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OPTIMIZATION OF CULTURE CONDITIONS FOR THE PRODUCTION AND GERMINATION OF ARTIFICIAL SEED IN AN IMPORTANT MEDICINAL PLANT, *NOTHAPODYTES NIMMONIANA*

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ABSTRACT

Nothapodytes nimmoniana (Icacinaceae) is an endangered tree species which is majorly found in the Western Ghats of India (400-2200m). It produces a secondary metabolite, camptothecin that holds a high commercial value owing to its anti-cancer properties. The large scale camptothecin demand has led to the overharvesting of this tree species. This study was done for mass propagation of N.nimmonian by optimizing the production and germination of its artificial seeds. The field grown plants of N.nimmoniana were taken and cultured on four different solid MS media for callus induction. The best result of callus induction was obtained on MS media amended with IBA (2mg/l) + KN (1 mg/l) in 50±5 days. Upon callus induction globular shaped somatic embryos were selected for artificial seed generation. The artificial seed were produced using the different concentrations of sodium alginate and calcium chloride solution followed by germination on MS media supplemented with (IBA)+KN+ (GA3) with and without activated charcoal. Better germination was observed in the media without charcoal within 13-15 days. The current study holds a great potential for providing an efficient means of easy transportation, germplasm conservation, secondary metabolite production and mass propagation of this tree.

Keywords: Artificial seeds, encapsulation, indirect somatic embryogenesis, mass propagation, N.nimmoniana.

I. INTRODUCTION

Nothapodytes nimmoniana Graham (Syn. Mappia foetida, common name – “Stinking tree”) is an endangered medium sized medicinal tree belonging to the family Icacinaceae. It is distributed in the evergreen forest of Western Ghats of India, North-East India, Sri Lanka, Myanmar and Thailand [1].The tree growth is slow, and propagation is usually achieved by seeds. The tree needs 7–8 years from seedling to reach maturity before start of flower production [2]. The seeds are recalcitrant due to high sensitivity to desiccation, freezing, and have a short shelf life [3][4]). This tree is a rich source of camptothecin, isoquinoline alkaloidis one of the most promising anti-cancer drugs of the twenty-first century [5][6]. CPT is currently being used for treating colorectal

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and ovarian cancer [7][8]. Worldwide annual sales of the analogs of camptothecin reached $1,000 million which is about 1 ton of camptothecin raw material representing about 1,000–1,500 tons of N. nimmoniana wood chip [9][2]. In Western Ghats of India, whole N. nimmoniana trees are cut to generate biomass for extraction and export [2]. Annual N. nimmoniana biomass demand reached 500–700 metric tons in 2001, wood chips trade volume reached 1,600 tons in 2002, reported trade volume exceeded 1,000 tons between 2006–2008. In the absence of synthetic sources, the global demand for this alkaloid is met by natural populations of N. nimmoniana. This has led to decrease in the population of this species in the Western Ghats, India, and**,** in fact, due to the extremely high pressure, the species has been declared as endangered [9]. Camptothecin has been reported to be isolated at higher yield from *N. nimmoniana* with greater quantity in stem and root of the tree than other natural sources. The recalcitrant seeds produced by the plants may be considered an alternative source of camptothecin when they lose viability as many could be found on forest floor few months after seed set [10]. Tissue culture techniques can help in its conservation. An efficient method has been developed for the in-vitro mass propagation of *N.nimmoniana* through indirect organogenesis using semisolid and liquid cultures [11]. Studies have been done to increase the response of the plant through seed and also clonal propagation has been carried out. Cryopreservation of embryonic axes is also employed in short term preservation of the species [12]. Effects of elicitor concentration and time course changes of camptothecin production after elictor addition has also studied [13]. The micro propagated plants have also been evaluated for their chemical potency using high performance liquid chromatography (HPLC) for analysis of CPT content [14]. All the above techniques hold promise in meeting demand for camptothecin and conservation of *N. nimmoniana* natural populations. This study possesses a robust potential in increasing the viability rate and germination potential of *N.nimmoniana* by optimizing the culture conditions for the artificial seed generation and germination through somatic embryogenesis as embryo culture helps in decreasing the breeding cycle of the plants. Not only this, artificial seed generation will help in germplasm conservation, large scale propagation of endangered species, and can be used as an alternative for camptothecin extraction [15].

II. MATERIAL AND METHODS

2.1 Selection Of Plant Material

Nothapodytes nimmoniana plantlets were procured from GKVK, University of Agricultural Sciences Bangalore The plant was planted and grown in greenhouse for 3-4 years at the laboratory area of the Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology Waknaghat, India.

2.2 Media Preparation And Culture Conditions

MS medium supplemented with different concentrations and combinations of plant growth hormones with 3% (w/v) sucrose was prepared. The pH of the media was adjusted to 5.7 using 0.1 N Hydrochloric acid (HCl) and 0.1 N sodium hydroxide (NaOH) and finally agar-agar 0.8% (w/v) was added as a solidifying agent before autoclaving at 121°C.

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Table 1: MS media supplemented with different concentrations of growth hormones for callus induction in *Nothapodytes nimmoniana*.

IBA(mg/L)	KN (mg/L)	TDZ(mg/L)	Parameters for callus induction	
			Number of days for calli formation	Percent explants forming calli
1	-	1.5	50-55	80%
1.5	-	2.5	60-64	70%
-	-	1.5	65-70	60%

*Data shown are the mean of triplicates (repeated thrice)

2.3. Surface Sterilization of Explants

Leaves chosen as explants were washed with distilled water to remove dirt and debris. Thereafter it was surface sterilized with 0.5% Bavistin and 0.1 % Mercuric Chloride followed by 4-5 washings with autoclaved distilled water in laminar air flow. Both Bavistin and mercuric chloride are known to have antibacterial and antifungal activity. Explants were then cut into small segments using autoclaved blades. Whole procedure was carried out in laminar air flow chamber.

2.4. Callus Induction And Regeneration

Small incisions were given to the surface sterilized explants. These explants were cultured on MS media (as shown in "Fig.1 (A)" comprising of different growth hormone concentrations as stated above in "Table1". The cultures were kept in the growth chambers for 50-55 days. The callus mass was obtained which was sub-cultured on shooting MS media comprising of different growth hormone concentrations as stated below in "Table 2". The cultures were incubated at 16hrs light/ 8hrs dark cycle at 25±2°C in growth chamber of the Biotechnology Department of Jaypee University of Information Technology.

Table 2: MS media supplemented with different concentrations of growth hormones for shoot regeneration in *Nothapodytes nimmoniana*.

MS media + growth hormones			Parameters of shoot formation	
TDZ(mg/L)	IBA(mg/L)	KN(mg/L)	No. Of days for shoot formation	Percent of shoot formation
2	1	-	20-25	1- 2
2.5	1.5	-	18-20	2-3
-	1.5	2	12-15	3-4
-	2	1	10-15	4-5

*Data shown are the mean of triplicates (repeated thrice)

2.5. Encapsulation Of Mature Embryos

The embryos in the callus obtained after sub culturing was chosen for somatic seed generation. 3% sodium alginate solution was prepared in lukewarm which was later autoclaved. Similarly, Calcium chloride solution

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was prepared by dissolving 1.10gm of calcium chloride in 100ml of chilled autoclaved water in laminar air flow. Developed somatic embryo obtained by culturing were selected and put into the sodium alginate solution under sterile conditions and then transferred to the chilled calcium chloride solution causing formation of beads. Calcium chloride solution containing the beads was left undisturbed for 30 minutes. The whole process was carried out in laminar air flow.

Somatic Seed Germination

MS media supplemented with growth hormones in the ratio IBA+KN+GA3 (1:3:2) and 0.5% activated charcoal as indicated in "Table 3" was used as the germination medium for the encapsulated beads formed. The cultures were kept at 25±1C temperature, 16 hrs light to initiate the germination from the somatic beads formed. Data was recorded for the seed germination.

Table 3: MS media supplemented with different concentrations of growth hormones for seed germination in *Nothapodytes nimmoniana*.

MS media+ growth hormones				Parameter for seed germination
IBA	KN	GA3	%Activated charcoal	No. Of days for shoot formation
1	3	2	-	13-15
1	3	2	0.5%	10-13

*Data shown are the mean of triplicates (repeated thrice)

III. RESULT

3.1 Callusing and shoot regeneration

Best results for callus induction was seen in MS medium supplemented with growth hormones with ratio of IBA (1mg/L) and TDZ (1.5mg/L) as shown in " Fig.1B". 80% callusing was observed within 50-55 days on the media mentioned above. The callus obtained was sub cultured on shooting media where the best results for shoot regeneration i.e. 5-6 shoot primordia was obtained on MS medium containing IBA(1 mg/L) and KN (2 mg/L) within 10-15 days as shown in "Fig.1 C".

3.2 Artificial seed germination

Mature embryos form the callus were taken and used for the artificial seed formation using 3% sodium alginate and 100mM calcium chloride which was found best for the seed formation. The seeds so formed were germinated using MS medium containing growth hormones (IBA+KN+GA3) in the ratio (1:3:2) mg/L and (IBA+KN+GA3) in the ratio (1:3:2) mg/L and 0.5% activated charcoal [15]. Although the germination was observed in both the medium but the best suited growth was in medium IBA+KN +GA3 (1:3:2) mg/L without activated charcoal. In 10-13 days the seeds were successfully germinated as shown in "Fig.2 E, F".

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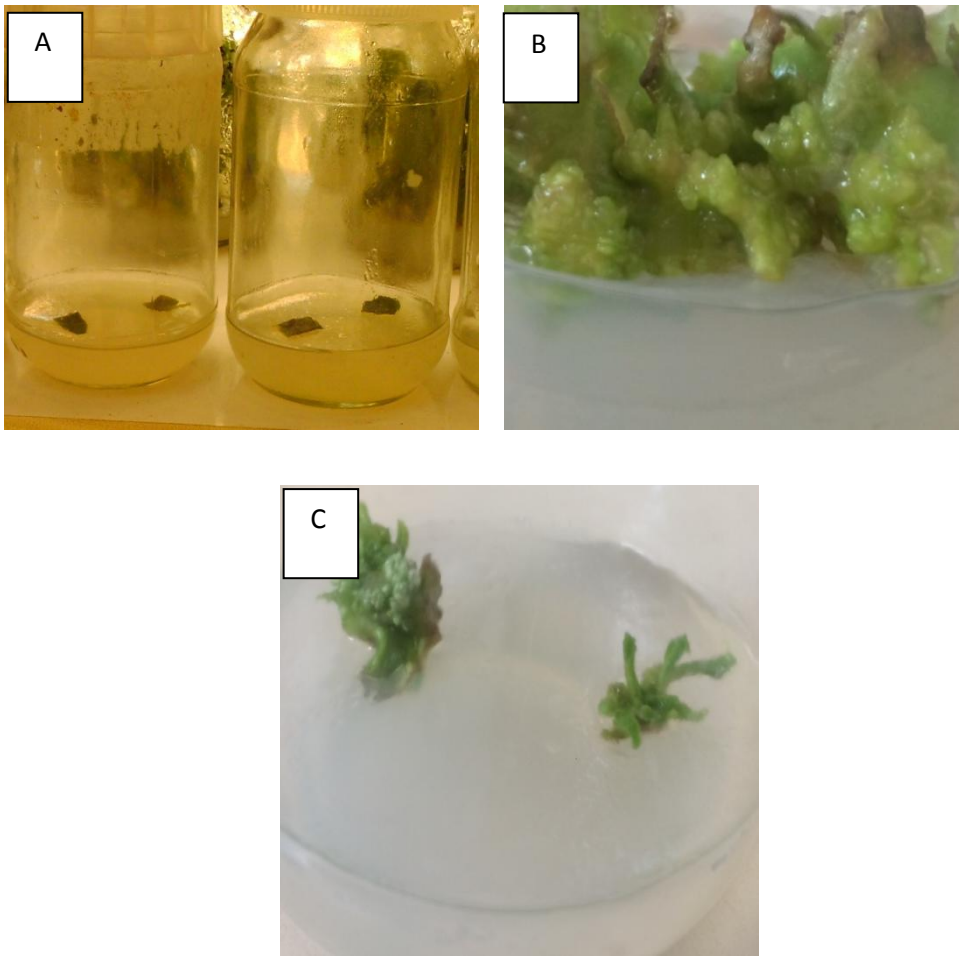

Fig. 1: Callus induction and regeneration of *Nothapodytes nimmoniana*, (A)Leaf explants on callusing medium, (B) Callus obtained on MS media supplemented with TDZ(1.5)mg/L+ IBA(1)mg/L within 50-55 days, (C) 4-5 shoot primodia on regeneration MS medium containing IBA(2)mg/L +KN(1)mg/L.

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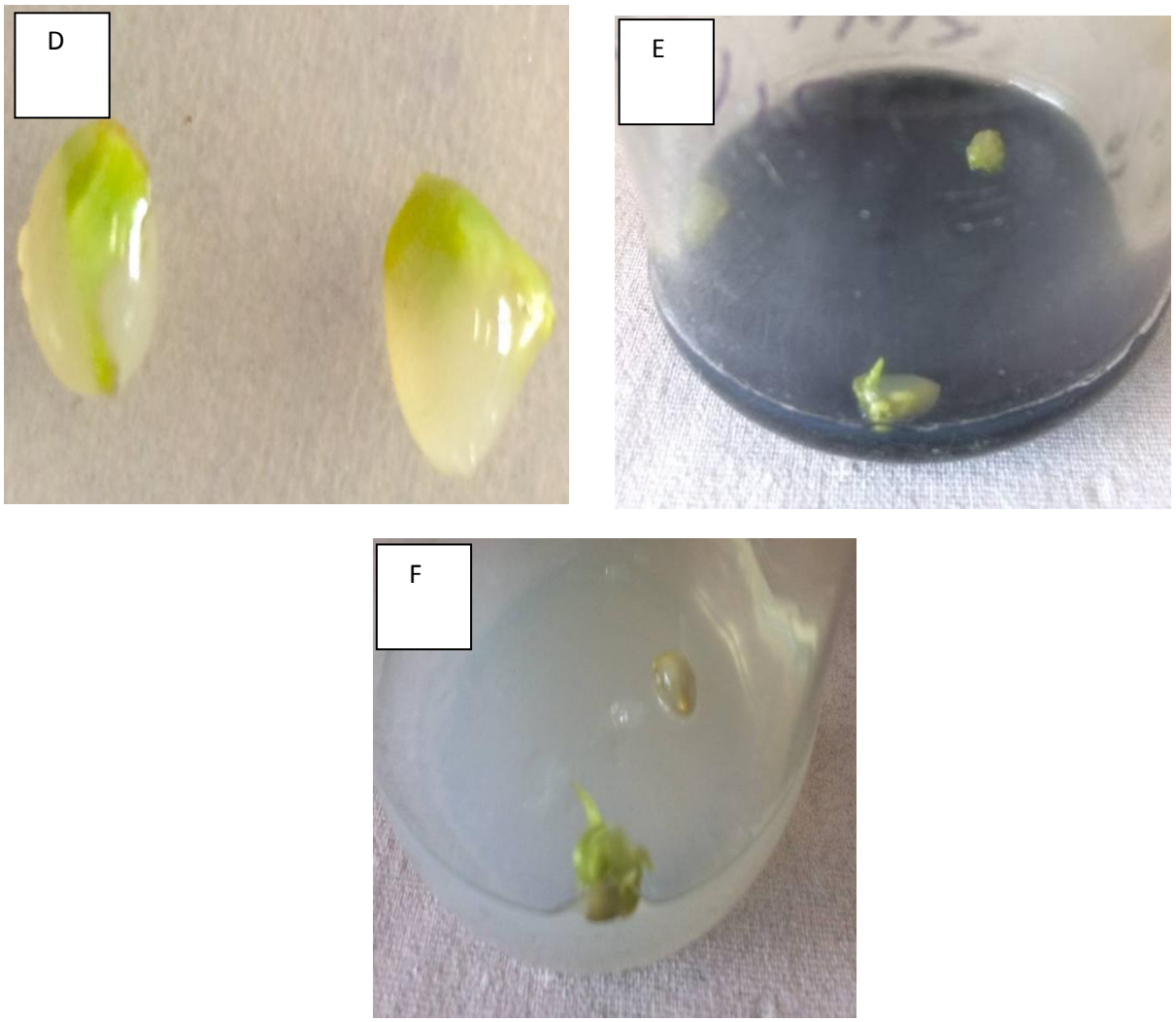

Fig. 2 (D) Encapsulated seed with the help of 3% sodium alginate and 100mM calcium chloride solution, (E) Emergence of shoot primordia from artificial seed in MS media supplemented with IBA+KN+GA3(1:3:2)mg/L and 0.3% activated charcoal, (F) Shoot initiation from artificial germinating seeds on MS+IBA+KN(1:3:2)mg/L.

IV. CONCLUSIONS

The present study is aimed at optimizing the culture conditions for the production of artificial seeds and their germination of a medicinally important plant *Nothapodytes nimmoniana*. To the best of our knowledge, this is the first report of artificial seed production and germination of *Nothapodyte nimmoniana*. Cellular differentiation properties of the somatc embryo cells into other plant organs have been exploited to make artificial seed and further its germination into shoots. Various hindrances have been reported in the cultivation of *Nothapodytes nimmoniana* such as its low seed germination rate even under the favourable environmental conditions attributed to its hard seed coat which makes the uptake of water and air difficult for the seeds resulting in low seed germination rate. The seeds produced through somatic embryogenesis will help in quick germination of the seeds due to the absence of hard seed coat which is not present in the somatic embryos.

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Artificial seeds production provides us with the advantage of readily available source of seeds throughout the year contrary to the natural process where seeds are formed once in a season. Apart from achieving the rapid mass propagation of *Nothapodytes nimmonina* seeds produced through somatic embryogenesis, it can also be used as an alternative for the camptothecin production which is one of the medicinally important alkaloid and is of great demand in the pharmaceutical world as it is used for the formulation of anti-cancer drugs. The optimized protocol for the production of artificial seeds and its germination can be utilized as an alternative technology of plant tissue culture for the conservation of the tree.

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