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Effects of various plant growth regulators and ampicillin activity on *in vitro* propagation of *Plumbago zeylanica*: An important medicinal plant of south India-Kolli hills

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Abstract
Plumbago zeylanica belongs to the family Plumbaginaceae grows widely in India and has many medicinal values. The aim of this study is to produce one of the valuable medicinal plants (*Plumbago zeylanica*) in large quantities through plant tissue culture technique. *In-vitro* propagation was attempted through micro propagation and direct organogenesis. In Micro propagation, Nodal explants were used and micro propagation was achieved using BAP and KIN with various concentrations. Nodal explants were produced at a concentration of 3 mg/l BAP with 10 % of Coconut Water and 5 mg/l of Ampicillin. With reference to direct organogenesis, 4 mg/l BAP produced maximum results from Internodal explants. The final survival rate of hardening percentage was ranged from 85 - 90 obtained from various explants. The highest percentage of the survival was 90% were observed in nodal explants after four weeks.

Keywords: *Plumbago zeylanica*, *micro* propagation, *In-vitro*, nodal explants, ampicillin

1. Introduction
Over the past twenty years, interest in medicinal plants has grown enormously from the use of herbal products as natural cosmetics and for self-medication for their biological effects in human beings [1]. Beyond this pharmaceutical approach to plants, there is a wide tendency to utilize herbal products to supplement the diet and to improve the quality of life and preventing the diseases in elderly people [2]. *Plumbago zeylanica* is a wild herb which is used as a medicine for much disease. *Plumbago* consists of 12 species of flowering plants in the family Plumbaginaceae, native to warm temperate to tropical regions of the world [3]. It has various pharmacological activities such as anti-malarial, anti-plasmodial, anti-microbial, anti-fungal, anti-inflammatory and anti-hyperglycemic [4]. The roots of *P. zeylanica*, have been used extensively in China and other Asian countries for the treatment of cancer, rheumatoid arthritis and dysmenorrhea [5].
Plant tissue culture is based on the principle of totipotency [6]. Plant tissue culture technique allows mass multiplication and propagation under aseptic conditions [7]. Tissue culture technique helps us to obtain mass number of plantlets within short span of time [8]. Micro propagation has many advantages over conventional methods of vegetative propagation [9]. The most significant feature of micro propagation process is the production of large number of healthy and disease free plants in relatively short span of time and also has the potential of producing propagules throughout the year [10, 11]. Micro propagation from nodal segments was considered to be one of the most promising methods for mass multiplication of selected medicinal plant [12]. Auxins and cytokinins were the hormones used in the experiments [13, 14]. 2, 4 – D, IAA, NAA, and IBA, were used as auxins. BAP and kinetin were used as cytokinins [15]. The various concentrations BAP and KIN combination with Coconut water and ampicillin were used [16, 17].
Antibiotic compounds have been used to eliminate or prevent microbial growth in *in vitro* propagation studies [18]. Generally antimicrobial compounds are frequently used in *in vitro* propagation techniques to obtain contaminated free elite clones [19]. The contaminants are originating from explants or arise during inoculation and incubation process. The ultimate aim of using Ampicillin was not only to eliminate microbial contamination and to enhance the high frequency of the rapid regeneration of *Plumbago zeylanica*, an important medicinal plant [20]. Other than micro propagation, direct organogenesis method was used to produce propagules, the propagules are produced without undergoing callus formation [21]. The aim of this study is to produce large number *Plumbago zeylanica* plantlets from Nodal and Internodal explants.

2. Materials and Methods
2.1 Collection of plant materials
The explants of *Plumbago zeylanica* were collected from hills of kollimalai. The explants were kept under running tap water for half an hour in order to remove the soil debris from the surface of the explant.

2.2 Sterilization of plant material
The explants were surface sterilized with teepol and bavistin for 15 minutes and then the explants are surface sterilized with 0.1% of mercuric chloride for 3 minutes. Finally explants were washed for 4 to 5 times with sterile distilled water.

2.3 Micro propagation from nodal explants
The nodal part of *Plumbago zeylanica* supplemented with different concentrations of Growth hormones like BAP (0.2-1.0mg/L) NAA (0.2-1.0mg/L) BAP+NAA (0.2-1.0mg/L). The nodal explants of *P. zeylanica* in MS medium supplemented with 1.0mg/L BA and 1.0 mg/L GA_3 was

achieved after 1 week of incubation. Nodal explants were cultured in modified MS ($NaNO_3$ instead of NH_4NO_3) media containing BAP (benzyl amino purine) (1.0 mg/L, 1.5 mg/L and 2.0 mg/L) The sterilized explants were cultured on MS basal medium (Murashige & Skoog, 1962) supplemented with 3% of sucrose, 6% of agar and growth hormones BA (0.5 -1.5 mg/L) and GA_3 (05-1.5 mg/L). It is then transferred to a half strength rooting media supplemented with BAP (0.5-1.5 mg/L) and IAA (0.5-1.5 mg/L).

2.4 Direct organogenesis from inter-nodal explants

The inter-nodal explants were tested using MS medium supplemented with IAA – GA_3 and NAA - GA_3 in very low concentrations (0.02 mg/L, respectively). Then for rooting the medium was supplemented with IAA and IBA. The culture were incubated at 25±2°C under 2000 lux light intensity provided by fluorescent lamp for 16 hours and the pH was adjusted to 5.7±0.1.

2.5 Hardening

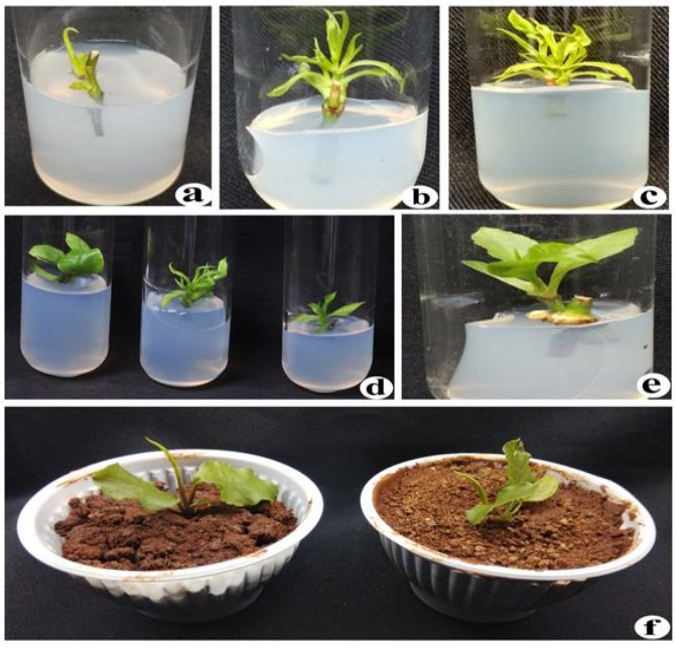
After 12 hours of treatment, the *in vitro* propagated shoots were placed in small poly bags or plastic cups containing mixture of Red soil, Vermiculate and Coconut husk in the ratio of 1:1:1 combination to facilitate the root formation. After the development of shoots and roots, the plantlets were transferred to the field condition.

3. Result and discussion

Micro propagation was carried with Nodal explants and indirect organogenesis was achieved from Internodal explants. Different concentration of BAP and KIN combinations with 10% of Coconut water and Ampicillin antibiotics were used for *in vitro* shoot regeneration from Node and Indernodal explants. The nodal explants produced the highest number of shoot multiplication that is 12 shoots per explant with 6.0 cm mean shoot length with very less level of microbial contamination (Table - 1and Fig - 1).

Table 1: Effect of various concentrations of Cytokinins with 10% Coconut Water and 5 mg/l of Ampicillin on Shoot differentiation from Nodal Explant of *Plumbago zeylanica*

Hormone Concentration mg/l	No. of Explants Cultured	No. of Explants Responded	Percentage of Response	No. Shoots / Explant Mean ± SE	Mean Shoot Length (cm)
BAP + Coconut Water					
Control	20	1	5	1.0 ± 0.14	1.2
1.0 + 10%	20	10	50	4.4 ± 0.54	4.2
2.0 + 10%	20	16	80	9.3 ± 0.40	5.2
3.0 + 10%	20	19	95	12.6 ± 0.34	6.0
4.0 + 10%	20	15	75	8.7 ± 0.55	4.4
5.0 + 10%	20	11	55	3.3 ± 0.14	3.6
KIN + Coconut Water					
Control	-	-	-	-	-
1.0 + 10%	20	3	15	2.0 ± 0.18	2.5
1.5 + 10%	20	11	55	4.5 ± 0.23	3.0
2.0 + 10%	20	16	80	7.4 ± 0.54	4.7
2.5 + 10%	20	12	60	5.0 ± 0.15	3.3
3.0 + 10%	20	8	40	2.5 ± 0.22	2.6



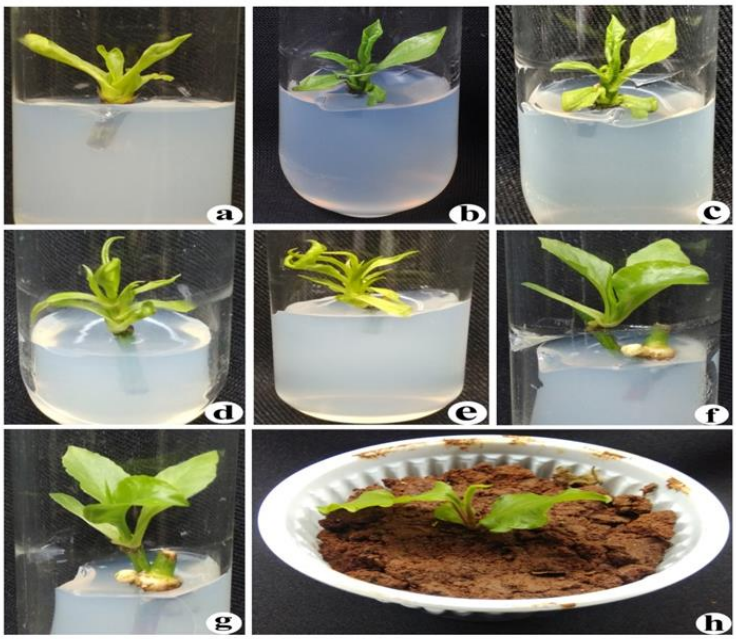
a. Microshoot initiation
b & c. High frequency of Multiple shoot formation
d & e. Various stages of shoot elongation
f. *Ex vitro* Hardened plantlet

Fig 1: Different stages of micropropagation from nodal explants

Considering the KIN the optimum hormone concentration was 2.0 mg/l KIN with 10 % of Coconut Water and 5 mg/l Ampicillin antibiotic in which the response was 80 % and the average number of micro-shoots was 7 shoots per explant with 4.7 cm mean shoot length. When comparing BAP and KIN, BAP is more reliable for high frequency multiple shoots production from nodal explants. Similar results were also observed in *Phalaenopsis (L.)* at 5mg/l BAP utilized for maximum shoot production. The highest number of shoots (55.3) with 100% response was obtained from nodal explants of *Simarouba glauca* on MS medium fortified with 3 mg/l BAP. Direct Organogenesis of *Plumbago zeylanica* was also tried with Internodal explants. The observations are expressed in Table- 2 and various stages are shown in Figure - 2).

Table 2: Effect of various concentrations of Cytokinins with 10% Coconut Water and 5 mg/l of Ampicillin on Shoot differentiation from Internodal Explant of *Plumbago zeylanica*

Hormone Concentration mg/l	No. of Explants Cultured	No. of Explants Responded	Percentage of Response	No. Shoots / Explant Mean ± SE	Mean Shoot Length (cm)
BAP + Coconut Water					
Control	20	-	-	-	-
1.0 + 10%	20	4	20	1.4 ± 0.43	1.0
2.0 + 10%	20	8	40	2.0 ± 0.30	3.0
3.0 + 10%	20	12	60	3.5 ± 0.62	3.3
4.0 + 10%	20	15	75	5.2 ± 0.12	4.0
5.0 + 10%	20	9	45	2.1 ± 0.10	2.7
KIN + Coconut Water					
Control	-	-	-	-	-
1.0 + 10%	20	2	10	1.0 ± 0.10	1.4
1.5 + 10%	20	5	25	2.0 ± 0.13	2.7
2.0 + 10%	20	9	45	2.4 ± 0.15	3.4
2.5 + 10%	20	10	50	3.4 ± 0.10	3.8
3.0 + 10%	20	8	40	1.2 ± 0.24	3.0



a & b. Microshoot initiation
c, d & e. High frequency of multiple shoot formation
f & g. Well elongated shoots
h. *Ex vitro* Hardened plantlet

Fig 2: Different stages of direct organogenesis from Inter - nodal explants

Maximum response 75% at 4 mg/l BAP with 10% of Coconut water and 5 mg/l Ampicillin. In KIN the maximum response was in 2.5 mg/l with 10% of Coconut water and 5 mg/l Ampicillin. With reference to internodal explant, maximum frequency of multiple shoot production is obtained from internodal explant at 4.0 mg/l BAP. The role of BAP and KIN in multiple shoot production through direct organogenesis has been reported in several other reports. In this present study various concentrations of Ampicillin (2 mg, 5 mg and 10 mg) was used for preventing the microbial contamination on *in vitro* cultures of *Plumbago zeylanica*. Among the various concentrations 5 mg/l Ampicillin provides very less contamination percentage (Node - 5% & Internode - 10%) and not harmful to the cultured cells. Both lower and higher concentrations of Ampicillin shows many harmful symptoms like high level of contamination and burnings on cultured cells respectively (Table - 3 and Figure - 3).

Table 3: Effect of Ampicillin on controlling the microbial contamination in various explants cultures of *Plumbago zeylanica*

Type of Explants	Concentration of Ampicillin (mg)	Number of Tubes Inoculated	Number of Tubes Contaminated	% of Contamination	Observations
Node	2	20	16	80	*High level of Contamination *No further growth
	5	20	1	5	*Very minimum level of Contamination * Healthy Cultures * Highest Results
	10	20	Nil	0	*No further growth *Buds and Shoots shows burning in culture
Internode	2	20	17	85	*High level of Contamination *No further growth
	5	20	2	10	*Very minimum level of Contamination * Healthy Cultures
	10	20	1	5	*No further growth *Buds and Shoots shows burning in culture *Cut end region become completely try

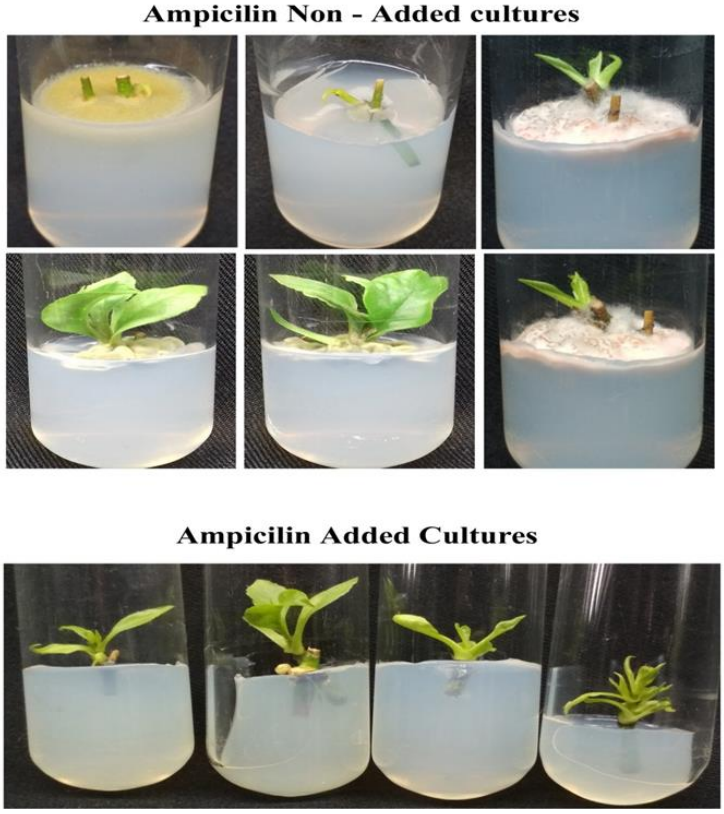


Fig 3: Effect of Ampicilin on various *in vitro* cultures of *Plumbago zeylanica*

After sufficient elongation of *in vitro* propagated shoots i.e. shoots exceeding 5 cm in length were excised and treated with different concentrations of IBA and IAA for 12 hours to induce the *ex vitro* rooting [22, 23]. After the 12 hours treatment of IBA and IAA well elongated *in vitro* propagated shoots were placed in the small poly bags or plastic cups containing mixture of Red soil, Vermiculate and Coconut husk in the ratio of 1:1:1 combination to facilitate the root formation. The potted shoots were fortified with quarter strength of M.S. liquid solution for a week (Table-4).

Table 4: Effect of various Concentrations of IBA and IAA on *Ex vitro* Root Induction

PGR mg/l	% of Root Induction	
	Micropropagation	Direct Organogenesis
	Node	Internode
IBA		
1.0	16	14
2.0	64	50
3.0	75	66
4.0	90	82
5.0	70	54
6.0	58	32

IAA		
1.0	0	0
2.0	12	10
3.0	44	35
4.0	52	40
5.0	60	44
6.0	46	35

The root incitation was visually observed only after 15 days and the full plantlet with sufficient root growth could be produced in 30 days IBA and IAA were used in the range of 1 - 6 mg/l. IBA at 4.0 mg/l was found to be ideal for root induction.

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