African Journal of Biotechnology Vol. 8 (16), pp. 3761-3768, 18 August, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Micropropagation of *Plumbago zeylanica* L.

Iyyakkannu Sivanesan[1] and Byoung Ryong Jeong[1, 2]*

[1]Department of Horticulture, Division of Applied Life Science (BK21 Program), Graduate School, Gyeongsang National University, Jinju, Korea 660-701.
[2]Institute of Agriculture and Life Science, Gyeongsang National University, Jinju, Korea 660-701.

Accepted 9 June, 2009

***In vitro* propagation of *Plumbago zeylanica* was investigated to develop reliable protocols for direct and indirect shoot regeneration. Axillary shoot multiplication, callus induction and shoot regeneration from callus culture was obtained on Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of plant growth regulators. A maximum number of shoots (38 ± 1.3 per explant) was obtained from nodal explants when cultured on MS liquid medium supplemented with 1.0 mg·L^{-1} BAP, 0.5 mg·L^{-1} IBA and 2.0 mg·L^{-1} adenine sulfate. The highest percentage of callus induction was obtained when stem explants cultured on MS medium supplemented with 2.0 mg·L^{-1} BAP and 1.5 mg·L^{-1} IAA. The greatest percentage of shoot induction (100%) with a mean of 34.2 shoots obtained from callus was cultured on MS medium supplemented with 0.75 mg·L^{-1} BAP, 1.0 mg·L^{-1} IAA, NAA and adenine sulfate each. Regenerated shoots were rooted best on half-strength MS medium containing 0.5 mg·L^{-1} NAA and 3% (w/v) sucrose. The regenerated plantlets were acclimatized in the culture room and successfully transferred in soil.**

Key words: Adenine sulfate, callus proliferation, direct regeneration, liquid culture, *Plumbago zeylanica*, shoot multiplication.

INTRODUCTION

Higher plants are valuable sources of industrially important natural products, which include flavors, fragrances, essential oils, pigments, sweeteners, feedstocks, antimicrobials and pharmaceuticals. In most instances these chemical compounds belongs to a metabolic group collectively referred to as secondary metabolites and are usually biosynthesized in smaller quantities. This has resulted in ruthless exploitation of medicinally important plants creating imbalance in supply and demand. An alternative technology could be the application of *in vitro* culture of desirable medicinal plants to increase the plantation propagules and enhance the yield of specific drug compounds. *Plumbago zeylanica* L. is a valuable medicinal plant widely used in treatments for rheumatism, piles, diarrhea, leprosy and anasarca (Anonymous, 1989). It is reported to have anticancer, antibacterial, and antifungal properties (Krishnaswamy and Purushothaman, 1980) and also shown to have antitumor activity (Kavimani et al., 1996).

The root of *P. zeylanica* has been reported to be a powerful poison when given orally or applied to ostium uteri, causing abortion (Azad Chowdhury et al., 1982; Premakumari et al., 1977). Leaves and roots of *P. zeylanica* contain an alkaloid, plumbagin (2-methoxy-5hydroxy-1,4-napthoquinone), which externally is a strong irritant but a powerful germicide; stimulates muscular tissue in smaller doses and paralyzes in larger ones; stimulates the contraction of the muscular tissues of the heart and intestines; stimulates the secretion of sweat, urine and bile; and also has a stimulant action on the nervous system (Chopra et al., 1996). Plumbagin has showed to possess a wide variety of pharmaceutical activities such as anticancer (Aziz et al., 2008), antioxidant (Tilak et al., 2004), antimicrobial (Pavia et al., 2003), radiosensitizing (Prasad et al., 1996) and antifertility (Bhargava, 1984).

Conventional propagation of the plant is rather difficult and insufficient to meet the growing demand owing to the poor germination of seeds and death of young seedlings under natural conditions (Anonymous, 1989). Micropropagation is currently applied to a large number of medici-

*Corresponding author. E-mail:brjeong@gnu.ac.kr. Tel.: +82-55-751-5489. Fax: +82-55-757-7542.

nally important plant species for conservation and multiplication, but is still costly due to intensive manual manipulation throughout the various culture phases. Commercially useful *in vitro* plant propagation for mass production requires the development of efficient micropropagation, based on rapid and extensive proliferation of cultures combined with inexpensive large scaled cultivation for the multiplication phase (Ziv, 1995). Compared to agar-based systems, liquid systems are more adaptable to automation and, are therefore, suitable for the reduction of labor and costs, as media can be changed easily during scaling-up.

Therefore, establishment of cultures in liquid medium is an important step towards automation. In order to propagate plants in bioreactors, plantlets should be firstly cultured in liquid medium. *In vitro* propagation of *P. zeylanica* on solid medium has been reported earlier (Rout et al., 1999; Das and Rout, 2002; Wei et al., 2006; Sivanesan, 2007). In this study, we report the use of liquid culture for large scale propagation of *P. zeylanica*.

MATERIALS AND METHODS

Surface disinfection, media preparation and culture condition

Actively growing healthy shoots were used as the explant source. The shoots were separated from mother plants (one year old) and cut into 4-6 cm. The explants were washed thoroughly in running tap water for 30 min and wash with distilled water then surface sterilized with 70% (v/v) ethanol for 1 min, followed by 0.1% (w/v) mercuric chloride for 10 min, followed by four washes for 3 min each in sterile distilled water and were cut in to smaller (0.5 – 1.0 cm) segments (internodes, nodes and shoot tips) inoculated aseptically on Murashige and Skoog, 1962 (MS) medium supplemented with various concentrations of plant growth regulators. Unless otherwise mentioned all the media contained 3% (w/v) sucrose and 0.8% (w/v) agar. The pH of the medium was adjusted to 5.7 with 0.1 N NaOH or 0.1 N HCl before autoclaving at 15 lb pressure and 121°C for 15 min. Unless specified, all cultures were maintained at 25 ± 2°C under 16 h photoperiod (45 µmol m^{-2} s^{-1}) using cool, white fluorescent light.

Direct shoot regeneration

Shoot tip and nodal explants were cultured on MS medium alone or supplemented with 6-benzylaminopurine (BAP) or kinetin (Kn) (0.5, 1.0, 1.5, 2.0 and 3.0 mg·L^{-1}) for axillary shoot induction. For shoot multiplication MS solid or liquid medium (with out agar) supplemented BAP 1.0 mg·L^{-1} along with different concentrations of IAA, IBA and NAA (0.5 and 1.0 mg·L^{-1}), and adenine sulfate (ADS) (1.0 and 2.0 mg·L^{-1}). For one experiment, 25 explants (shoot tip, node) were used, and the experiment was repeated thrice. The number of shoots and percentage of shoot induction was recorded after 45 days.

Callus induction, proliferation and regeneration

Segments of internodes were placed on MS medium supplemented with different concentration of BAP (0, 0.5, 1.0, 1.5, 2.0 and 3.0 mg·L^{-1}) alone or combined with indole-3-acetic acid (IAA) or indole-3-butric acid (IBA) or α-napthaleneacetic acid (NAA) (0, 0.5, 1.0 and 1.5 mg·L^{-1}). Callus induction, proliferation and, nature of callus were recorded after 30 days. The calli were transferred onto MS medium supplemented with different concentration and combinations of BAP, IAA, NAA and adenine sulfate (ADS) to induce plant regeneration. After 5 weeks of culture regenerated shoots were transferred to fresh medium containing same composition of ingredients with out agar. For one experiment, 25 callus (approximately 500 mg) were used, and the experiment was repeated thrice. The number of shoots and percentage of shoot induction was recorded after 35 days.

Rooting and acclimatization

Individual shoots, which were 3 - 4 cm long were excised from the shoot clump and transferred to MS and half strength MS medium containing IAA, IBA and NAA (0.1, 0.5, 1.0, 1.5 and 2.0 mg·L^{-1}). For one experiment, 25 shoots were used, and the experiment was repeated thrice. After 35 days rooted plantlets were removed from tubes, washed thoroughly with sterile distilled water to remove traces of agar and planted in small plastic pots filled with mixture of sterile soil, sand, vermiculite (1:1:1). The potted plants were irrigated with MS basal salts solution (1/4 strength) devoid of sucrose and myo-inositol every 4 days for 4 weeks. After 4 weeks, the plants were transferred to polythene bags, which were placed in greenhouse till the new leaves emerged. Then they were transferred into the field.

Statistical analysis

All experiments were repeated thrice. Data were analyzed by analysis of variance (ANOVA) to detect significant differences between means using SAS computer package (SAS Institute Inc., Cary, NC, USA, Release 9.1). Means differing significantly were compared using the Duncan multiple range test at the 5% probability level.

RESULTS AND DISCUSSION

Influence of cytokinins on axillary shoot induction

Direct regeneration of shoots from the node and shoot tip explants were tested using MS medium supplemented with different concentration of cytokinins (BAP and Kn), ranging from 0.5-3.0 mg·L^{-1}. The MS medium devoid of cytokinins was used as control. The explants cultured on the MS medium without cytokinins did not produce any shoots; however, both explants induced axillary shoots within a period of 2 weeks when the MS medium was supplemented with either BAP or Kn. A maximum number of shoots were obtained from nodal (8.0) and shoot tip (5.2) explants when cultured on MS medium supplemented with 1.0 mg·L^{-1} BAP and the percentage of shoot induction were 81.4% and 72.8%, respectively (Table 1). At higher concentrations shoot induction and number of shoots were reduced significantly in both explants. At 1.5 mg·L^{-1} Kn, 5.4 shoots were induced from nodal explants and 2.8 from that of shoot tip explants and the percentage of shoot induction were 73% and 65%, respectively. The above results revel that nodal explants was better than the shoot tip explants. Of the two cyto-

Table 1. Effect of cytokinins on direct shoot regeneration of *Plumbago zeylanica*.

Conc. ($mg \cdot L^{-1}$)	Shoot induction (%)				No. of shoots/explants			
	Shoot tip		Node		Shoot tip		Node	
	BAP	Kn	BAP	Kn	BAP	Kn	BAP	Kn
0.5	62.6c	45.6d	67.0c	47.2e	3.6c	1.8bc	5.6b	3.2c
1.0	72.8a	59.8b	81.4a	54.6d	5.2a	3.0a	8.0a	4.8ab
1.5	68.4b	65.0a	76.8b	73.0a	4.4b	2.8ab	6.0b	5.4a
2.0	57.2d	53.4c	69.6c	60.2b	3.2cd	2.2b	4.6c	4.2b
3.0	42.8e	37.0e	62.0d	58.0c	3.0d	1.4c	3.8d	2.0d

Means having same letter(s) in a column are not significantly different by Duncan's comparison test $p \angle 0.05$ level.

kinins tested, BAP was superior for multiple shoot induction than Kn. This result was in agreement with the previous studies (Rout et al., 1999; Rout and Das, 2002).

Effect of combination of plant growth regulators on axillary shoot induction

Effect of combination of BAP, IAA, IBA, NAA and ADS on shoot induction was tested on explants such as node and shoot tip. A combination of BAP and auxins maximized axillary shoot multiplication significantly; however, shoot induction percentage and number of shoots developed per explants significantly different in each treatment. When the explants were cultured on MS medium containing BAP with IBA enhanced the frequency of shoot induction and the number of shoots per explants, as compared to BAP with IAA or NAA. At 1.0 $mg \cdot L^{-1}$ BAP and 0.5 $mg \cdot L^{-1}$ IBA, 15.6 shoots induced from nodal explants and 9.8 from that of shoot tip explants, and the percentage of shoot induction were 91.6 and 87.2, respectively (Figure 1A and B).

These results are better than earlier studies (Rout et al., 1999; Selvakumar et al., 2001; Rout and Das, 2002). Inclusion of ADS to shoot multiplication medium (MS with 1.0 $mg \cdot L^{-1}$ BAP and 0.5 $mg \cdot L^{-1}$ IBA) significantly enhanced the number of shoots per explant. The nodal explants cultured on the MS medium with 1.0 $mg \cdot L^{-1}$ BAP, 0.5 $mg \cdot L^{-1}$ IBA, and 2.0 $mg \cdot L^{-1}$ ADS yielded the highest percentage of shoot induction (100%) with an average of 24 shoots per explants (Table 2).

Similar results were also reported in other plant species like *Pentanema indicum Sida cordifolia* (Sivanesan and Jeong, 2007a, 2007b). In order to reduce the intensive labor requirement along with the production cost during plant propagation by tissue culture technique, there is an immense need of developing scale-up systems and automation (Aitkin-Christie, 1991). For scale-up cultures using bioreactors, the use of liquid culture medium has been recommended (Kim et al., 2004; Ewelina et al., 2005). 25 ml of liquid MS medium with 1.0 $mg \cdot L^{-1}$ BAP, 0.5 $mg \cdot L^{-1}$ IBA, and 2.0 $mg \cdot L^{-1}$ ADS was dispensed in 100 ml Erlenmeyer flask or 150 ml saline bottle and nodal explants were cultured for 5 weeks. The number of shoots significantly increased from 24 to 38 when nodal explants were cultured in 100 ml Erlenmeyer flask, whereas in 150 ml saline bottle 30 shoots per explant was recorded after 5 weeks of culture (Figure 1C and D). The above results revels that type of culture vessel important for liquid culture, hence, further studies are needed. The growth of the shoots in liquid medium was also high as compared to solid medium (data not shown). Advantages of liquid media for maximizing shoot proliferation has also been reported for other plant species like tea (Indra et al., 2001), *Centaurium erythraea* (Ewelina et al., 2005), and day lily (Jianxin et al., 2005).

Callus induction, proliferation and regeneration

Initiation of callus was observed on the cut ends of stem explants after about 7 - 10 days when MS medium with BAP alone or in combination with auxins (IAA, IBA or NAA). The percentage of callus varied from 18 - 71% when stem explants were cultured in the presence of BAP, and callus was light brown and friable. A maximum callus induction (71%) observed when MS medium containing 2.0 $mg \cdot L^{-1}$ BAP. Callus induction and growth were further enhanced by addition of auxins. The highest percentage of callus induction was obtained when stem explants cultured on MS medium supplemented with 2.0 $mg \cdot L^{-1}$ BAP and 1.5 $mg \cdot L^{-1}$ IAA (Table 3).

The primary calli were subcultured twice successively, and calli obtained in each subculture were tested further for shoot regeneration. In both subcultures, the shoot regenerative ability of the callus was not changed significantly. Four weeks old callus was transferred to regeneration medium mainly supplemented with BAP, IAA and NAA. A mean of 21.6 shoots were regenerated from callus cultured on MS medium with 0.75 $mg \cdot L^{-1}$ BAP, 1.0 $mg \cdot L^{-1}$ IAA and NAA each, and the percentage of shoot induction was 87.6% (Table 4). Low frequency of regeneration of shoot buds was marked in the medium with higher concentrations of BAP. Similar findings have been

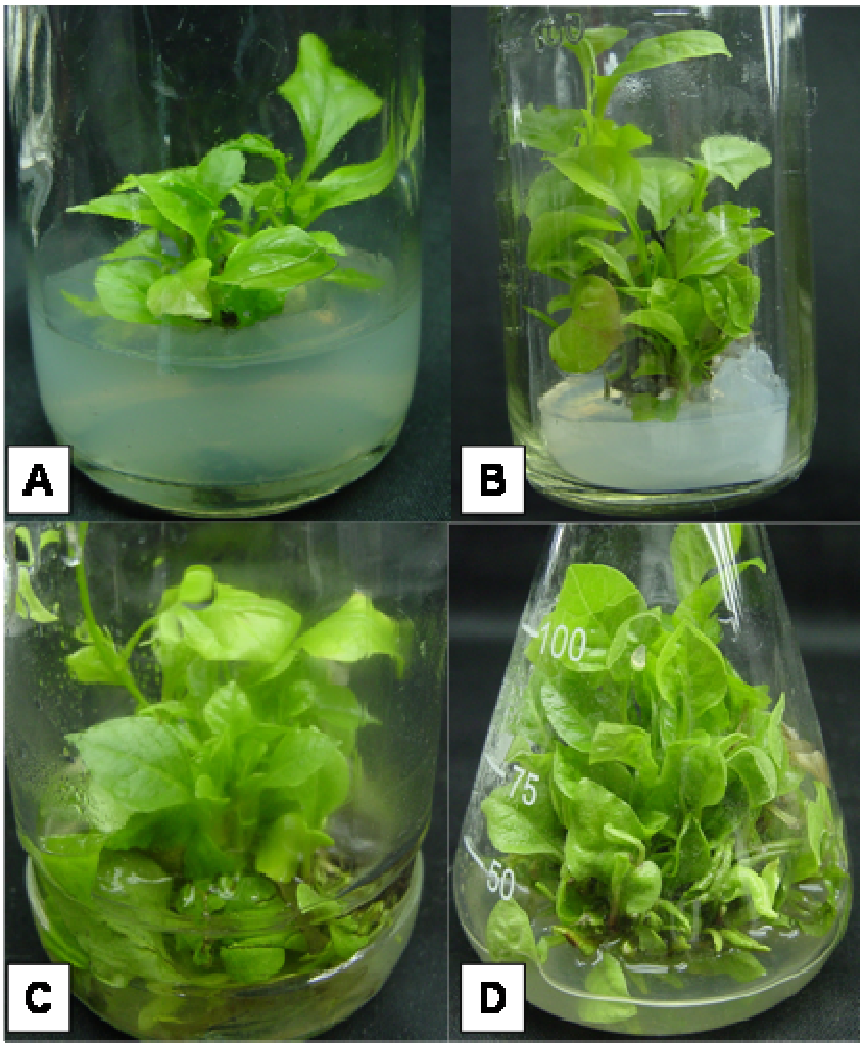

Figure 1. Multiple shoot induction from explants of *P. zeylanica*. A) Multiple shoot induction from shoot tip explants after 3 weeks,. B) Multiple shoot induction from nodal explants after 6 weeks. C) Nodal explants were cultured in 150 ml saline bottle with 25 ml of MS liquid medium containing PGRs. D) Nodal explants were cultured in 100 ml Erlenmeyer flask with 25 ml of MS liquid medium containing PGRs.

Table 2. Effect of plant growth regulators (PGRs) on direct shoot regeneration of *Plumbago zeylanica*.

Plant growth regulators (mg·L^{-1})					No. of shoots/explants		Shoot induction (%)	
BAP	**IAA**	**IBA**	**NAA**	**ADS**	**Shoot tip**	**Node**	**Shoot tip**	**Node**
1.0	0.5	0	0	0	6.3f	8.7f	76.0e	83.0e
1.0	1.0	0	0	0	7.6e	10.2e	78.4d	88.4d
1.0	0	0.5	0	0	9.8c	15.6c	87.2b	91.6c
1.0	0	1.0	0	0	7.8d	10.6e	83.4c	90.2c
1.0	0	0	0.5	0	8.0d	12.8d	81.6cd	87.0d
1.0	0	0	1.0	0	7.2e	11.0de	78.9d	85.2de
1.0	0	0.5	0	1.0	12.4b	19.6b	93.0a	96.4b
1.0	0	0.5	0	2.0	16.7a	24.0a	94.8a	100a

Means having same letter(s) in a column are not significantly different by Duncan's comparison test p∠0.05 level.

Table 3. Effect of PGRs on callus induction.

Plant growth regulators (mg·L^{-1})				Callus induction (%)
BAP	IAA	IBA	NAA	
0.5	0	0	0	18.0j
1.0	0	0	0	39.2i
1.5	0	0	0	62.6h
2.0	0	0	0	71.0f
3.0	0	0	0	66.8g
2.0	0.5	0	0	83.0d
2.0	1.0	0	0	97.2ab
2.0	1.5	0	0	100a
2.0	0	0.5	0	79.2e
2.0	0	1.0	0	84.0d
2.0	0	1.5	0	90.6c
2.0	0	0	0.5	88.0cd
2.0	0	0	1.0	90.2c
2.0	0	0	1.5	95.8b

Means having same letter(s) in a column are not significantly different by Duncan's comparison test p∠0.05 level

Table 4. Effect of PGRs on indirect shoot regeneration.

Plant growth regulators (mg·L^{-1})				Shoot induction (%)	No. of shoots/callus
BA	IAA	NAA	ADS		
0.5	0.5	0.5	0	56.2h	9.4f
0.5	1.0	1.0	0	71.0e	10.8f
0.75	0.5	0.5	0	64.4f	17.0d
0.75	1.0	1.0	0	87.6c	21.6c
1.0	0.5	0.5	0	60.0g	13.6e
1.0	1.0	1.0	0	74.0d	16.2d
0.75	1.0	1.0	1.0	100a	34.2a
0.75	1.0	1.0	2.0	96.2b	29.0b

Means having same letter(s) in a column are not significantly different by Duncan's comparison test p∠0.05 level

observed by Rout et al. (1999). Since previous studies (Rout et al., 1991; Das and Rout, 2002; Wei et al., 2006) reported that high concentration of ADS greatly enhanced shoot regeneration. The greatest percentage of shoot induction (100%) with a mean of 34.2 shoots obtained from callus when cultured on MS medium was supplemented with 0.75 mg·L^{-1} BAP, 1.0 mg·L^{-1} IAA, NAA and ADS each (Figure 2A and B). In this study, we report that low concentration of ADS (1.0 mg·L^{-1}) was effective for *P. zeylanica*.

Rooting and acclimatization

Regenerated shoots were transferred to ½ MS medium containing 3% (w/v) sucrose with or without IAA, IBA and NAA for root induction. The medium without auxins did not show any root induction. In contrast, root induction was observed when regenerated shoots were cultured on medium with low concentrations of auxins, whereas at higher concentrations shoots formed callus at the cut end of the stem. These results are in agreement with earlier published work (Rout et al., 1999). Of the three auxins tested, NAA was found to be best followed by IBA and IAA (Table 5).

A maximum root induction (100%) was obtained on ½ MS medium with 0.5 mg·L^{-1} NAA and an average of 12.5 roots per shoot with a mean root length of 4.4 cm (Figure 3A and B). Rooted plantlets were transferred to plastic pots containing mixture of sterile soil, sand, vermiculite (1:1:1) and were acclimatized in the culture room for 4 weeks. Acclimatized plantlets were transferred to the field with 100% survival rate, which was an improvement over 90% survival rate obtained by Rout and Das (2002). The

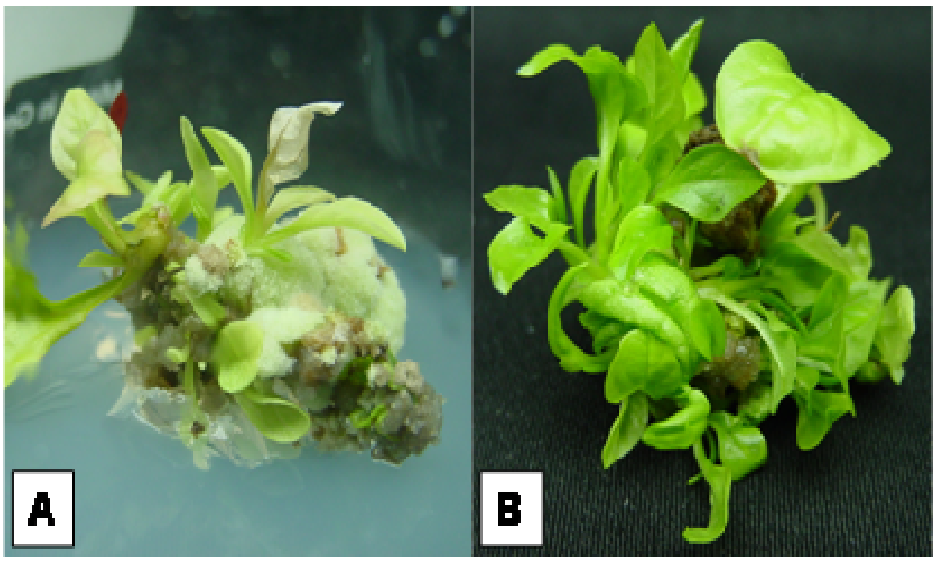

Figure 2. Different stages of shoot regeneration from callus cultures of *P. zeylanica*. A) 3 weeks old, and B) 5 weeks old.

Table 5. Effect of PGRs on root induction.

Plant growth regulators (mg·L^{-1})			Root induction (%)	No. of roots/shoot	Root length (cm)
IAA	IBA	NAA			
0.1	0	0	74.6e	5.4de	2.0d
0.5	0	0	83.2d	7.3c	3.2bc
1.0	0	0	C+R	C+R	C+R
2.0	0	0	C	C	C
0	0.1	0	69.0f	4.4e	1.8d
0	0.5	0	88.4c	6.2d	2.6c
0	1.0	0	96.0b	9.0b	3.0bc
0	2.0	0	C	C	C
0	0	0.1	67.0g	7.1c	3.6b
0	0	0.5	100a	12.5a	4.4a
0	0	1.0	81.3d	9.5b	4.2a
0	0	2.0	C	C	C

C = Callus; R = root.
Means having same letter(s) in a column aere not significantly different by Duncan's comparison test p∠0.05 level.

regenerated plantlets were morphologically similar to donor plants, and exhibited normal flowering (Figure 3C and D).

Conclusion

We have developed for first time a simple and efficient protocol for direct regeneration from shoot tip explants of *P. zeylanica* and also we developed protocols for indirect regeneration. Further, we demonstrated advantages of liquid culture medium for maximizing shoot multiplication. The shoots which were grown in liquid medium rooted well in solid medium and did not show any morphological variation revels that liquid culture medium can be useful for large scale propagation of this important plant species.

ACKNOWLEDGEMENTS

Iyyakkannu Sivanesan was supported by a scholarship from the BK21 Program, the Ministry of Education, Science and Technology, Korea.

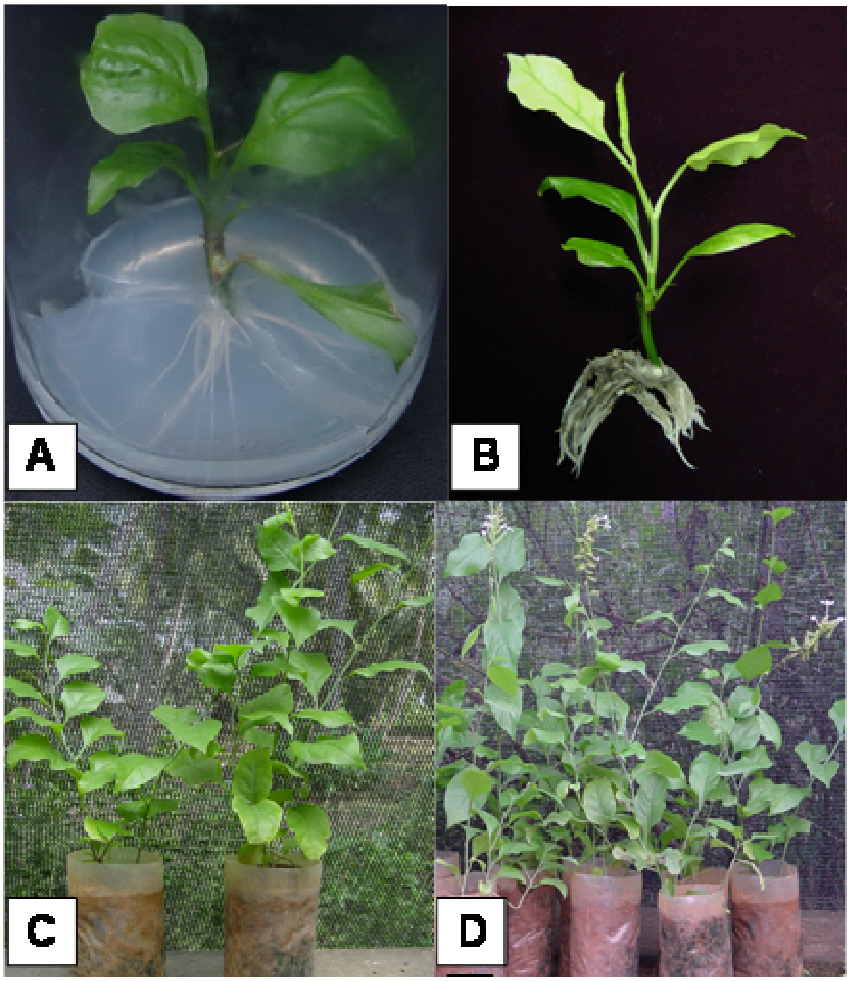

Figure 3. Rooting and acclimatization of *P. zeylanica.* A) regenerated shoot rooted on ½ MS medium with 0.5 mg·L^{-1} NAA. B) Regenerated plantlets removed from the culture medium showing well developed shoot and roots. C) Acclimatized plantlets. D) Plantlets showing normal flowering.

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