

*Full Length Research Paper*

# Micropropagation of *Plumbago zeylanica* L. (Plumbaginaceae) in Ibadan, Southwestern, Nigeria

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Accepted 5 January, 2010

The root of *Plumbago zeylanica* is widely used by traditional Yoruba healers in Ibadan, Southwestern Nigeria, in the management and treatment of various infections and diseases. The plant is mainly harvested from the wild. The indiscriminate collection of the roots and non-cultivation of the plant has many implications for biodiversity. The plant is becoming scarce due to increasing demand for its use in ethnobotanical practice. These factors necessitate the study of micropropagation of *P. zeylanica* via tissue culture to ensure its sustainability. The embryos and nodal cuttings of *P. zeylanica* were used to evaluate the effect of culture media and growth regulators on the *in-vitro* shoot production and growth. The embryos were significantly viable on Nitrogen - Phosphorus Potassium (NPK) basal media. The highest multiplication rate of the explants was obtained using Murashige and Skoog (MS) medium supplemented with naphthalene acetic acid (NAA) (0.01 - 0.05 mg/l) and benzyl amino purine (BAP) (2.0 - 4.5 mg/l). The single nodes of established plantlets were repeatedly sub-cultured on MS-NAA-BAP media at 4 week intervals for six months; the media enabled multiple shooting, rooting and mass multiplications without decline. The phytochemicals found in the *in-vitro* plantlets were saponins and tannins. The rooted plants which were successfully acclimatized in a green-house, then transferred to soil, showed a normal growth.

**Key words:** Micropropagation, natural media, phytochemical screening, *Plumbago zeylanica* L.

## INTRODUCTION

*Plumbago zeylanica* is a spreading or subscandent, herbaceous, suffrutescent plant, 1 - 2 m in length. The root contains an acrid crystalline, principle called 'Plumbagin'. Plumbagin is present in all the variety of plumbago to a maximum of about 0.91%. This active chemical is vesicant, has properties of vitamin K, and is antibiotic on several human pathogens (Burkill, 1985). The plant is continuously collected from the wild and over exploited. In view of this, there is an urgent need for alternative means of propagation with a view to preventing the plant from extinction.

Biotechnological tools are important for multiplication and genetic enhancement of medicinal plants by adopting techniques such as *in-vitro* regeneration and genetic transformations. It can also be harnessed for production of secondary metabolites using plants as bioreactors

(Leena et al., 2003). Tissue culture offers means not only for rapid and mass multiplication of existing stock of germplasm but also for conservation of important, elite and endangered plants (Razdan, 2003).

Although there is dearth of information on the micropropagation of *P. zeylanica* in Nigeria, a survey of literature indicates that many investigators have studied *in-vitro* propagation of medicinal plants. Mohammed et al. (2006) reported the highest amount of *Stevia rebaudiana* Bert. Callus in MS supplemented with 3.0 mg/L 2,4-D. Sang et al. (2009) obtained the highest number of shoots of *Rehmannia glutinosa* L. on MS medium containing 1 mg/l TDZ. Nerman et al. (2009) investigated the micropropagation of endangered Sinai hawthorn *Crataegus sinaica* Boiss. Rapid shoot multiplication of *Nyctanthes arborescens* L. was achieved from axillary meristems on MS supplemented with 1.0 - 1.5 mg/l BA, 50 mg/l adinine sulfate and 3% (m/v) sucrose (Rout et al., 2007). Bohidar et al. (2008) also reported the effect of plant growth regulators on micropropagation of *Ruta graveolens* L.

This work studied the micropropagation of *P. zeylanica*

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**Table 1.** The constituents of the media used for the micropropagation of *P. zeylanica*.

Medium code	MS	NaH <sub>2</sub> P0 <sub>4</sub> .H <sub>3</sub> O (mg/ l)	2,4-D (mg/ l)	NAA (mg/ l)	BAP (mg/ l)	NPK (mg/ l)	Citrus sinensis juice (ml/ l)	Sugar (mg/ l)
A (control)	+	-	-	-	-	-	-	30
B	+	170	-	-	-	-	-	30
C	+	-	0.5	-	-	-	-	30
D	+	-	-	0.01	0.05	-	-	30
E	+	-	-	0.02	30	-	-	30
G1	-	-	-	-	-	10	20	30
G2	-	-	-	-	-	10	30	30
G3	-	-	-	-	-	10	40	30
G4	-	-	-	-	-	15	20	30
G5	-	-	-	-	-	15	30	30
G6	-	-	-	-	-	15	40	30
G7	-	-	-	-	-	20	20	30
G8	-	-	-	-	-	20	30	30
G9	-	-	-	-	-	20	40	30
M1	+	-	-	0.01	2.0	-	-	30
M2	+	-	-	0.02	2.5	-	-	30
M3	+	-	-	0.03	3.0	-	-	30
M4	+	-	-	0.04	3.5	-	-	30
M5	+	-	-	0.05	4.5	-	-	30

+ = Present; - = Absent; MS = Murashige and Skoog salt base (Murashige and Skoog, 1962); NaH<sub>2</sub>P0<sub>4</sub>.H<sub>2</sub>O = Sodium dihydrogen ortho phosphate; NAA = 1-napthalene acetic acid; BAP = Benzyl aminopurine; NPK = Nitrogen – Phosphorus – Potassium; 2, 4-D = 2, 4-dichlorophenoxyacetic

to ensure its availability for frequent collection and use in ethnobotanical practice and research.

## MATERIALS AND METHODS

### Micropropagation

The experiment was performed in the tissue culture laboratory of National Center for Genetic Resources and Biotechnology, Ibadan, Nigeria in 2008. Fresh and healthy plant materials of *P. zeylanica* collected during the raining season from University of Ibadan campus were used as explants in the experiments. Explants include embryos and nodal cuttings of the plant. The constituents of the media used are as shown in Table 1. The pH of each medium was adjusted to 5.7 prior to the addition of 0.7% agar (Difco, USA). Media and instruments were sterilized by autoclaving for 15 - 30 min at 121°C (1 atm).

Aseptic transfers were performed in a laminar flow hood (Bottino, 1981). The explants were washed thoroughly with liquid detergents and rinsed properly with sterile distilled water. They were washed with 70% ethanol solution for 5 min subsequently surface-sterilized with 10% sodium hypochlorite for 20 min and 5% sodium hypochlorite for 10 min. The explants were properly rinsed with sterile distilled water 3 times, leaving the explants in the third rinse. The seed coats were removed with sterile forceps in sterile Petri dishes. The seeds were opened to remove the embryo from the seeds. The embryo/ nodal cuttings were aseptically implanted vertically on the sterile culture medium in glass tubes (20 x 150 mm) using sterile forceps. After inoculation in the different nutrient media, the glass-tubes were sealed with paraffin wax and labelled. The cultures were incubated at 27 ± 1°C with a photoperiod of 16 h at an intensity of

of 10 - 20 µmol m<sup>-2</sup> S<sup>-1</sup> (Phillips ThD 36W/84) in the growth room for a period of 30 - 90 days. Each treatment was replicated at least three times.

Plantlets were repeatedly sub-cultured on MS supplemented with NAA and BAP media at 4-week intervals for six months. Rooted plantlets in MS supplemented with NAA and BAP media were washed in tap-water and transferred to plastic chambers containing sterile mixture of soil and sand (1:1). The plastic chambers were covered with a plastic cap that was gradually opened during the acclimatization period of fifteen days. Successfully acclimatized plants were transferred to the greenhouse and then to outdoor conditions during the raining season.

The parameters evaluated were: the percentage viability, the number of shoots per explants, shoots length, the number of leaves, leaf length, and the percentage of callus formation.

### Phytochemical screening

Phytochemical screening of powdered field plants (roots) and the *in vitro* plantlets were carried out using standard procedures. The plant materials were tested for the presence of alkaloids, tannins, saponins, anthraquinones and cardenolides in the laboratory of the Department of Pharmacognosy, University of Ibadan, Ibadan, Nigeria.

### Statistical analysis

Analysis of variance and comparison of means were carried out on all data using Statistical Analysis System (SAS). Differences between means were assessed for significance at P ≤ 0.05 by

**Table 2.** Leaf formation and growth of *Plumbago zeylanica* embryos in MS-basal media.

Media	% viability	No. of leaf	Leaf length	% callus formation
A	10	*0.50 ± 0.70 <sup>a</sup>	0.50 ± 0.70 <sup>a</sup>	0
B	20	1.50 ± 0.70 <sup>ab</sup>	4.00 ± 1.41 <sup>a</sup>	0
C	30	2.00 ± 0.00 <sup>bc</sup>	17.50 ± 3.53 <sup>b</sup>	30
D	60	3.00 ± 0.00 <sup>c</sup>	21.00 ± 000 <sup>b</sup>	0
E	30	1.00 ± 0.00 <sup>ab</sup>	3.50 ± 2.12 <sup>a</sup>	0

Evaluation was made after 30 days in culture.

\*Mean of three readings ± standard deviation.

\*Values in the same column followed by the same letter are not significantly different ( $p > 0.05$ ) from each other. They differ significantly ( $p \leq 0.05$ ) with values that do not share a similar letter.

**Table 3.** Shoot production and growth of *Plumbago zeylanica* embryos in NPK- basal media

Media	% viability	No of shoot per explant	Shoot length (mm)	No of leaf primodial	% callus formation
G1	60	*0.00 ± 0.00 <sup>a</sup>	3.00 ± 0.60 <sup>a</sup>	0.25 ± 0.18 <sup>a</sup>	0
G2	100	1.00 ± 0.81 <sup>abc</sup>	1.80 ± 0.40 <sup>b</sup>	4.25 ± 2.06 <sup>bc</sup>	0
G3	100	0.25 ± 0.50 <sup>ab</sup>	12.25 ± 0.64 <sup>ab</sup>	2.25 ± 0.50 <sup>ab</sup>	40
G4	100	0.00 ± 0.00 <sup>a</sup>	4.50 ± 0.10 <sup>a</sup>	2.00 ± 0.00 <sup>ab</sup>	30
G5	100	2.50 ± 1.29 <sup>cd</sup>	21.50 ± 0.59 <sup>bc</sup>	4.25 ± 2.62 <sup>bc</sup>	30
G6	100	0.00 ± 0.00 <sup>a</sup>	16.25 ± 0.47 <sup>b</sup>	2.00 ± 0.00 <sup>ab</sup>	0
G7	100	3.50 ± 2.38 <sup>d</sup>	28.25 ± 1.27 <sup>c</sup>	7.00 ± 4.25 <sup>c</sup>	0
G8	100	1.75 ± 0.95 <sup>bc</sup>	15.75 ± 0.55 <sup>b</sup>	4.50 ± 1.73 <sup>bc</sup>	60
G9	100	0.00 ± 0.00 <sup>a</sup>	3.25 ± 0.12 <sup>a</sup>	2.0 ± 0.00 <sup>ab</sup>	30

Evaluation was made after 30 days in culture.

\*Mean of three readings ± standard deviation.

\*Values in the same column followed by the same letter are not significantly different ( $p > 0.05$ ) from each other. They differ significantly ( $p \leq 0.05$ ) with values that do not share a similar letter.

**Table 4.** Shoot production and growth of *Plumbago zeylanica* embryos in MS-BAP-NAA media.

Media	% Viability	No. of shot per explant	Shoot length (mm)	No. of leaf primodial	% Callus
M1	80	*2.00 ± 0.81 <sup>b</sup>	22.50 ± 0.86 <sup>c</sup>	4.50 ± 2.51 <sup>a</sup>	0
M2	80	1.25 ± 0.50 <sup>ab</sup>	23.00 ± 1.16 <sup>c</sup>	4.00 ± 3.36 <sup>a</sup>	0
M3	40	1.00 ± 1.15 <sup>ab</sup>	12.50 ± 1.50 <sup>ab</sup>	2.25 ± 2.62 <sup>a</sup>	0
M4	20	0.25 ± 0.50 <sup>a</sup>	4.50 ± 0.71 <sup>a</sup>	0.50 ± 1.00 <sup>a</sup>	0
M5	80	0.75 ± 0.95 <sup>ab</sup>	1.52 ± 0.80 <sup>ab</sup>	3.00 ± 2.16 <sup>a</sup>	0

Evaluation was made after 30 days in culture. \*Mean of three readings ± standard deviation.

\*Values in the same column followed by the same letter are not significantly different ( $p > 0.05$ ) from each other. They differ significantly ( $p \leq 0.05$ ) with values that do not share a similar letter.

Duncan's multiple range test (DMRT).

## RESULTS

The growth of *Plumbago zeylanica* embryos in MS- basal media (A-E) and NPK basal media (G1 - G9) are as shown in Tables 2 and 3 respectively. Tables 4 and 5 show the growth and shoot production of *P. zeylanica* embryos and nodal cuttings in MS supplemented with

BAP and NAA. The phytochemical properties of the field plant and *in-vitro* cultured plantlets of *P. zeylanica* are shown in Table 6. Plates 1 and 2 show the growth of *P. zeylanica* in various media.

## DISCUSSION

The initial experiments on the growth of *P. zeylanica* (Table 2) in MS- basal-media showed that medium D (MS

**Table 5.** Shoot production and growth of *P. zeylanica* (nodal cuttings) in MS-BAP-NAA media

Media	% Viability	No of shoot per explant	Shoot length (mm)	No of leaf primodial	% Callus
M1	15	*1.25 ± 0.50 <sup>a</sup>	21.20 ± 0.62 <sup>a</sup>	2.50 ± 1.29 <sup>a</sup>	0
M2	40	2.00 ± 0.00 <sup>ab</sup>	40.25 ± 0.05 <sup>b</sup>	7.50 ± 2.64 <sup>b</sup>	0
M3	47	2.00 ± 0.00 <sup>ab</sup>	35.50 ± 0.31 <sup>b</sup>	6.75 ± 0.50 <sup>b</sup>	0
M4	53	2.25 ± 0.05 <sup>ab</sup>	33.75 ± 0.47 <sup>ab</sup>	6.75 ± 1.25 <sup>b</sup>	0
M5	40	2.75 ± 1.70 <sup>b</sup>	45.00 ± 1.75 <sup>b</sup>	7.75 ± 4.57 <sup>b</sup>	0

Evaluation was made after 30 days in culture.

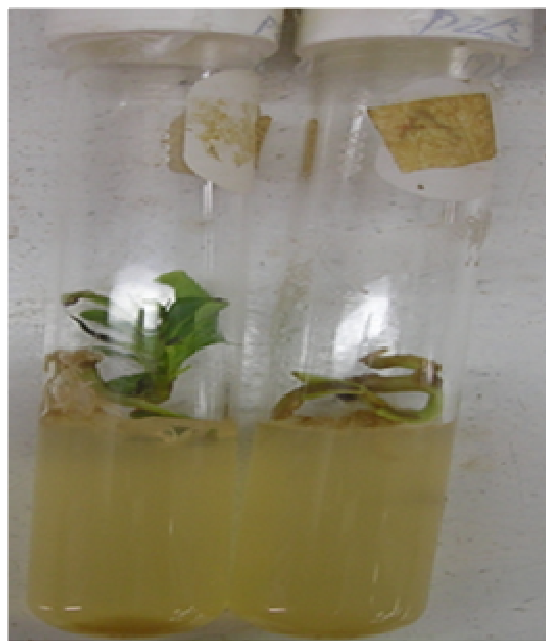
\*Mean of three readings ± standard deviation.

\*Values in the same column followed by the same letter are not significantly different ( $p > 0.05$ ) from each other. They differ significantly ( $p \leq 0.05$ ) with values that do not share a similar letter.

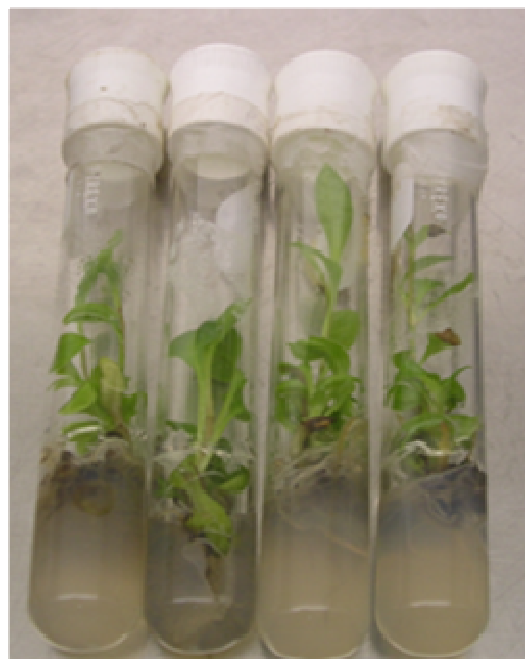
**Table 6.** Phytochemical properties of the field plant and *in-vitro* cultured plantlets

Natural product	<i>Plumbago zeylanica</i>	
	Field plant(root)	In-vitro plantlets
Alkaloids	+	-
Tannins	+	+
Saponins	+	+
Anthraquinones	+	-
Cardenolides	+	-

- = negative reaction. + = positive reaction.



**Plate 1.** Shoot production and growth of *Plumbago zeylanica* embryos in NPK-basal medium G5 after 30 days in culture.



**Plate 2.** Shoot production and growth of *Plumbago zeylanica* (nodal cuttings) in MS-BAP-NAA media. Picture was taken after 90 days in culture.

+ 0.05 mg/l BAP + 0.01 mg NAA) significantly enhanced leaf formation and elongation, whereas the growth was

poor in medium A (control). The present results conform to the results of other authors, George (1993) reported

that the combination of cytokinins and auxins stimulate the *in-vitro* multiplication and growth of shoots of several plant species. Sang et al. (2009) reported that auxin treatments in basal medium marginally increased the shoot regeneration and growth rate of *Rehmannia glutinosa* leaf culture. A common feature of auxins is the property of inducing cell division. In nature the hormones of this group are involved with such activities as elongation of stem, internodes, tropism, apical dominance, abscission and rooting. Cytokinins are adenine derivatives which are mainly concerned with cell division, modification of apical dominance and shoot differentiation in the tissue culture (Razdan, 2003). Rout et al. (2007) reported optimal rooting and growth of microshoots of *Nyctanthes arbotristis* in medium containing 0.25 mg/l IBA, 0.10 mg/l IAA with 2% sucrose after 14 days of culture. The results in Table 2 led to further cultures in MS supplemented with NAA and BAP with varied concentrations of auxin and cytokinin in media M1 - M5.

In Table 3, media G2 - G9 gave 100% viability of explants. Medium G7 supported shoot production and elongation, whereas medium G8 gave the highest callus of 60%. The results showed that tissue culture could be established on natural media. The natural materials are cheap and will be readily available for researchers. Only 2 out of 9 NPK-basal media were significantly effective on the growth of *P. zeylanica*, the right combination and adequate proportion of materials (base, vitamin and sucrose) in media formula are required for effective *in-vitro* propagation of explants.

The embryos (20 - 80%) were more viable than the nodal cuttings (15 - 53%) in MS supplemented with NAA and BAP media (Tables 4 and 5), the nodal cuttings gave multiple shoots, longer shoots and many leaves compared to the embryos. The nodal cuttings of *P. zeylanica* are better as explants for regeneration and mass propagation, but effort must be made to ensure higher viability.

As shown Table 6, the *in-vitro* cultured plantlets of *P. zeylanica* contained saponins and tannins. The field plant contained alkaloids, tannins, saponins, anthraquinones and cardenolides. Although there are more active compounds in the field plants than the plantlets, with maturity there may be more active compounds in the *in-vitro* cultured plantlets. Considering economical and pharmacological importance of secondary metabolites, industries are deeply interested in utilizing plant tissue culture technology for large-scale production of these substances (Misawa, 1994).

## Conclusion

*P. zeylanica* has been collected by the indigenous people without replanting; this study provides a rapid method of propagation to supplement natural propagation and prevent the plant from extinction due to frequent collection. The study produced an insight into the use of natural vitamin (*Citrus sinensis* juice) in media formula.

## ACKNOWLEDGEMENT

The authors are grateful for the scientific assistance provided by the entire staff of the Tissue Culture Laboratory, National Center for Genetic Resources and Biotechnology, Ibadan, Nigeria.

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