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Research Article

IN VITRO PROPAGATION OF *PLUMBAGO INDICA* L. THROUGH DIRECT ORGANOGENESIS

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ABSTRACT
Plumbago indica is a medicinal plant belonging to family Plumbaginaceae. It is widely used in Ayurveda, Siddha, Unani, Homeopathy and also in unmodified ethnic preparations of the rural folks. In this study, reliable protocols were developed for direct organogenesis via nodal and inter nodal segments. Different concentrations of BAP were tested for shoot bud induction from nodal segments and among them MS medium supplemented with 2.0 mg/L BAP gave highest mean number of multiple shoots (3.40 ± 0.91) while MS medium supplemented with 3.0 mg/L BAP gave highest mean shoot length (2.41 ± 0.21 cm**)**. Multiple shoot buds induced from inter nodal segments were cultured on different concentrations of GA_3 and BAP. Out of different concentrations, best medium for shoot elongation (1.03 ± 0.45 cm) was MS medium supplemented with 4.0 mg/L GA_3. Highest mean number of roots (26.53 ± 8.21) and highest mean root length (2.49 ± 0.31 cm) were recorded in half MS medium supplemented with 0.4 mg/L IBA. Plantlets showed 100% survival percentage in jiffy palates and 90% survival percentage when transfer to pots filled with top soil : compost (1:1) mixture. Stem cuttings that have treated by dipping in 0.25 mg/L IBA solution for fifteen minutes and dipped in water for 15 minute before covering cutting edges with 0.3% IBA powder were shown 100% of survival while control has shown 80% survival. Conventionally propagated (stem cuttings) has higher growth rate, early flowering and higher fresh (56.90 ± 11.19 g) and dry root weight (17.33 ± 3.15 g) when compare with same age tissue cultured plants.

Keywords: *Plumbago indica, Plumbaginaceae, Direct organogenesis, Multiple Shoot Buds, Nodal Segments*

INTRODUCTION

Nearly one fourth of world's population (about 1.42 billion people) depend on traditional plant base medicine for prevention and cure of different kind of diseases and seek for more and more eco-friendly and bio-friendly remedies which have less side effects than the synthetic chemical base drugs (Saha and Paul, 2012). *Plumbago indica* has a wide range of pharmacological activities against many diseases (Panichayupakaranant and Tewtrakul, 2002). *P. indica* is used to treat different kind of diseases from decades (Bhadra *et al*, 2009) including rheumatoid arthritis, dysmenorrhea, cancer, leprosy, syphilis, headache (Jose *et al*, 2014), rheumatism, paralysis, scabies (Jayaweera, 1982), leucoderma, enlarged glands and scorpion-sting, ophthalmia (Saha and Paul, 2012) etc.

This plant is originated in Sikkim and Khasi hills of India and migrated to other neighbouring countries including Sri Lanka (Schmelzer and Gurib-fakim, 2008). *P. indica* is cultivated in South India, Philippine, Kenya, Tanzania, Zimbabwe, Mozambique, Madagascar (Jose *et al*., 2014), Africa, Europe, Indonesia, China, Malaysia and Arabian Peninsula for harvesting its tuberos roots (Sharma and Yadav, 2019).

P. indica contains different types of alkaloids, flavonoids, saponins, glycosides and tannins (Eldose *et al*, 2013). Plumbagin (5-Hydroxy-2-methyl-1, 4-naphthoquinone) is a main naturally occurring active organic compound which is isolated from *P. indica* roots (Gangopadhyay *et al*, 2010). It is a simple hydroxy naphthoquinone which is commercially important for its broad range of pharmacological activities (Silja *et al*, 2014). This plant does not produce seeds. Therefore shoot cuttings are used for the vegetative propagation (Yogananth and Basu, 2009). However as this species has slow growth rate and

considerably long period for roots production, it is not considered as a feasible method to obtain propagules (Panichayupakaranant and Tewtrakul, 2002). *P. indica* is rapidly decline from their natural environment due to over exploitation for commercial uses (Gangopadhyay *et al*, 2010). Normally distractive harvesting is done due to the root system of this plant is used to prepare traditional medicines (Yogananth and Basu, 2009) as plumbagin is mainly exploited from roots (Panichayupakaranant and Tewtrakul, 2002). Thus developing techniques for *in vitro* propagation as well as finding attractive techniques such as cell culture to obtain plumbagin is a current need.

METERIALS AND METHODS

P. indica plants were obtained from Commercial medicinal plant cultivation. All plants were grown in the shade house at University of Sri Jayewardenepura. Voucher herbarium specimens were prepared and handed over to National Herbarium at Royal Botanical Garden, Peradeniya and authenticated. Murashige and skoog (MS) medium was used as the basal medium in all experiments and growth regulator free medium was used as the control. Cultures were maintained at $24 \pm 1\ ^0$C in 24 h photoperiod. Number of replicates was fifteen unless otherwise stated. Completely Randomized Design (CRD) was used in all experiments.

Determination of best growth regulator concentration for direct shoot induction

Surface sterilized nodal segments (1.0 – 1.5 cm) were cultured on MS basal medium supplemented with different concentrations of BAP (1.0 -5.0 mg/L). Percentage shoot induction, mean number of shoots per explant and mean shoot length were measured after eight weeks.

Determination of best growth regulator concentration for shoot elongation

Surface sterilized inter nodal segments were cultured on MS medium supplemented with 3.0 mg/L BAP for multiple shoot bud induction. Six weeks old multiple shoot buds were transferred into MS basal media supplemented with different concentrations of GA_3 (1.0 - 5.0 mg/L) and BAP (0.5 - 2.5 mg/L) for shoot elongation separately. Initial shoot length was recorded. Shoot length was measured after eight weeks and the highest shoot length increment was calculated.

Determination of best growth regulator concentration for root induction

Shoots (2.0 - 3.0 cm) were taken out from shoot elongation medium and carefully separated from the cluster. Shoots were transferred in to ½ MS medium supplemented with different concentrations of IBA (0.2 – 0.8 mg/L). Root induction was observed after eight weeks and mean number of roots per shoot, percentage of rooting response and mean root length was recorded.

Acclimatization of in vitro propagated plantlets

Well-developed, rooted plantlets were selected, root systems of plantlets were washed under running tap water to remove all trace of agar and transferred in to well wetted Jiffy coir pallets. Plants were covered with transparent polythene after watering. Transparent polythene covers were removed only for two to three hours every day after two weeks and removed permanently when plants stop withering within exposer time to outer environment over three hours. Survival percentage was observed after one month. Plants were transferred into pots which contain top soil : compost (1:1) for further development.

Comparison of survival percentage of conventionally propagated plants

Matured stems with at least one node were separated from healthy mother plants. Twenty stem cuttings were dipped in 0.25 mg/mL IBA solution for fifteen minutes and planted in Jiffy coir pallets. Another twenty stem cuttings were dipped in water for 15 minute and cutting edges were covered with 0.3% IBA powder before planted in Jiffy coir pallets for rooting. Stem cuttings which dipped in water for 15 minutes were planted in Jiffy coir pallets use as a control. All plants were covered with transparent polythene cover and kept in the lab under controlled sunlight for one month and observed the survival percentage of each cultivation method over a period of eight weeks.

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Comparison of growth, flowering age, fresh and dry root weight of tissue cultured and conventionally propagated plants

Acclimatized two month old twelve tissue cultured plants and twelve conventionally propagated plants were transferred in to pots filled with a mixture of compost: top soil (1:1). Plants were kept in the shade house and watered in every other day. Mean plant height, mean number of branches and flowering started week were recorded. Plants were uprooted after eight months of growth and measured the fresh and dry weight.

RESULTS AND DISCUSSION

Determination of best growth regulator concentration for direct shoot induction

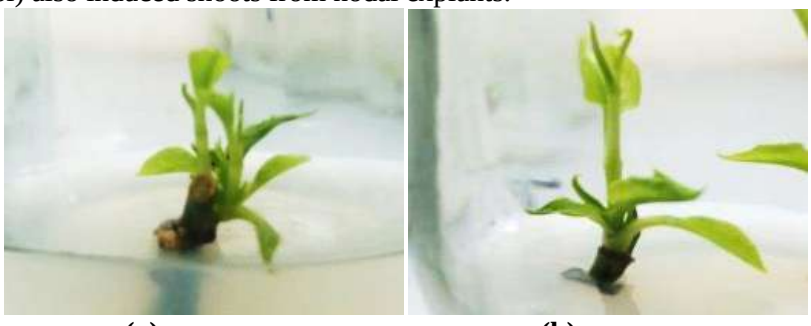
Nodal explants cultured on all tested media including the control showed a positive response in shoot induction. Highest number of shoots was observed in MS medium supplemented with 2.0 mg/L BAP and highest mean shoot length was observed in the presence of 3.0 mg/L BAP after eight weeks of incubation.

Table 01: Development of shoots from nodal segments in MS medium supplemented with different concentrations of BAP after eight weeks of incubation

BAP concentration (mg/L)	Mean number of shoots ± SD	Mean shoot length ± SD (cm)
0.0	1.33 ± 0.48^{c}	1.30 ± 0.17^{c}
1.0	1.33 ± 0.48^{c}	1.36 ± 0.11^{c}
2.0	$\mathbf{3.40 \pm 0.91^{a}}$	2.17 ± 0.35^{b}
3.0	2.20 ± 0.56^{b}	$\mathbf{2.41 \pm 0.21^{a}}$
4.0	1.13 ± 0.35^{c}	1.32 ± 0.11^{c}
5.0	1.00 ± 0.00^{c}	1.03 ± 0.10^{d}

The values marked with the different letters are significantly different with each other ($P<0.05$)

According to the Jindaprasert *et al*, (2010), highest mean number of shoots from nodal explant (5.2 ± 0.4) was observed in MS medium supplemented with 2.0 mg/L BAP. In the present study highest mean number of shoots (3.40 ± 0.91) was observed at the same BAP level confirming the findings of Jindaprasert *et al*, (2010). However, there was a difference between mean numbers of shoots produced *in vitro* in the present study which was low (3.40 ± 0.91). Controlled environmental conditions within the culture room and the status of mother plants are always different from one research to another and that might be the possible reason for obtaining different mean number of shoots at same BAP concentration in two studies. On the other hand, MS medium with no added BAP or Kinetin (control) didn't proliferate shoots from nodal explants (Jindaprasert *et al*, 2010), However in the present study, MS medium with no added BAP (control) also induced shoots from nodal explants.



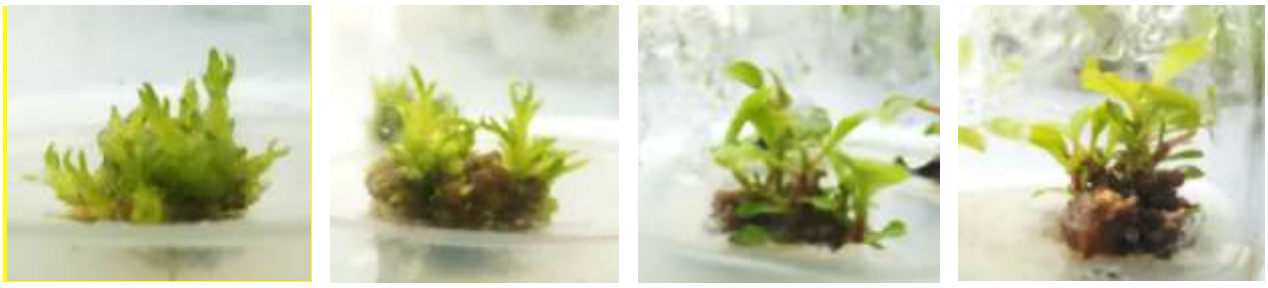
(a) **(b)**

Figure 01: (a) Single shoot induction in control (b) Multiple shoot induction in MS medium supplemented with 2.0 mg/L BAP after eight weeks.

Among different individual concentrations of Kinetin, 2ip, BAP and combinations from these cytokines with IAA, MS medium supplemented with 3.5 mg/L BAP has given 95% shooting response with highest mean number of shoots (2.41 ± 0.14) and mean shoot length (0.87 ± 0.14) within three weeks. In this research, highest mean number of shoot induction BAP concentration was higher than in present study. However this research has proven that the best plant growth regulator for direct shoot induction was BAP alone (Jose *et al*, 2007). According to statistical analysis of mean shoot height, there is a statistically significant difference between the mean shoot heights in different BAP concentrations (P=0.00).

Determination of best growth regulator concentration for shoot elongation

Highest mean shoot length increment (1.03 ± 0.45 cm) was observed in MS medium supplemented with 4.0 mg/L GA_3 when compare with other concentrations of GA_3 and BAP. Highest shoot length increment (0.79 ± 0.38) cm has been observed in the presence of 0.5 mg/L BAP. There was a shoot elongation in control but the different was significantly low. There is a statistically significant different between the highest mean shoot length increment GA_3 concentration and other GA_3 concentrations (P=0.01). According to Gopalakrishnan *et al*, (2009), cytokine like BA was combined with GA_3 to elongate *P. indica* shoot buds. Highest shoot length (2.0 cm ± 0.0 cm) was observed in MS medium supplemented with 0.12 mg/L BA and 0.50 mg/L GA_3. Present study showed that GA_3 alone has the ability to elongate shoot buds. However the combine effect of GA_3 and BA is significantly higher.



(a) (b) (c) (d)

Figure 02: Growth of multiple shoot buds (a) Just after culturing (b) After four weeks (c) After eight weeks (d) After twelve weeks in MS medium supplemented with 4.0 mg/L GA_3.

Table 02: Shoot length increment in MS medium supplemented with different concentrations of GA_3 and BAP after eight weeks

GA_3 Treatment		BAP treatment	
GA_3 (mg/L)	Mean shoot length increment ± SD (cm)	BAP (mg/L)	Mean shoot length increment ± SD(cm)
0.0	0.51 ± 0.21^{b}	0.0	0.35 ± 0.16^{b}
1.0	$0.78 \pm 0.43^{a,b}$	0.5	$\mathbf{0.79 \pm 0.38^{a}}$
2.0	$0.71 \pm 0.28^{a,b}$	1.0	$0.57 \pm 0.26^{a,b}$
3.0	$0.87 \pm 0.57^{a,b}$	1.5	$0.56 \pm 0.38^{a,b}$
4.0	$\mathbf{1.03 \pm 0.45^{a}}$	2.0	$0.63 \pm 0.30^{a,b}$
5.0	$0.73 \pm 0.18^{a,b}$	2.5	0.47 ± 0.18^{b}

The values marked with the different letters are significantly different with each other (P<0.05)

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Determination of best growth regulator concentration for root induction

In vitro grown *P. indica* shoots (1.0 – 2.0 cm height) showed 100% root induction response in every tested treatment including control. Highest mean number of roots (26.53 ± 8.21) and mean root length (2.49 ± 0.31) was observed in ½ MS medium supplemented with 0.4 mg/L IBA after eight weeks (Table 3). It was observed that increased levels (over 0.5 mg/L) of IBA retarded the root induction. It is reported that the best root induction (11.76 ± 0.3) was observed in ½ MS medium supplemented with 0.4 mg/L IBA with 90% rooting response. *In vitro* grown shoots grown in growth regulator free ½ MS medium was only gave 40 % root induction response (Kumar *et al*., 2014). Present study confirms the results of the previous report suggesting that ½ MS medium supplemented with 0.4 mg/L IBA is the most suitable growth regulator concentration for *in vitro* root induction from *in vitro* grown shoots of *P. indica*. However, in contrast to that present study reported 100% rooting response in control instead of low rooting percentage reported by Kumar *et al*., (2014).

Table 3: Development of roots in *in vitro* grown shoots on MS basal medium supplemented with different concentrations of IBA

IBA concentration (mg/L)	Mean number of roots ± SD	Mean root length ± SD
0.0	5.53 ± 4.00^{c}	1.48 ± 0.20^{c}
0.2	$9.13 \pm 4.07^{b,c}$	$1.85 \pm 0.54^{b,c}$
0.4	$\mathbf{26.53 \pm 8.21^{a}}$	$\mathbf{2.49 \pm 0.31^{a}}$
0.6	10.87 ± 5.19^{b}	$1.81 \pm 0.51^{b,c}$
0.8	9.80 ± 5.63^{b}	1.98 ± 0.58^{b}

The values marked with the different letters are significantly different with each other (P<0.05)

Figure 03: Root induction in ½ MS basal medium supplemented with 0.4 mg/L IBA after eight weeks

(a) (b) (c)

Figure 04: (a) Tissue cultured plant just after planting on Jiffy pallet (b) Four weeks old acclimatized tissue cultured plant on Jiffy pallet (c) Eight weeks old acclimatized tissue cultured plant on top soil : compost (1:1) mixture

Acclimatization of in vitro propagated plantlets

Hundred percent survival was observed when tissue cultured plantlets planted on Jiffy pallets after one month. Plants were covered with transparent polythene cover to reduce transpiration by increasing humidity and reducing the moments of air around the plants. Leaves of these plants easily withered as cuticle on leaves are not developed well.

Plants have small, easily breakable and soft leaves when introduce into Jiffy coir pallets. Plant leaves stared to expand after two weeks when root system established well throughout the coir pallets. Acclimatized plants have shown 90% Survival percentage when plants were transferred into top soil : compost (1:1) mixture and survived plants were healthy and shoot elongation occurred with increasing number of leaves after one month. According to the Gopalakrishnan *et al*, (2009) *in vitro* grown *P. indica* plantlets were directly introduced into red soil, vermiculite and farmyard manure (1:1:1) mixture, survival rate was reduced to 10% after three weeks acclimatization. Present study revealed that, survival percentage can be increased using Jiffy pallets before introduce plants into media containing soil. Tissue cultured plantlets have less developed and less adapted root system which can affect easily by soil microbes and dryness. Therefor culturing plantlets on Jiffy pallets before transferring to soil containing media allow plants to get their root system adjusted and grow further. Jiffy pallets have high water holding capacity as they are made out of coir dust. They hold water to the amount that immature roots can absorb yet not holding excessive water for microbes to grow and cause root damage.

According to Lenora *et al*, (2012), Tissue cultured *P. indica* plantlets were first transferred into polythene bag filled with coir dust and sand (1:1) potting mixture before they were transplanted in pots containing Soil: coir dust: compost (1:1:1) potting mixture. Survival percentage of acclimatized plantlets was not mentioned. Higher survival percentage (90%) was recorded in tissue cultured *Plumbago zeylanica* when tissue cultured plantlets were planted in culture bottles with sterile soilrite. These culture bottles were covered with plastic papers and kept in the culture room for two weeks before transferring to the pots containing vermicompost and sand (50:50) mixture. These plants were maintained in a greenhouse before transferring to the field (Ceasar *et al.*, 2013).

Comparison of survival percentage of conventionally propagated plants

(a) (b) (c)

Figure 05: (a) Just after planting (b) Axillary bud growth after four weeks (c) Shoot cutting growth after eight weeks.

Stem cutting is a most simple and abundantly used propagation method for many plant species. Stem cuttings with 0.25 mg/L IBA treatment, cuttings dipped in water for 15 minute before covering cutting edges with 0.3% IBA powder showed 100% survival. Control showed 80% survival.

Stem segments with 2-3 nodes and fully open leaves were used for planting. Axillary buds started to emerge from the nodes within 14 days and mature leaves were removed to provide the space when newly

emerged axillary bud has one to two fully open leaves. White color immature roots can be observed all over the Jiffy coir pallet after one month.

(a) **(b)**

Figure 06: Roots of eight month old (a) tissue culture plants (b) conventionally propagated plants

Comparison of growth, flowering age, fresh and dry root weight of tissue cultured and conventionally propagated plants

Slow growth of tissue cultured plants was observed comparing to conventionally propagated plants. Mean height of tissue cultured plants was 116.80 ± 9.03 cm and 7.50 ± 1.84 mean number of branches were observed while conventionally propagated plants showed 145.00 ± 12.90 cm mean height and 5.30 ± 1.42 mean number of branches showing the different was statistically significant (P=0.00). All conventionally propagated plants started flowering in 15th or 16th week of growth in soil however tissue cultured plants stared flowering in between 15th to 24th week indicating flowering age was shorter in conventionally propagated plants than tissue cultured plants. Plants were uprooted after flowering (eight month after growing in soil containing medium) and mean fresh and dry root weight were observed. It was observed that mean fresh root weight of conventionally propagated plants was higher (56.90 ± 11.19 g) while tissue cultured plants has 53.21 ± 16.85 g. Mean dry root weight of conventionally propagated plants also was higher (17.33 ± 3.15 g) than tissue cultured plants (16.04 ± 5.05 g). However the difference was not statistically significant in fresh and dry root weight (P>0.05).

CONCLUTIONS

Best BAP concentration to induce highest mean number of shoots was 2.0 mg/L BAP while highest mean shoot length was observed in MS medium supplemented with 3.0 mg/L BAP. GA_3 gave highest mean shoot length increment (1.03 ± 0.45 cm) at 4.0 mg/L while highest shoot length increment has been given by BAP was 0.79 ± 0.38 cm at 0.5 mg/L. Therefore GA_3 was more suitable for shoot elongation. IBA 0.4 mg/L was the best PGR concentration for *in vitro* root induction. Survival percentage of tissue cultured plants were high when jiffy palates were used for acclimatization before transferring into soil: compost

(1:1) mixture. Conventionally propagated plants have higher growth, early flowering and fresh and dry root weight when compare with tissue cultured plants.

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