitro* plantlets produced fruits on the 2nd week and 80% of them produced fruits when they were culture for 4 weeks on the same medium. When the amount of sucrose supplemented in the MS medium was increased to 50 and 70 g L^{-1}, abnormal plantlets were formed and *in vitro* flowering and fruiting were inhibited. Less than 20% of the plantlets produced flowers even after 4 weeks of culture on MS medium supplemented with 20 g L of sucrose and these flowers eventually did not bear fruits (Fig. 3 and 4).

P. niruri in vitro plantlets derived from the nodal segments cultured on MS medium supplemented with GA_3 (0.5-1.5 mg L^{-1}) started to flower as early as one week

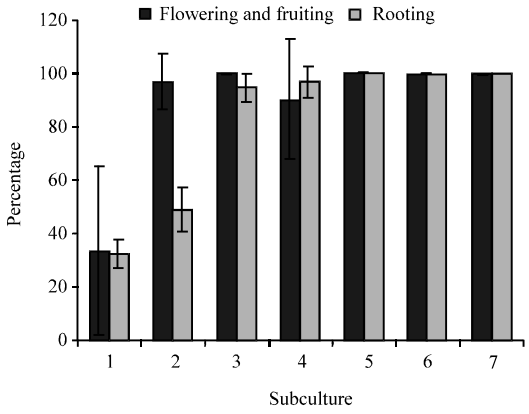

Fig. 2: Effect of subculturing on *in vitro* flowering and fruiting of Phyllanthus niruri on basic MS medium

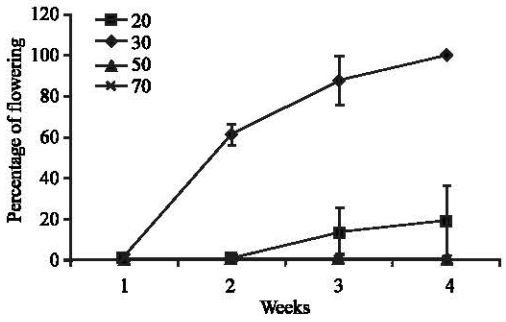

Fig. 3: Effect of sucrose supplemented into the MS basic medium on *in vitro* flowering of *Phyllanthus niruri*

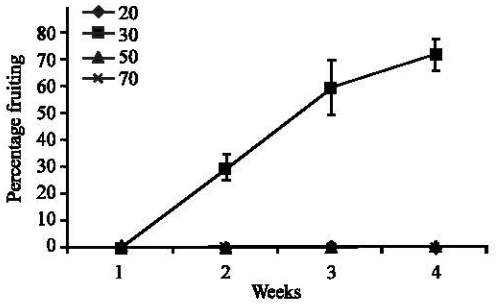

Fig. 4: Effect of sucrose supplemented into the MS basic medium on *in vitro* fruiting of *Phyllanthus niruri*

after culture. After 2 weeks of culture, 96.7% of the *in vitro* plantlets produced flowers on MS medium supplemented with 0.5 mg L^{-1} GA_3 while only 53.3-63.3% of the plantlets produced flowers when they were cultured on basic MS medium without plant growth regulator or MS medium supplemented with 1.0 or 1.5 mg L^{-1} GA_3. Between 80 and 100% of the plantlets produced flowers after 4 weeks of culture on MS medium with or without the addition of any of the GA_3 (Fig. 5). However, *in vitro* fruiting only occurred on the plantlets that were cultured

on MS medium without GA_3 with 35% of them produced fruits on the 2nd week. The number of plantlets produced fruits became double after 4 weeks of culture (Fig. 6). This study showed that GA_3 could shorten the period of flowering but inhibited fruiting. Observation from light microscope showed that the flower (Fig. 7) and fruit (Fig. 8) morphology of the mother plant and the

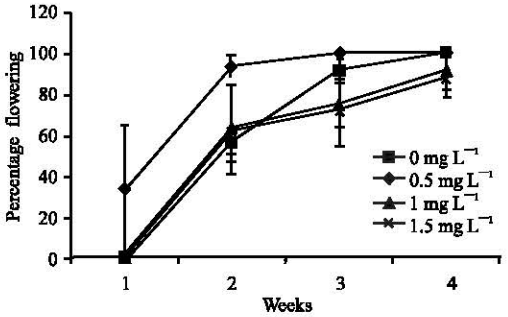

Fig. 5: Effect of GA3 supplemented into basic MS medium on *in vitro* flowering of *Phyllanthus niruri*

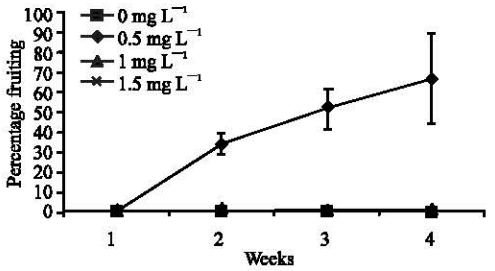

Fig. 6: Effect of GA_3 supplemented into basic MS medium on *in vitro* fruiting of *Phyllanthus niruri*

Fig. 7: The Flower of *Phyllanthus niruri* A, *in vitro* plant B, mother plant

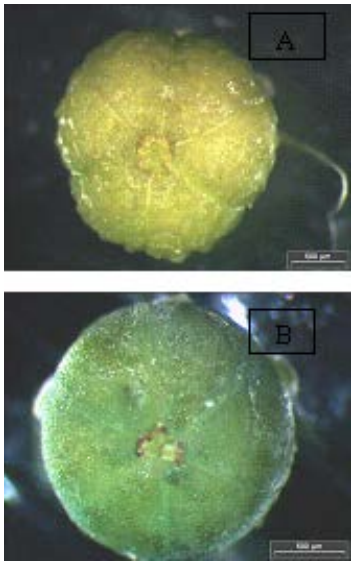

Fig. 8: The fruit of *Phyllanthus niruri* A, mother plant B, *in vitro* plant

in vitro plantlet were similar except the flower and the fruit of the mother plant were bigger. Rajasubramaniam and Saradhi (1997) reported that *in vitro* plantlets of *P. frartenus* only flowered after *ex vitro* transfer. Catapan *et al.* (2000) also reported the same phenomena occurred in *P. caroliniensis*. Our study was the first to report the success of inducing *in vitro* flowering and fruiting for *P. niruri*. Therefore *P. niruri* could be a very good model for further investigation on regulation of *in vitro* flowering and fruiting. Many investigators incorporate growth regulators into the culture medium for initiating *in vitro* flowering and fruiting. However, our study showed that basic MS medium without any plant growth regulator could induce *in vitro* flowering and fruiting for *P. niruri*.

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