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An Improved Micropropagation Protocol for *Plumbago zeylanica* L. An Important Medicinal Plant

¹S. Antony Ceasar, ²M. Ayyanar and ¹S. Ignacimuthu

¹Entomology Research Institute, Nungambakkam, Loyola College, Chennai-600 034, India

²Post Graduate and Research Department of Botany, Pachaiyappa's College, Chennai-600030, India

Corresponding Author: M. Ayyanar, Post Graduate and Research Department of Botany, Pachaiyappa's College, Chennai-600030, India

ABSTRACT

An improved protocol was developed for the micropropagation of *Plumbago zeylanica* L. from nodal explants. The best response of shoot induction was observed on Murashige and Skoog (MS) basal medium supplemented with 1.0 mg L⁻¹ thidiazuron (TDZ) and 1.0 mg L⁻¹ Kinetin (KN). The regenerated shoots rooted best on half strength Murashige and Skoog basal medium containing 1.0 mg L⁻¹ indole-3-butyric acid (IBA). *In vitro* propagated plants were transferred to soil with 90% survival rate. The *in vitro* obtained plants did not show any detectable morphological variation compared to the normal plants. This system will help in the mass production of *Plumbago zeylanica* and other medicinally important plants.

Key words: Micropropagation, thidiazuron, root induction, shoot induction, synergistic effect

INTRODUCTION

Plumbago zeylanica L. (Plumbaginaceae) is an important medicinal shrub grown in many parts of Asia. The root of *P. zeylanica* has been used extensively in many Asian countries for the treatment of many ailments. The roots of *P. zeylanica* contain plumbagin, a natural naphthoquinone possessing antimalarial and antimicrobial activities (Didry *et al.*, 1994). The propagation of *P. zeylanica* through seeds is unpredictable due to poor seed viability, improper germination and higher rate of seedling mortality under field conditions. These characteristics of *P. zeylanica* demand for an efficient micropropagation protocol for the mass production of *P. zeylanica*.

So far only a few tissue culture studies have been reported in *P. zeylanica* such as rapid plant regeneration from callus cultures (Rout *et al.*, 1999a), rapid clonal propagation (Rout *et al.* 1999b), micropropagation (Sahoo, 1986; Selvakumar *et al.*, 2001; Chaplot *et al.*, 2006) and direct plant regeneration from leaf explants (Rout, 2002). Extreme and destructive harvesting of *P. zeylanica* by pharmaceutical industries for the procurement of naturally occurring secondary metabolite (plumbagin) and insufficient attempts to either allow its replenishment or its cultivation led to depletion of natural plant population.

Only few reports are available on cultivation, breeding and improvement of *P. zeylanica* despite its commercial importance. It necessitates further improvement in micropropagation system for the mass multiplication of *P. zeylanica* to make this plant available for the use of future generation. The objective of this study was to develop an improved regeneration protocol from nodal explants of *P. zeylanica* by manipulating concentrations and combinations of phytohormones. We used

thidiazuron (TDZ) and gibberellic acid (GA₃) separately and in combination with naphthalene acetic acid (NAA) and kinetin (KN) on shoot induction of *P. zeylanica* from nodal explants which has not been attempted so far.

MATERIALS AND METHODS

Plant material: Nodal explants of *P. zeylanica* were collected from herbal garden. They were immersed in running tap water for 10 min, then transferred to 1% Tween 20, maintained for 30 min and repeatedly washed with sterile distilled water. The explants were then immersed in 70% alcohol for 40 sec, followed by washing with distilled water and immersion in 0.1% HgCl₂ for 7 min and repeated washing with sterile distilled water before transferring into the shoot induction medium.

Shoot induction: Cotyledonary nodal explants were placed vertically on MS medium (Murashige and Skoog, 1962) supplemented with different concentrations (0.5, 1.0, 1.5 and 2.0 mg L⁻¹) of BAP, TDZ and GA₃ to find the hormone type and concentration for efficient shoot induction (Table 1). The culture tubes were incubated at 25±2°C in light with 16 h photoperiod. The percent explants responding (explants producing at least one shoot), total number of shoots per responding explant and mean length of shoots were calculated after 4 weeks of incubation in the light.

Combined effect of hormones on shoot induction: In the second experiment, combined effect of NAA or KN with BAP, TDZ and GA₃ was tested on shoot induction of *P. zeylanica*. Four different concentrations (0.5, 1.0, 1.5 and 2.0 mg L⁻¹) of NAA and TDZ were added separately to the shoot induction medium containing 1.0 mg L⁻¹ BAP, TDZ and GA₃; total number of shoots produced by each explant was counted after 4 weeks of incubation in the light.

Table 1: Effect of TDZ, BAP and GA₃ on shoot induction from nodal explants of *Plumbago zeylanica* after four weeks of incubation in the light

Concentration of cytokinins (mg L ⁻¹)	(%) of explants responding	Mean no. of shoots	Shoot length (cm)
BAP	0.0	NR	NR
	0.5	25±2.34 ^a	06.30±0.21 ^a
	1.0	57±1.97 ^b	13.23±0.32 ^b
	1.5	43±3.43 ^c	10.43±0.19 ^b
	2.0	20±2.76 ^a	03.42±0.43 ^a
GA ₃	0.0	NR	NR
	0.5	34±4.12 ^a	08.22±0.51 ^a
	1.0	67±2.34 ^b	16.48±0.32 ^b
	1.5	51±3.67 ^b	12.62±0.23 ^b
	2.0	29±4.23 ^a	08.22±0.71 ^a
TDZ	0.0	NR	NR
	0.5	43±2.67 ^c	14.82±0.37 ^b
	1.0	86±3.19 ^d	20.24±0.42 ^c
	1.5	63±4.12 ^b	16.61±0.59 ^b
	2.0	37±3.15 ^a	10.84±0.64 ^a

NR: No Response; Values are Mean±SD of 20 explants per treatment of 3 replications.; Means followed by the same letter are not significantly different at 0.05% level based on fisher's LSD test

Table 2: Effect of IBA, IAA and MS strength on root induction of *Plumbago zeylanica* after four weeks of incubation in the light

Concentration of (mg L ⁻¹)		Half strength MS		Full strength Auxin MS	
		No. of roots	Root length (cm)	No. of roots	Root length (cm)
IBA	0.0	NR	NR	NR	NR
	0.5	11.8±0.11 ^a	4.8±0.13 ^a	09.4±0.12 ^a	3.7±0.12 ^a
	1.0	16.5±0.26 ^b	8.7±0.18 ^b	13.7±0.15 ^b	6.3±0.10 ^b
	1.5	09.6±0.18 ^a	6.7±0.14 ^c	07.3±0.13 ^a	5.3±0.13 ^c
	2.0	07.0±0.22 ^c	5.9±0.16 ^a	05.9±0.17 ^c	4.7±0.16 ^c
IAA	0.0	NR	NR	NR	NR
	0.5	12.0±0.17 ^a	3.7±0.11 ^a	09.0±0.11 ^a	7.4±0.19 ^b
	1.0	13.5±0.25 ^b	6.9±0.19 ^c	10.2±0.16 ^a	5.9±0.17 ^c
	1.5	10.0±0.17 ^a	5.4±0.13 ^a	07.5±0.12 ^a	4.3±0.13 ^c
	2.0	08.0±0.20 ^c	4.0±0.16 ^a	05.2±0.16 ^c	3.0±0.18 ^a

NR: No Response; Values are Mean±SD of 10 explants per treatment of 3 replications; Means followed by the same letter are not significantly different at 0.05% level based on fisher's LSD test

Root induction: Shoots obtained from shoot induction medium were transferred to the rooting medium amended with 0.5, 1.0, 1.5 and 2.0 mg L⁻¹ IBA and IAA. The hormones were added separately to full strength and half strength MS media (Table 2) to find the effect of MS salt concentration on root induction. The cultures were incubated in the light at 25±2°C; total number of roots produced per shoot and length of the root were calculated after 4 weeks of incubation in the light.

RESULTS AND DISCUSSION

Shoot induction: Nodal explants of *P. zeylanica* were inoculated on MS medium supplemented with different concentrations of BAP, GA₃ and TDZ. Initiation of auxiliary buds was observed on the nodal parts of the explants after 10 days of inoculation in the light and later these buds developed into shootlets after 2 weeks. Shoot induction was observed in all the tubes with hormones. The best response was observed at 1.0 mg L⁻¹ for all the three hormones tested. TDZ produced superior response over GA₃ and BAP (Table 1) it produced the best response of shoot induction (86%), maximum number of shoots (20) and lengthier shoots (3.8 cm).

In an earlier study KN and BAP were used individually on shoot induction in *P. zeylanica* (Sahoo, 1986); BAP produced more shoots than KN. In this study, we used GA₃ and TDZ for the first time on shoot induction in *P. zeylanica*. TDZ has been used most frequently in recent times for efficient shoot induction and regeneration of many plant species. TDZ, a synthetic phenylurea, is considered to be one of the most active cytokinins for shoot induction in plant tissue culture (Huetteman and Preece, 1993; Murthy *et al.*, 1998). Several reports suggest that TDZ promoted shoot regeneration better than other cytokinins (Barna and Wakhlu, 1995).

In this study, we observed that TDZ was superior to BAP and GA₃ on shoot induction of *P. zeylanica*. TDZ showed high cytokinin activity in promoting growth of cytokinin-dependent cultures (Mok *et al.*, 1982). TDZ stimulated conversion of cytokinin nucleotides to more biologically active nucleotides (Capelle *et al.*, 1983) and stimulated accumulation of endogenous purine cytokinins (Thomas and Katterman, 1986). Proficient role of TDZ for the efficient micropropagation of *Pterocarpus marsupium* was reported by Husain *et al.* (2007).

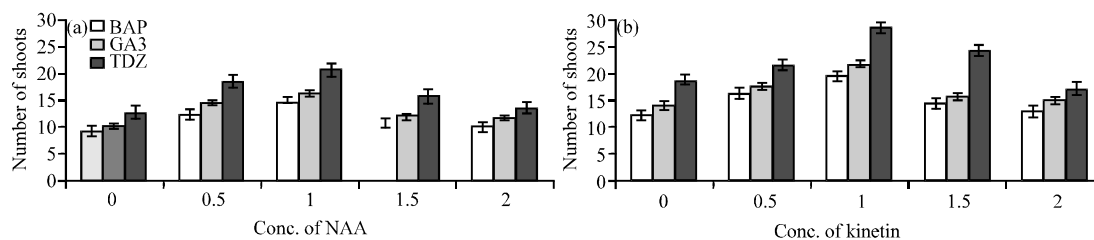


Fig. 1(a-b): Combined effect of hormones on shoot induction, at 1.0 mg L⁻¹ BAP, TDZ and GA₃, supplemented with (a) NAA and Kinetin (b) After four weeks of incubation in the light

Combined effect of hormones on shoot induction: Four concentrations (0.5, 1.0, 1.5 and 2.0 mg L⁻¹) each of NAA and KN were added separately to 1.0 mg L⁻¹ TDZ, GA₃ and BAP to assess the combined effect of hormones on shoot induction of *P. zeylanica*. Addition of NAA and KN to either of the hormones had improved the rate of shoot induction. The optimum concentration of NAA and KN was 1.0 mg L⁻¹. KN produced superior response over NAA; KN improved the number of shoots compared to NAA at all the four concentrations (Fig. 1a, b). Addition of KN to TDZ produced more shoots (27.2) compared to the addition of KN to the other two hormones (GA₃ and BAP). KN and NAA also increased the length of the shoots; KN produced lengthier shoots compared to NAA.

Synergistic effect of hormones on efficient shoot induction has been well documented and proved in many plant species. Addition of two different hormones in the regeneration medium has augmented the shoot induction and produced more shoots than addition of single hormone (Kantia and Kothari, 2002; Dimitrov *et al.*, 2003; Ewa and Halina, 2004). We have already reported this effect in *Curcuma amada* (Prakash *et al.*, 2004); this was also proved in *Santolina canescens* (Casado *et al.*, 2002), *Bupleurum fruticosum* (Fratenale *et al.*, 2002) and *Acacia albida* (Gassama and Duhoux, 1986). Selvakumar *et al.* (2001) obtained shoots on MS medium supplemented with IBA+KN and IBA+Adenine sulphate in *P. zeylanica*. BAP was mostly used for shoot induction in *P. zeylanica*; it was used either with IAA (Rout *et al.*, 1999a).

Root induction: Well elongated shoots were separated from the shoot induction medium and transferred to the root induction medium (Fig. 2d). Root induction medium consisted of different concentrations (0.5, 1.0, 1.5 and 2.0 mg L⁻¹) of IAA and IBA added separately to half strength and full strength MS basal salts. Hormones added with half strength MS medium produced better response of rooting compared to hormones added with full strength MS medium (Table 2). Many plant species are rooted best in the half strength MS medium for *in vitro* propagation including *Tylophora indica* (Faisal and Anis, 2003), *Solanum melongena* (Magioli *et al.*, 1998), *Trapa japonica* (Hoque and Arima, 2002) and *Rhodiola fastigiata* (Liu *et al.*, 2006). Of the two hormones (IBA and IAA) tested for root induction, the best response was observed in IBA at all the four concentrations (Table 2). The best response was observed with 1.0 mg L⁻¹ IBA. Most number of roots (16.5) and lengthier roots (8.7 cm) were obtained on half strength MS medium supplemented with IBA. In earlier reports on *P. zeylanica*, the root was induced with IBA on half strength MS (Verma *et al.*, 2002; Rout *et al.*, 1999b) and with IAA on full strength MS (Rout *et al.*, 1999a). But from this study it is apparent that half strength MS is superior over full strength MS for efficient *in vitro* root induction in *P. zeylanica*.



Fig. 2(a-d): Micropropagation of *Plumbago zeylanica*. (a) Shoot bud initiation from cultured nodal segment after 10 days of incubation (b) Formation of multiple shoots after 2 weeks (c) Shoot elongation and (d) Root induction

IBA and IAA are the most commonly used hormones for *in vitro* root induction of many shrubs (Gonzalez *et al.*, 1991; James, 1983). IBA produced superior response over IAA on root induction; this was confirmed in many plant species including *Echinacea purpurea* (Kristen *et al.*, 2000), *Arabidopsis* (Ludwig-Muller *et al.*, 2005) and *Picea sitchensis* (Selby *et al.*, 1992).

Hardening and transplantation: Rooted plants were carefully removed from the culture tubes and rinsed with double distilled water to remove agar media. The plants were then placed in the culture bottle containing sterile soilrite; the mouth of the bottle was then covered with plastic paper to maintain high humidity and kept in the culture room. After two weeks of incubation the plants were then transferred to the pots containing vermicompost and sand (50:50) and maintained in the green house before transferring to the field with 90% survival rate. The plants treated with different concentrations of growth regulators grew well and survived like normal plants. The regenerants did not show any morphological variations compared to mother plants.

CONCLUSION

From this study it is apparent that, the use of TDZ with other cytokinins has increased the rate of shoot induction in *P. zeylanica* than all the other combination of hormones reported earlier. This may be helpful for the efficient shoot induction and mass multiplication of this important medicinal plant. The diverse medicinal and others uses of the plant *P. zeylanica* and restrictions imposed by

biological and natural propagation parameters force the need to adopt alternative means for multiplication/resource augmentation. The protocol developed in the present study will help in the mass production of *Plumbago zeylanica* and other medicinally important plants.

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