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In vitro tissue culture studies and synthetic seed formation from *Plumbago zeylanica* L.

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Received 09 June 2017; revised 18 July 2018

Synthetic seeds and *in vitro* propagation are the need of the hour especially for conservation of medicinal plants which are under the threat of extinction due to extensive exploitation. The plumbagin, *Plumbago zeylanica* L., is one such highly exploited medicinal plant. Here, we attempted *in vitro* propagation of its roots by tissue culture and also synthetic seed development towards conservation of this plant. Its leaves were used as the explant. Surface sterilized explants were aseptically cultured on MS medium supplemented with different plant growth hormones. The embryoid callus produced from tissue culture was then used to produce synthetic seed for large scale production of the plants and to reduce the risk of maintenance, storage and transportation of the cultured plants. For the production of synthetic seed, the embryoid callus were chopped aseptically and were encapsulated with sodium alginate and liquid MS medium without CaCl₂ supplemented with growth hormones of similar concentration as used in tissue culture. Best result of callus induction and root regeneration was observed on MS medium supplemented by 2 ppm NAA (naphthalene acetic acid).

Keywords: Auxin, Callus, *In vitro* propagation, NAA, Plumbagin, Root regeneration

Plumbago zeylanica L., commonly known as Ceylon Leadwort or Doctorbush (U.S. Dept. of agriculture 1996), belongs to the family Plumbaginaceae. Ceylon Leadwort is a plant with glabrous stems that are prostrate. The plant needs full sun to partial shade with intermediate to warm temperature ranges¹. It is native to warm temperate-tropical regions of the world and grows wild in India (especially in Jharkhand, Bengal, Uttar Pradesh, and South Indian states) and Sri Lanka. In India it is commonly known as *chitraka*. *P. zeylanica* is widely used by rural and tribal people for hundreds of years as traditional medicine. Its roots and leaves contain plumbagin, a

major component that constitutes about 0.03% of dry weight of the roots, which has been identified as significant bioactive component related to several pharmacological activities, like antitumor, antimicrobial, anticancer, wound healing, anti-inflammatory, antioxidant, insecticidal, antidote for snake bite, mental disorder, scabies, body pain, gonorrhoea, altered T-cell proliferative activities and antifertility actions²⁻⁶. Leaves are used to treat leprosy and skin diseases. The root part is widely used traditionally in the treatment of various infections and diseases. The *Bhil* tribe of Bibdod, Madhya Pradesh using its root extract is taken with tea to cure headache, cough and cold⁷. The *Thottianaickans* communities of Tamil Nadu, mixed the dried powdered root of *P. zeylanica* with goat milk for administered to arrest frequent urination^{8,9}.

P. zeylanica is a wild growing plant and is not cultivated so the indiscriminate collection of roots and non-cultivation has impact on biodiversity. Nowadays, many pharmacological companies also utilize this plant for the preparation of variety of drugs so the number of plants is decreasing and it needs to be continuously harvesting as it is slow growing perennial shrub. Hence, only continuous harvesting is not a solution to save the *P. zeylanica* from exploitation. In such a situation, *in vitro* propagation of roots by tissue culture and synthetic seed development is a good solution for pharmaceutical companies as well as naturopathic treatment of various diseases.

Production of synthetic seeds, endowed with high germination rate under *in vitro* and *in vivo* conditions, bears immense potential as an alternative of true seeds¹⁰. It can be defined as the artificial encapsulation of somatic embryo, shoot buds or aggregates of cell or any tissues which has the ability to grow into a plant under both, *in vivo* and *in vitro* conditions. Synthetic seed can be stored for a long time in appropriate condition. These are used to provide protection to the artificially produced propagules. It can be used to propagate hybrid plant, genetically modified plants, endangered species, and elite genotype, moreover synthetic seed production is cost effective when compared to traditional method and can be directly used in fields. Artificial seed can be transported from one country to another without obligation from

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quarantine department. They are small therefore they are easy to handle^{11,12}. The seeds provide aseptic condition to the plant material or explant, which is present inside the capsule. Synthetic seed crops are easy to maintain because of uniform genetic constituent. This technology improves the production and also produces environment friendly plantation. In several plants, conversion of artificial seeds into plantlets was reported^{13,14}.

Materials and Methods

Collection and identification of the explants

The plant material was collected from the well botanically leveled medicinal garden of Ranchi University, Ranchi in 2016. The botanical identity of the plant was confirmed by comparing with the authentic samples at the Herbarium of Taxonomy Department of Ranchi University, Ranchi.

Callus induction and regeneration

MS media with different concentration of hormones were used for callus induction and regeneration. The required amount of MS media was dissolved in distilled water heated till the media becomes transparent. The pH was adjusted to 5.8 with the help of N/10 NaOH or HCl and dispensed into the test tubes (15.0 × 2.5 cm), each containing about 10-15 mL, which are plugged with cotton plugs. The medium containing tubes were autoclaved at 121°C for 15-20 min and allowed to cool gradually. The slant were prepared and left for 2-3 days then inoculated. Before inoculation the explant was sterilized with distilled water and then treated with 0.1% w/v aqueous solution of HgCl₂ (mercuric chloride) for 2 min and then thoroughly washed with autoclave double distilled water (DDW) in Laminar Air Flow (LAF) for 3-4 times to remove traces of HgCl₂ which were then immersed in 70% ethyl alcohol for 30 s and finally washed with autoclaved DDW thoroughly (3-4 times) under LAF to remove traces of alcohol¹⁵.

LAF was thoroughly wiped with 70% ethyl alcohol. All the sterilized glassware, DDW and test tube and UV light were switched on for 20-30 min. Sterilized instruments were flamed and inoculation was done near the flame as quick as possible with the help of sterilized forceps under LAF cabinet.

The inoculated culture tubes were kept in sterilized condition in culture room under controlled conditions of temperature 25±3°C, light period 16 h, light intensity 1500-3000 lux and relative humidity 60%. Screening was done regularly, every week and

infected tubes were discarded subcultures maintain on the same medium¹⁶.

Encapsulation of embryos

Preparation of solution A, B and C

Solution A: About 3 g of sodium alginate was mixed with 50 mL of DDW using Vortex; *Solution B:* Similarly, about 3.2 g of liquid MS media without CaCl₂ was mixed with 50 mL of DDW.; and *Solution C:* For the preparation of 50 mM concentration of CaCl₂, 2.77 g of CaCl₂ was dissolved in 500 mL of DDW.

Sterilization

All the required glasswares, equipments and solution A, B and C were autoclaved at 121°C and 15 lb pressure for 15-20 min.

Preparation of synthetic seed

Before the preparation, the LAF was wiped with 70% ethyl alcohol. All the glasswares, solution A (sodium alginate), solution B (MS media) and solution C (50 mM CaCl₂) was transferred to LAF. After that, solution A and B were mixed. The embryonic callus was chopped with the help of sterilized scalpel in Petri dishes in front of flame and they were transferred to the sodium alginate- MS medium mixture and dropped one by one into 50 mM CaCl₂ for encapsulation with the help of spatula. Precaution should be taken that each drop must contain one piece of chopped callus. Then the seeds were left overnight for encapsulation. Next day the seeds were taken out and placed in sterilized Petri dishes and sealed.

The artificial seeds were maintained same as in tissue culture and the screening of the seeds was done at the interval of 4 days. Total number of seeds, number of nonviable seed and number of germinating seeds were counted. The number of plants developed from each capsule was determined by counting the plants under stereomicroscope.

Results and Discussion

Callus induction

Various concentration and combinations of phytohormones were used for the development of callus in various plant parts of *Plumbago zeylanica*. In this study, the best performance was observed at MS medium supplemented with 2 ppm naphthalene acetic acid (NAA) (Figs. 1 & 2). In this concentration the nodal explants showed the production of whole plant whereas the leaf explants produced profuse amount of root induction whereas when BAP, KN and

NAA, 0.5 ppm each was supplemented on the leaf explants only brown callus was observed with no further growth and in all the other combination of hormones only curling, swelling and disappearance of colour was observed in the leaf explants with no appearance of further growth (Table 1).

Embryo encapsulation

The best growth was observed after 8 days in which 6 seeds showed callusing and 22 seeds showed rooting and the growth percentage was 36.8% after which the growth rate decreased, after 12 days three

seeds showed the production of white roots, after 19 days two seeds showed callusing and rooting and one seed became non-viable. The seeds showed growth till 25 days and after 25 days showed a decline in the percentage of germination. (Table 2, Fig. 3)

The initial experiment on the growth of *Plumbago zeylanica* in MS basal medium supplemented with 2 ppm NAA significantly enhances root formation

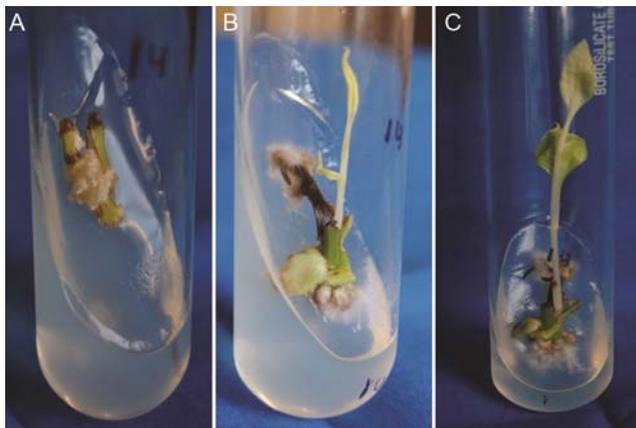


Fig. 1 — Whole plant regeneration from nodal explant of *Plumbago zeylanica* at 2 ppm NAA. (A) Callus induction after 15 days of inoculation; (B) Nodal explant initiation of root and shoot after 25 days; and (C) Regeneration of whole plant after 35 days.

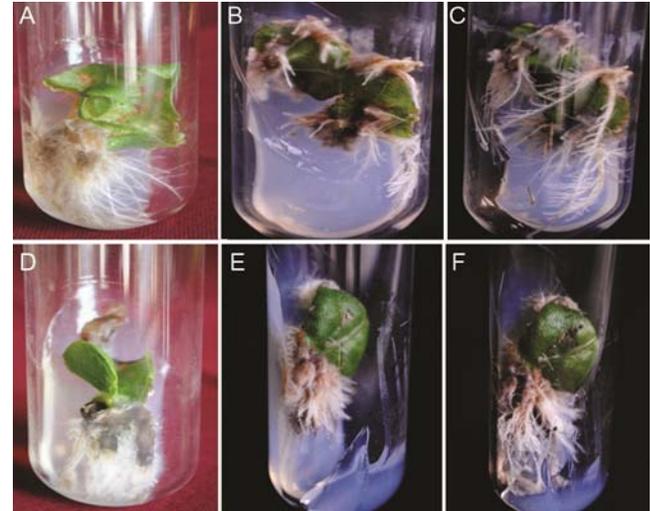


Fig. 2 — Profused amounts of root regeneration from leaf explant of *P. zeylanica* at 2 ppm NAA. (A) Callus induction after 15 days of inoculation; (B) Initiation of root after 20 days; and (C-F) Observation of profused amounts of root appearance after 24, 28, 32 and 36 days, respectively from leaf explant.

Table 1 — Effect of different concentration and combination of growth hormones on morphogenic response of *Plumbago zeylanica* L.

Conc. of hormones (ppm)	Type of explant	Morphogenic changes observed			Degree of callusing	Organogenesis	Remarks
		Curling	Swelling	Disappearance of colour			
BAP+KN+NAA (0.5 ppm each)	leaf	+	+	+	+	∅	brown callus
2,4-D (1 ppm)+ KN (0.1 ppm)	leaf	++	++	+	∅	∅	∅
2,4-D (1 ppm)+ KN (0.5 ppm)	leaf	+++	++	+++	∅	∅	∅
NAA (1.5 ppm)+ KN (0.25 ppm)	leaf	++	++	+	∅	∅	∅
NAA (2.0 ppm)	leaf	++	++	+	++	profuse roots	white and brown sticky callus. multiple hairy roots.
NAA (2.0 ppm)	nodal	∅	+	∅	+++	regeneration of complete plants (shoot and root)	formation of white, hairy roots.
BAP (2.0 ppm)	leaf	++	++	++	∅	∅	∅

[+, poor; ++, medium; +++, abundant amount; and ∅, no growth. BAP, 6-benzylaminopurine; KN, kinetin, NAA, naphthalene acetic acid; and 2,4-D, 2,4-dichlorophenoxyacetic acid]

Table 2 — Percentage growth of synthetic seed of *Plumbago zeylanica* L.

Interval of days	No. of growing seeds			No. of non-viable seeds	Percentage growth
	Callusing	Rooting	Total		
0	0	0	0	0	0
4	0	6	6	0	7.89
8	6	22	28	0	36.8
12	7	22 (3 white root)	29	0	38.15
19	7	23 (2 callusing+ rooting)	30	1	39.47
25	10	25	35	4	46.05

Total number of seeds = 76

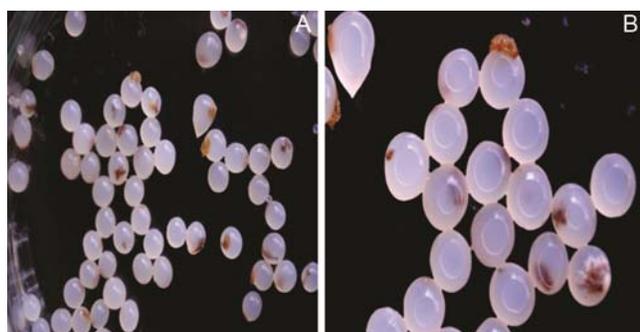


Fig. 3 — Synthetic seed of *P. zeylanica* and its regeneration. (A) Formation of synthetic seeds by encapsulation of embryonic callus into calcium alginate beads; and (B) Germination of synthetic seeds and appearance of roots from the seeds.

before its elongation from leaf explant whereas the growth was poor in other hormone concentration. Selvaraju *et al.*¹⁷ reported that the combination of cytokinin and auxin stimulates the *in vitro* multiplication and growth of shoot and root. Lin *et al.*¹⁸ reported that relatively high auxin- cytokinin ratio induces root formation in tobacco callus whereas a low ratio of the same hormone favoured shoot production. A common property of auxin is inducing cell division. The maximum conversion rate of encapsulated shoot tips into plantlets was 76.6% after 6 weeks of culture on MS medium supplemented with 0.25 mg/L NAA plus 1.0 mg/L BAP¹⁹.

The synthetic seed was produced from the somatic callus of *Plumbago zeylanica* supplemented with the same hormone concentration as in tissue culture and liquid MS medium (Moquamoel Haque) produced synthetic seed of micro shoot with their *in vitro* germination and hardening upto establishment on field condition²⁰. As a general rule the younger the explanted material, the more possibilities there are of obtaining a growth response²¹. Therefore *in vitro* callus induction and regeneration are often used as starting material.

Conclusion

From the above result it was observed that 2 ppm of NAA was the best concentration of hormone for

callusing and regeneration (rooting from leaf explants and whole plant from nodal explants) in *Plumbago zeylanica* so this protocol will be used for the large scale production of plantlets for the extraction of useful pharmaceutical compounds. In the present investigation synthetic seed were produced by somatic embryonic callus which is a good source for easy transportation, maintenance, production or regeneration of large amount of plantlets and conservation of germplasm.

The production of uniform, firm beads following a relatively short duration exposure to CaCl₂ and the high rate of subsequent germination suggest that synthetic seeds in *P. zeylanica* may be suitable for practical application as it is a medicinal plant.

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