Somatic Embryogenesis and Plantlet Regeneration in Red Sandalwood (Pterocarpus Santalinus)

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Research Article

Keywords: Fabaceae, cotyledon, Histology, somatic embryo, callogenesis

Posted Date: February 13th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2007849/v2

Version of Record: A version of this preprint was published at Plant Cell, Tissue and Organ Culture (PCTOC) on March 22nd, 2023. See the published version at https://doi.org/10.1007/s11240-023-02491-w.

Abstract

Cotyledonary segments from the germinated immature zygotic embryos were used for somatic embryogenesis of red sandalwood (*Pterocarpus santalinus*). It was established on Murashige and Skoog (MS) medium containing 5% sucrose and amalgamation of 6-benzylaminopurine (BAP), 2,4-Dichlorophenoxyacetic acid (2,4-D), and α-Naphthaleneacetic acid (NAA). All treatments were responsive for callus induction with the frequency range between 36–97%. The maximum embryogenic frequency (69.44%) was obtained when 0.1 mg/l BAP + 2 mg/l 2,4-D and 0.1 mg/l BAP + 4 mg/l 2,4-D combinations were used. When explants were treated individually with growth regulators, the maximum embryogenic frequency (58.33%) was produced by 4 mg/l 2,4-D. BAP was completely ineffective for somatic embryogenesis when used individually. The average number of globular-staged somatic embryos ranged between 1–5 (irrespective of the treatments). The maximum number of the cotyledonary-staged somatic embryos (2.85) were obtained with treatment 0.1 mg/l BAP and 2 mg/l 2,4-D. The maximum plantlets were developed (1.30) when the cotyledonary-staged embryos from 0.1 mg/l BAP and 2 mg/l 2,4-D were transferred to MS basal medium. The plantlets obtained were acclimatized and showed 100% survival in the greenhouse condition. The embryonic cells have been histologically distinguished from non-embryonic cells with dense cytoplasm and a long suspensor. The induction, maturation and germination of somatic embryos were challenging, suggesting the need for molecular approaches through proteomic expression for mass production and understanding the evolution, structure, and genetic organization of the plant species.

Key message

2,4-D and NAA, either individually or in combination with BAP has a significant effect on embryonic potential of cotyledon explant of *P. santalinus*.

Introduction

Pterocarpus santalinus is a woody tree of the *Fabaceae* family, endemic to the Deccan part of the Indian peninsula, however, it is cultivated in China, Pakistan, Sri Lanka, and Philippines (Ahmedullah et al. 2019). It is an endangered species, listed in Endangered A2cd ver 3.1 of IUCN Red List, with an exceptional timber quality. The plant has been smuggled over time, mainly in China, Japan, and Myanmar, and hence cataloged in Appendix II of CITES (UNEP-WCMC 2017; Ahmedullah 2021). The hardwood is 16% saturated with a red-colored dye, known as santalin, used as a coloring agent in pharmaceutical, food, and leather industries (Arunkumar and Joshi 2014). The species is also a rich source of numerous phytometabolites, including glycosides, flavonoids, alkaloids, tannins, phenols, saponins, sterols, and triterpenoids (Arunakumara et al. 2011; Navada and Vittal 2014; Bulle et al. 2016). Shree et al. (2019) have reported the presence of two antitumor molecules, savinin and calocedrin. The plant products are in use as a folk medicine to cure skin infections, blood diseases, vision problems, diabetes, bile problem, and insect bites (Arunakumara et al. 2011; Azamthulla et al. 2015; Keshavamurthy et al. 2018; Rao et al. 2019; Shree et al. 2019). The extracts of red sandalwood have the potential to treat diabetic neuropathy

(Halim and Misra 2011) and also help in angiogenesis (Jadhav et al. 2011) and vasculogenesis (Jadhav et al. 2012). Because of its curative potential, the plant has been considered for the 21st meeting of the plant committee by RST (Review of Significant Trade) as a priority species for review (UNEP-WCMC 2017).

The natural regeneration of the plant is difficult due to hard pod, low fruit formation, stringent habitat, lengthened dormancy, and impoverished seed development (Rao and Raju 2002; Hegde et al. 2012; Arunkumar and Joshi 2014). Conventional regeneration methods like grafting, root cutting, and air layering proved futile (Chaturani 2006; Vijayalakshmi and Renganayaki 2017; Renganayaki et al. 2020). In this context, *in-vitro* techniques offer an alternative to red sandalwood propagation. Micropropagation of *P. santalinus* through direct organogenesis has been reported by many researchers (Anuradha and Pullaiah 1999; Arockiasamy et al. 2000; Prakash et al. 2006; Rajeswari and Paliwal 2008; Padmalatha and Prasad 2008; Balaraju et al. 2011; Warakagoda and Subasinghe 2013; Chen et al. 2019). The difficulty of obtaining somatic embryos in woody species is already an established fact (Isah 2019; Gulzar et al. 2020). Despite the problems reported, somatic embryogenesis has been reported in many woody species (Hu et al. 2017; Nunes et al. 2018; Sun et al. 2021; Xia et al. 2021; Yan et al. 2021). Individually, it has also been achieved in many leguminous plants (Singh and Chand 2003; Buendı´a-Gonza´lez et al. 2012; Viji et al. 2012; Kaul et al. 2014; Nolan et al. 2014), including *P. marsupium* (Husain et al. 2010). To our knowledge, there is no report of somatic embryogenesis in *P. santalinus*. This study documents the regeneration of plantlets by establishment of somatic embryogenesis in red sandalwood and histological analysis to differentiate embryonic and non-embryonic cells from cotyledon explants.

Material And Methods

Plant material and surface sterilization

Immature green-winged pods of *P. santalinus* were collected after 45 days of anthesis from a 15-year-old plant growing in the Andhra Pradesh Red Sandalwood Forest Patch (Latitude N 17^{o} 53´ 9.955˝, Longitude E 83^{o} 19´ 42.648˝) (Fig. 1A). The wings were excised from the pods (Fig. 1B), and rinsed under tap water for 10–15 minutes followed by disinfection with 1% (v/v) Savlon and 20 drops of Tween 20 for 10 min. After washing under running tap water, the surfaces were sterilized with 70% ethanol for 2 minutes, followed by treatment with 0.1% $HgCl_2$ for 15 minutes inside Laminar Air Flow (LAF; KEMI, India). Finally, it was rinsed with sterilized double-distilled water 4–5 times for 30 sec to 1 min. The immature zygotic embryos were dissected out by removing the hard outer layer of the pods with the help of a wire cutter (Fig. 1C). It was then placed on MS (Murashige and Skoog, 1962) medium for germination. After 28 days, the cotyledon sections of the size 0.5–1 cm were sliced out from the 5–6 cm height plantlet and used as an explant to induce somatic embryos (Fig. 1D).

Culture media and culture condition

The explants were cultured in test tubes (25×150 mm, BOROSIL) containing 15 ml of MS basal media adjusted to pH 5.8 ± 0.2 using 1N NaOH before autoclaving at 121ºC for 15 minutes. The media was added with BAP (6-Benzylaminopurine; PCT0802, HIMEDIA) at 0.01, 0.1, 1.0, 2.0, 4.0 mg/l; 2,4-D (2,4-Dichlorophenoxyacetic acid; PCT0825, HIMEDIA) at 0.01, 0.1, 1.0, 2.0, and 4.0 mg/l; NAA (α-Naphthaleneacetic acid; PCT0809, HIMEDIA) at 0.01, 0.1, 1.0, 2.0, and 4.0mg/l or combination of all of them. 5% (w/v) sucrose (PCT0607, HIMEDIA) and 1% (w/v) agar powder (PCT0901, HIMEDIA) were added to all induction medium. The basal media without PGR (plant growth regulator) were kept as a control for comparing results. The cotyledons were placed in contact with the culture medium. All the cultures were kept in an air-conditioned tissue culture room at 25 ± 2°C, 60–65% relative humidity, and a 16/8 hr (light/dark) photoperiod under 40 W cool white fluorescent tube- light at 70 µM $m^{-2}s^{-1}$. Each treatment was replicated thrice with 12 cotyledon segments per replication, which were performed from July 2019 to January 2022, and the results were recorded after 12 weeks of culture.

Cotyledonary-staged somatic embryos were transferred to full-strength MS basal media as well as to the media containing BAP (0.5, 1.0, and 2.0 mg/l) and 2,4-D (0.5, 1.0, and 2.0 mg/l) individually and in combinations. All the treatments were fortified with 3% (w/v) sucrose and 1% (w/v) agar powder for germination and kept in air-conditioned tissue culture room maintained at, 25 ± 2°C, 16/8 hr (light/dark) photoperiod receiving 70 µM$m^{-2}s^{-1}$ with 40 W cool white fluorescent tube lights for 3–4 weeks.

Acclimatization

The plantlets obtained from conversion of somatic embryos from all the applied treatments were further acclimatized in the laboratory and greenhouse condition. First of all, the plantlets were drawn-out from the agar media very carefully so not to break its root or root hairs. The roots of the plantlets were then cleaned lightly under tap-water to remove any attached media to it. Thereafter, the roots were treated with an antifungal, Bavistine (0.5%, w/v), for approximately 3 minutes. After the treatment the plantlets were shifted to a transparent plastic glass of height 9 cm and width 7 cm containing a combination of garden soil and peat (1:1, w/w). To maintain the humidity the plantlets were covered with another plastic glass in an upside-down position and shifted to culture room (25 ± 2°C and 16/8 hr (light/dark) photoperiod) for 1–2 weeks. During this period humidity was progressively decreased by removing the upper glass cover. The well-acclimatized plantlets were selected for further acclimatization wherein the plantlets were shifted to laboratory condition for another 4 weeks. The soil mixtures used during this period was garden soil and peat in 1:1 (w/w) ratio. As the plants grew in height, the acclimatized plantlets were shifted to bigger pots progressively and finally transferred to greenhouse for further development.

Growth measurement

The growth for callus and somatic embryo development were measured based on the callogenic and embryogenic frequency respectively. The embryogenic responses were further measured as (i) average number of globular-staged embryos, (ii) average number of cotyledonary-staged embryos, and (ii) average number of plantlets per treatment. The response parameters were calculated as:

Callogenic frequency (%) = No. of explants that produced callus / Total number of explants × 100

Embryogenic frequency (%) = No. of explants that produce embryogenic callus / Total number of explants × 100

Histological studies

The embryonic cells were observed fresh by placing it on a glass slide with the help of an inoculating loop. The cells were then stained with 2% (w/v) acetocarmine for 30 sec. The staining solution was washed with distilled water and 1–2 drops of glycerol were added before mounting with a cover slip. The microscopy was performed under light microscopy (CH20 i, Olympus, Japan).

Statistical analysis

The results for all the experiments were statistically analyzed using SPSS (IBM, version 19.0). Mean with the standard error was calculated with Microsoft Excel (2016 version) and presented in tabular form. The average callogenic frequency, embryogenic frequency, globular-staged embryos per treatment, cotyledonary-staged embryos per treatment, and plantlets generated per treatment were subjected to analysis of variance (ANOVA). Significant differences among means were evaluated at $p \leq 0.05$ level by a Duncan multiple comparison test.

Results

Somatic embryogenesis induction

The experiments were divided into individual treatments or combinations of auxins and cytokinin (BAP, 2,4-D, and NAA). It was observed that though all the concentrations of BAP (when used alone) were significantly effective for callus formation, were unresponsive for somatic embryo formation. The maximum callogenic frequency (95.37 ± 2.44%) was obtained at 0.1 mg/l BAP (Fig. 2). The individual treatment with auxins either 2,4-D or NAA, was found effective (at significance $p < 0.05$) for induction of somatic embryogenesis as evident by the embryogenic callus formation (Fig. 1E). The maximum callogenic frequency (95.37 ± 2.44%) obtained at 1 mg/l 2,4-D showed the least frequency of embryogenic callus formation (19.44 ± 2.77%). On the contrary, though the callogenic frequency obtained at 4 mg/l 2,4-D was less (67.13 ± 2.82%) compared to the treatment of 1 mg/l 2,4-D, but it was more significant for embryogenic callus formation with 58.33 ± 4.81% frequency (Fig. 3A). NAA was found significantly productive for somatic embryogenesis at 2 mg/l and 4 mg/l concentration (Fig. 4A). The MS basal media without any growth regulator (control) was not significant in forming both non-embryogenic or embryogenic callus. It was observed that the explant (cotyledon) remained unresponsive in the absence of any growth regulator (Fig. 1F). Among all individually tested growth regulators, 4 mg/l 2,4-D proved to be significantly more efficient for induction of somatic embryogenesis.

The combination of cytokinin BAP with auxin 2,4-D or NAA was also tested for somatic embryogenesis induction. The treatments were responsive for callus induction with the frequency range between 50–

82%. The maximum callogenic frequency (82.41 ± 4.04%) was obtained at 0.1 mg/l BAP + 2 mg/l 2,4-D (Table 1). The highest embryogenic frequency (69.44 ± 2.77%) was obtained at 0.1 mg/l BAP + 2 mg/l 2,4-D which do not differ significantly from the result obtained at 0.1 mg/l BAP + 4 mg/l 2,4-D (Table 1). Independent of the concentrations of BAP used (0.01 and 0.1 mg/l), the presence of 2,4-D (1, 2, and 4 mg/l) or NAA (2 and 4 mg/l) significantly ($p < 0.05$) increased the frequencies of embryogenic induction, except for the combination 0.01 mg/l BAP + 4 mg/l 2,4-D (Table 1).

Table 1
Influence of combination of BAP × 2,4-D and BAP × NAA on frequency of callogenesis and somatic embryogenesis, average number of globular-staged embryos, average number of cotyledonary-staged embryos, and conversion to plantlets

Growth regulators (mg/l)			Frequency of callogenesis (%)	Frequency of embryogenesis (%)	Average No. of Globular-staged embryos per treatments	Average No. of Cotyledonary-staged embryos per treatments	Average No. of plantlets per treatments (after transferring to MS basal)
BAP	2,4-D	NAA					
0.00	0.00	-	0.00 ± 0.00^{f}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}
0.01	1.00	-	73.15 ± 0.93^{b}	50.00 ± 4.80^{c}	2.62 ± 0.10^{c}	1.83 ± 0.05^{c}	0.85 ± 0.02^{d}
0.01	2.00	-	71.30 ± 2.45^{b}	61.11 ± 2.78^{ab}	2.84 ± 0.10^{c}	2.62 ± 0.10^{a}	1.08 ± 0.05^{bc}
0.01	4.00	-	69.45 ± 2.78^{d}	52.77 ± 2.77^{bc}	4.85 ± 0.04^{a}	2.84 ± 0.09^{a}	1.24 ± 0.05^{ab}
0.10	1.00	-	57.41 ± 4.90^{e}	50.00 ± 4.80^{ab}	4.85 ± 0.04^{a}	2.38 ± 0.09^{b}	1.01 ± 0.07^{c}
0.10	2.00	-	82.41 ± 4.04^{a}	69.44 ± 2.77^{a}	3.62 ± 0.10^{b}	2.85 ± 0.04^{a}	1.30 ± 0.07^{a}
0.10	4.00	-	75.00 ± 4.81^{b}	63.89 ± 2.78^{a}	4.85 ± 0.04^{a}	2.18 ± 0.04^{b}	1.13 ± 0.06^{bc}
0.01	-	1.00	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}
0.01	-	2.00	50.00 ± 3.21^{c}	36.11 ± 2.78^{d}	1.83 ± 0.05^{c}	1.00 ± 0.08^{c}	0.92 ± 0.12^{a}
0.01	-	4.00	60.19 ± 3.34^{b}	47.22 ± 2.77^{c}	2.85 ± 0.04^{a}	1.84 ± 0.08^{a}	0.94 ± 0.04^{a}
0.10	-	1.00	0.00 ± 0.00^{d}	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}	0.00 ± 0.00^{d}
0.10	-	2.00	75.00 ± 4.81^{a}	52.77 ± 5.55^{b}	2.38 ± 0.09^{b}	1.54 ± 0.10^{b}	0.68 ± 0.09^{c}

Standard errors of means are indicated. Values followed by the same letter are not significantly different by Duncan multiple comparison test ($p \leq 0.05$).

Growth regulators (mg/l)			Frequency of callogenesis (%)	Frequency of embryogenesis (%)	Average No. of Globular-staged embryos per treatments	Average No. of Cotyledonary-staged embryos per treatments	Average No. of plantlets per treatments (after transferring to MS basal)
BAP	2,4-D	NAA					
0.10	-	4.00	72.22 ± 2.78[a]	61.11 ± 2.78[a]	1.83 ± 0.05[c]	1.11 ± 0.06[c]	0.74 ± 0.04[b]

Standard errors of means are indicated. Values followed by the same letter are not significantly different by Duncan multiple comparison test ($p \leq 0.05$).

Cream-colored, shiny, translucent, smooth-surfaced, and compact somatic embryos developed through callus formation from the cut edges of the cotyledonary explant. The development of somatic embryos was observed to be initiated by the end of 5th week. Somatic embryo formed and matured asynchronously through various developmental stages; globular (Fig. 1G), heart (Fig. 1H), torpedo (Fig. 1I), and cotyledonary (Fig. 1J). Some treatments, particularly with higher NAA concentration (individually or in combination), resulted in direct shoot formation.

It was observed that a smaller number of globular-staged somatic embryos were produced from the callus obtained on the surface of the explant, and still, the lesser number of the globular-staged embryos matured to the cotyledonary-staged somatic embryos. The average number of globular-staged somatic embryos ranged between 1–5 irrespective of the treatments (individual auxin treatments and an auxin and a cytokinin combination treatments). The maximum average number of a cotyledonary-staged embryos (1.53 ± 0.04) were obtained at 4 mg/l 2,4-D (Fig. 3B). The NAA produced a maximum of 1.12 ± 0.05 cotyledonary-staged somatic embryo at 4 mg/l (Fig. 4B). Combination of 0.1 mg/l BAP with 2 mg/l 2,4-D produced significantly ($p < 0.05$) higher (2.85 ± 0.04) number of cotyledonary-staged somatic embryos (Table 1). However, this data did not differ significantly from the data obtained with the treatment of 0.01 mg/l BAP + 2 mg/l 2,4-D or 0.01 mg/l BAP + 4 mg/l 2,4-D. The combination of two growth regulators, a cytokinin and an auxin, showed an enhanced effect for the induction and development of somatic embryos.

Somatic embryo conversion to plantlet

The conversion of somatic embryos, developed from cotyledons of *P. santalinus*, to plantlets were unproductive on the hormonal medium (data not shown). Therefore cotyledonary-staged somatic embryos were transferred to MS basal medium (Fig. 5A and 5B). The somatic embryos were converted into a complete plantlet with the concurrent development of a shoot and root (Fig. 5C, 5D, and 5E). It was also noticed that sometimes, instead of developing into complete plantlet, the matured cotyledonary-staged somatic embryos developed into either shoots or roots. Transferring embryos from individual PGR treatment to basal media did not support somatic embryo conversion. The plantlets were generated only

when embryos were transferred from combinations of PGR treatments to basal media with the highest number of average plantlets (1.30 ± 0.07) obtained at 0.1 mg/l BAP + 2 mg/l 2,4-D (Table 1).

Acclimatization of plantlets

Though on an average a maximum of only 1.30 plantlets were formed from somatic embryo conversion, it was successfully established during acclimatization in laboratory conditions and greenhouse environment. The plantlets produced from conversion of somatic embryos, generated from all the applied treatments, were acclimatized well and survived 100%. Under laboratory condition in plastic bottles the plantlets grew at the height of 6–7 cm, developed 2–3 leaflets, and a thick root (Fig. 5F). After transferring to a progressively bigger pots, it was observed that plants also grew progressively in height to 28 cm (Fig. 5G). The number of leaves also increased in number and size. Finally, after being transferred to greenhouse the plants grew slowly but developed healthy (Fig. 5H).

Histological studies

Histological analysis helped to differentiate between embryogenic and non-embryogenic cells with the former having dense cytoplasm and the latter having large vacuoles (Fig. 6A). Somatic embryos showed the presence of protoderm and accumulation of meristematic cells (Fig. 6B). Globular structures were developed after 5 weeks of induction (Fig. 6C). The transition from globular to heart-stage showed embryogenic cells attached with a suspensor (Fig. 6D). The polar migration of auxin with organization of embryogenic tissues towards periphery with globular embryo at the tip marked the importance of the mechanism called polar-auxin transport (Fig. 6E).

Discussion

The observations of the study highlighted the importance of growth regulators and induction period for the establishment of somatic embryogenesis in *P. santalinus*. The importance of auxins, especially 2,4-D, was already confirmed for somatic embryogenesis in case of woody (Mehta et al. 2011; Nunes et al. 2018) and *Fabaceous* trees (Han and Park 1999; Trigiano et al. 1999; Buendı´a-Gonza´lez et al. 2012). In *P. marsupium*, Husain et al. (2010) used hypocotyl explant and showed the formation of somatic embryos by 5 µM 2,4-D with 1 µM BAP. Lakshmi Sita et al. (1980) obtained embryogenic callus upon induction with 1 mg/l 2,4-D using shoot pieces as explant in sandalwood. Our observation also follows these results proving the efficiency of 2,4-D for somatic embryogenesis. Many researchers assessed the potency of other auxins for somatic embryogenesis in woody species (Dunstan et al. 1995; Kendurkar et al. 1995) and *Fabaceae* plants (Canhoto et al. 2006). Auxins proved to be an efficient group of PGRs for somatic embryogenesis, probably because of its role in cell cycle, division, and differentiation (Lemes da Silva et al. 2021). 2,4-D reported as one of the most suitable auxins for somatic embryogenesis might be because of its role in DNA hypermethylation (Ebrahimi et al. 2018). The result of the present study reported better somatic embryogenesis when cytokinin and auxins were used in combinations, whereas, cytokinin alone found to be inefficient for the induction. The similar result has also been reported by Canhoto et al. (2006) in carob, where they observed somatic embryogenesis when BAP and IAA have

been used in combinations, whereas, the individual use of hormones proved ineffective. Auxins and cytokinin both play a vital role to regulate the embryogenic response which also depends upon the internal hormonal supply that varies from species to species and explant to explant (Verma et al. 2016). In the current study cotyledon explants from germinated immature zygotic embryo were used for the production of somatic embryos. Immature cotyledons provide a developmental window that can be utilized for somatic embryogenesis (Trigiano et al. 1999). Canhoto et al. (2006) showed the importance of explant for induction of somatic embryogenesis while working with carob. The difference of *in-vitro* condition or the genotype of the plant and the explant have a significant effect on the successful induction and maintainance of somatic embryogenesis (Canhoto et al. 2006). Gulzar et al. (2020) reported that though with the advancement of technology the difficulty in establishment of somatic embryogenesis has been overcome, but still, most of the woody species are either remain recalcitrant or respond poorly for the same. They also mentioned the incapability of embryonic cells to develop into complete plantlets. The same has been validated by our results with lesser number of the globular-staged embryos, cotyledonary-staged embryos and plantlets.

The period for induction of somatic embryogenesis varies in different plant species. Some plants require a short period of induction (Sharry et al. 2006), whereas others require prolonged induction (Wang et al. 2003). In the current study the somatic embryos were obtained after prolonged induction of 12 weeks. Singh and Chand (2003) obtained 26.5 average number of somatic embryos after 15 weeks of culture in half-strength MS media. Sucrose is also an important factor for the induction of somatic embryogenesis because it acts as an osmotic stress (Verma et al. 2016). The author's group also worked with 3% sucrose on the same explant and resulted in non-embryonic callus, the results are already being published (Chakraborty et al. 2022). Our current observations and results are in accordance with Verma et al. (2016), who obtained a 10% increase in embryogenic callus frequency with 5% sucrose. Higher sucrose concentration was also reported in other plant species, e.g., grapevine (Li et al. 2014), *Hevea brasiliensis* (Srichuay et al. 2014), sandalwood (Herawan et al. 2014), *Amorphophallus konjac* (Li et al. 2021) and *Cambod tea* (Mishra et al. 2022). Sucrose interacts with PGRs and help in growth and development process of plants. It also helps in metabolic processes of plants (León and Sheen 2003; Skylar et al. 2011). Koch (1996) reported the function of sucrose in transcriptional, post-transcriptional, and post-translational processes. Sucrose enhances the expression of the genes controlled by promoters like patatin and phloem-specific rolC (Jeferson et al. 1990; Yokoyama et al. 1994).

The success of micropropagation through somatic embryo formation depends on its development and final conversion to plantlets, steps which remains a challenging work for many woody species (Isah 2019). The same has been noticed for *P. santalinus* which prevented from getting a large number of plantlets from the conversion of somatic embryos. Some somatic embryos evolved in shoots or roots on the conversion medium, indicating incomplete maturation and incapability in completing the morphogenic process. Weaver and Trigiano (1991) worked with a *Fabaceae* plant, *Cladrastis lutea*, and established the lack of somatic embryo conversion to plantlet. The conversion problem might be due to the anomalous shoot apex formation, which in turn depends on the type of auxin and the duration of PGR it is exposed to (Weaver and Trigiano 1991). Similar observations have also been reported by Canhoto et

al. (2006) in carob, a *Fabaceae* species stating the defect in embryo maturation and meristem formation. The mature cotyledonary stage somatic embryos germinated to complete plantlets with prominent roots and shoot growth in the basal medium supplemented with 3% sucrose. Singh and Chand (2003) worked with a timber-generating leguminous plant, *Dalbergia sissoo* Roxb., and successfully achieved 50% of embryo conversion using cotyledon as explant. They used 10% sucrose for the maturation and 2% sucrose for the germination of the matured somatic embryos. High concentration of sucrose prior to the germination improves the process as it acts as a signal for biogenesis of stored proteins (Singh and Chand 2003). On the contrary, the high sucrose concentration may inhibit germination, if present in the germination medium, due to osmotic shock (Verma et al. 2016). As observed by our experiment that the matured somatic embryos generated from the combinations of PGR treatments converted into plantlets showed the importance of induction medium on successful conversion of somatic embryos. Lemes da Silva et al. (2021) reported that the treatment of 18.1 µM 2,4-D + 4.5 µM BAP resulted in maximum somatic embryos and also being the only treatment that regenerated plantlets after their transfer to basal media. Hence, the development of somatic embryos and its successful conversion also depends on various factors like the explant, growth regulators, and duration of the use of growth regulators.

The histological analysis helped to differentiate between embryogenic and non-embryogenic callus formed simultaneously from the same explant. It showed the presence of a dense cytoplasm within the parent cell wall, a characteristic feature of somatic embryos. The same characteristics were also evident in embryogenic cells of other species (Nunes et al. 2018; Sun et al. 2021). The polar migration of embryonic callus towards the tip consisting of meristematic cells was also evident during somatic embryogenesis in zygotic embryo of *Cunninghamia lanceolata* (Hu et al. 2017). As acetocarmine is used to detect DNA and chromatin, it can easily differentiate embryonic cells from non-embryonic cells and also from the attached suspensor. It helps to tract the polarly movement of the embryonic callus towards the tip (Hasbullah et al. 2007). The attached suspensor with embryonic cells, a characteristic feature of somatic embryos, has also been shown by Xia et al. (2021) during the work on masson pine (*Pinus massoniana*). An early phase of somatic embryo development with dense cytoplasm at embryonal end with long suspensor has been shown by Arya et al. (2000) while working with *Pinus roxburghii* Sarg. Many other researchers also used acetocarmine to differentiate between proembryonal, embryonal, and non-embryonic tissue (Fráterová et al. 2013; Hazubska-Przybył et al. 2020).

Conclusion

The obtained result is the first approach for regeneration of *P. santalinus* through somatic embryogenesis. It showed that the red sandalwood can be regenerated from somatic embryos. However, future studies are required to increase the frequency of somatic embryos formed and converting them into plantlets. Apart from this, the molecular approaches through proteomic expressions are needed to optimize the protocol for mass production at a commercial scale and to understand the evolution, structure, and genetic organization of this plant species. In conclusion the present study can be a step ahead in the large-scale propagation regenerated through somatic embryogenesis to overcome the endangered status of *P. santalinus* species.

Abbreviations

2,4-D: 2,4-Dichlorophenoxyacetic acid

BAP: 6-Benzylaminopurine

MS: Murashige and Skoog

NAA: α-Naphthaleneacetic acid

PGR: Plant growth regulator

Declarations

ACKNOWLEDGEMENT

The financial support provided by SCIENCE & ENGINEERING RESEARCH BOARD (SERB), Department of Science and Technology, Government of India for the major research project under a core research grant to Dr. Nasim Akhtar (Principal Investigator) and Dr. K. Viswanatha Chaitanya (Co-investigator) (sanction order no. CRG/2018/000517 dated 24.6.2019) is gratefully acknowledged.

AUTHOR'S CONTRIBUTION

The manuscript's concept, layout, and design are conceived by the corresponding author Dr. Nasim Akhtar. The whole manuscript, including the table and text, is developed, written and revised by the first author Tanushree Chakraborty. The manuscript was reviewed and edited several times by authors Dr. K. Viswanatha Chaitanya and Dr. Nasim Akhtar.

DATA AVAILABILITY STATEMENT

Data will be made available by the corresponding author on reasonable reason request.

CONFLICT OF INTEREST

All the authors declared that there is no conflict of interest concerning any part of the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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Figures

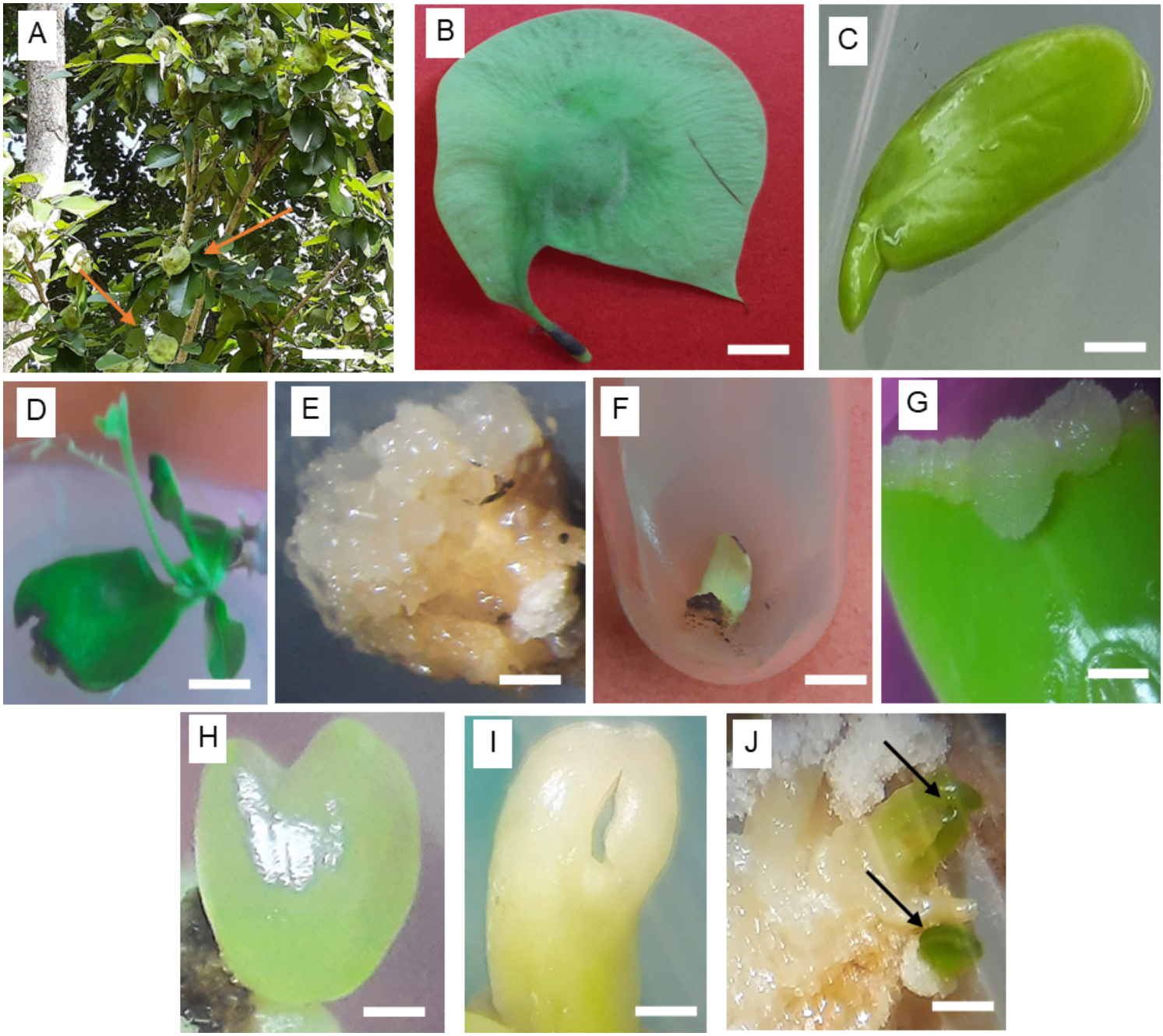

Figure 1

Morphological aspects of somatic embryo formation in *P. santalinus*. (A) Fifteen years old plants growing at Andhra Pradesh Red Sandalwood Forest Patch in Visakhapatnam showing pods (arrows) (bar = 6.38 cm), (B) Immature green pods collected to dissect immature zygotic embryo (bar = 0.81 cm), (C) Green-

colored immature zygotic embryo (bar = 0.27 cm), (D) Formed plantlet from immature zygotic embryo (bar = 0.29 cm), (E) Cream-colored shiny compact embryogenic callus (bar = 0.50 mm), (F) Unresponsive cotyledon in control treatment (bar = 0.50 cm), (G) Globular somatic embryos (bar = 0.30 mm), (H) Heart-shaped somatic embryos (bar = 0.80 mm), (I) Torpedo-shaped somatic embryo (bar = 0.60 mm), (J) Cotyledonary stage somatic embryo (bar = 0.30 mm).

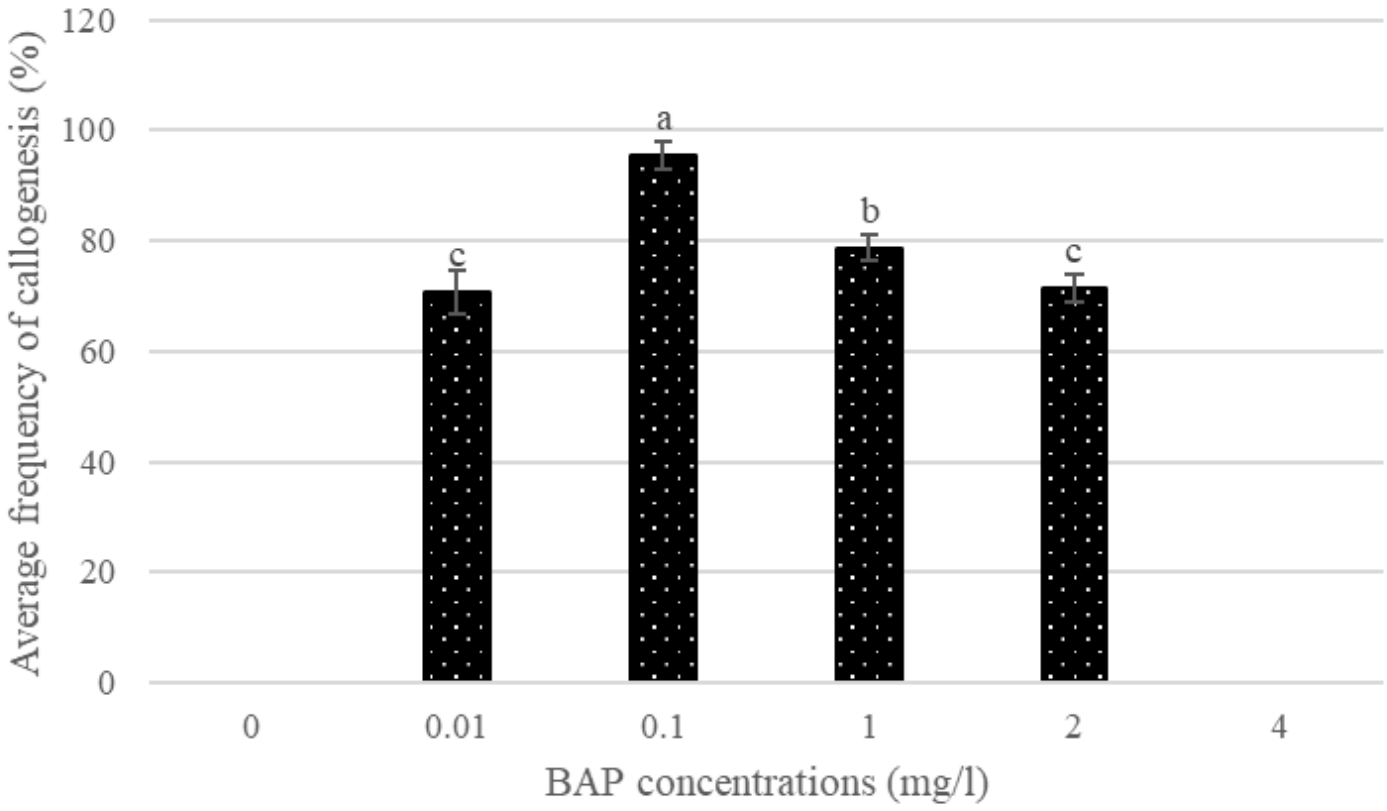

Figure 2

Influence of BAP on frequency of callogenesis. Standard errors of means are indicated. Values followed by the same letter are not significantly different by Duncan multiple comparison test ($p \leq 0.05$).

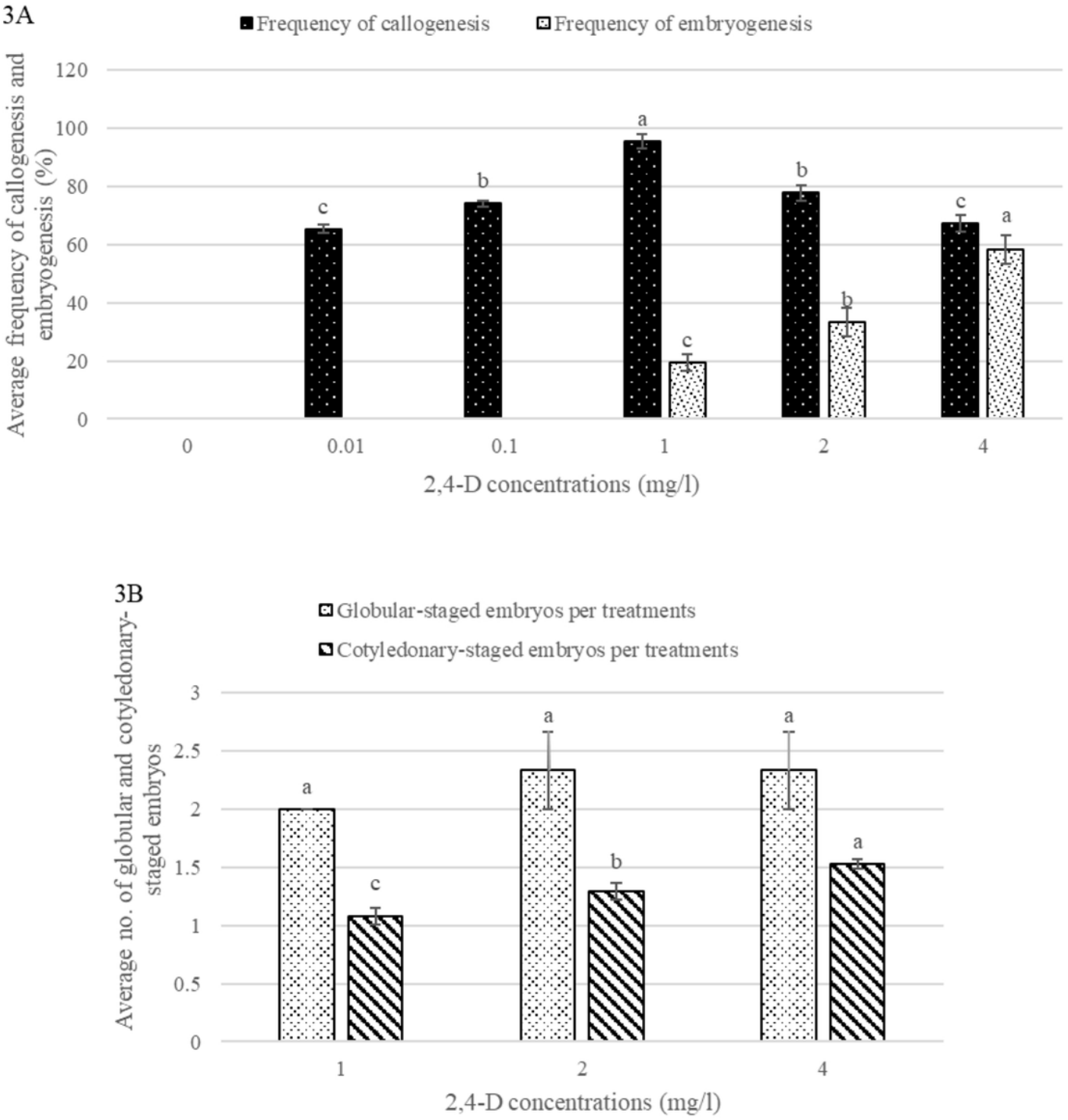

Figure 3

Influence of 2,4-D on frequency of callogenesis, somatic embryogenesis, and somatic embryo maturation. (A) Graph showing effect of 2,4-D on average frequency of callogenesis and somatic embryogenesis, (B) Graph showing effect of 2,4-D on the formation of average number of globular-staged and cotyledonary-staged somatic embryos. Standard errors of means are indicated. Values followed by the same letter are not significantly different by Duncan multiple comparison test ($p \leq 0.05$).

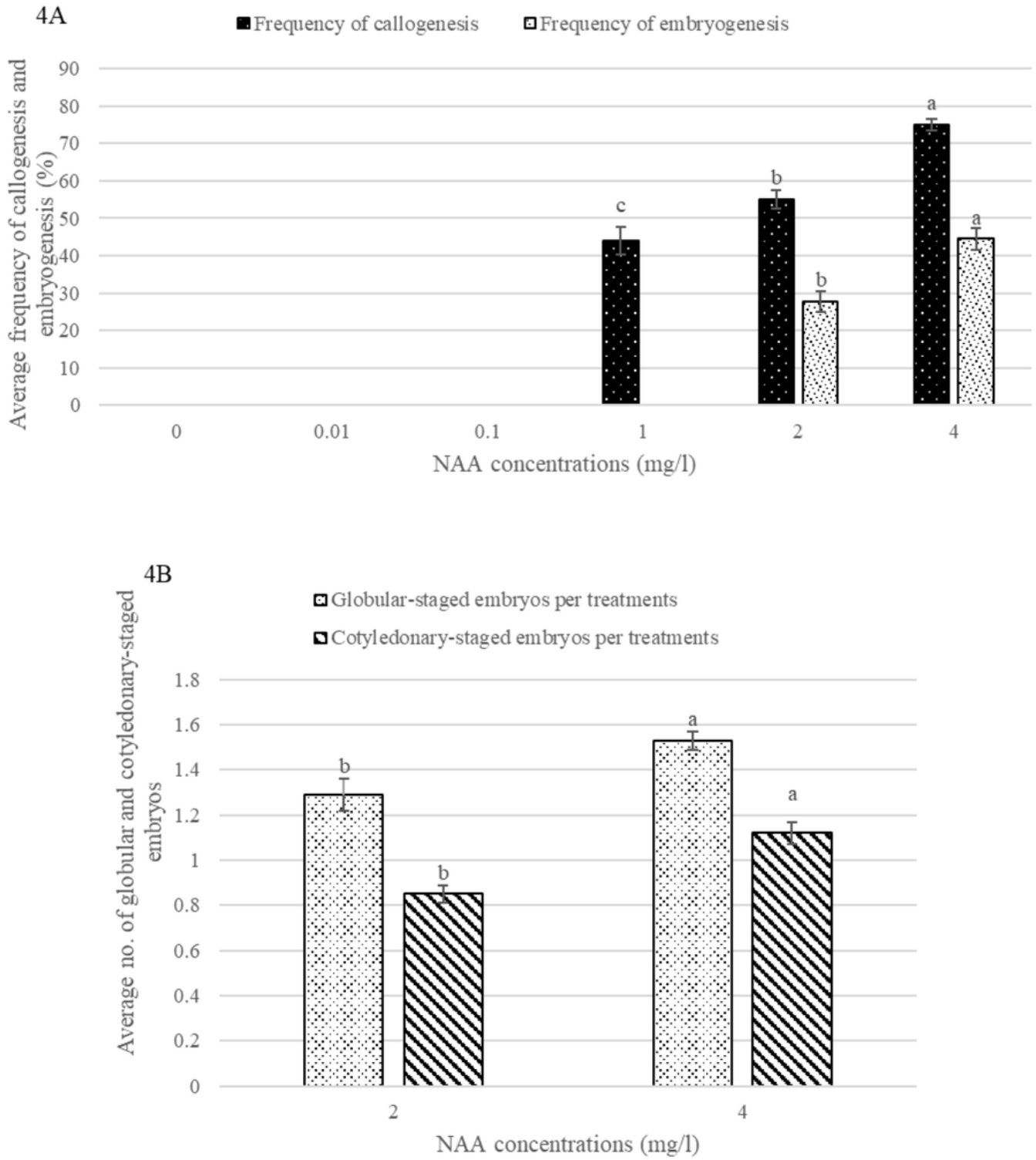

Figure 4

Influence of NAA on frequency of callogenesis, somatic embryogenesis, and somatic embryo maturation. (A) Graph showing effect of NAA on average frequency of callogenesis and somatic embryogenesis, (B) Graph showing effect of NAA on the formation of average number of globular-staged and cotyledonary-staged somatic embryos. Standard errors of means are indicated. Values followed by the same letter are not significantly different by Duncan multiple comparison test ($p \leq 0.05$).

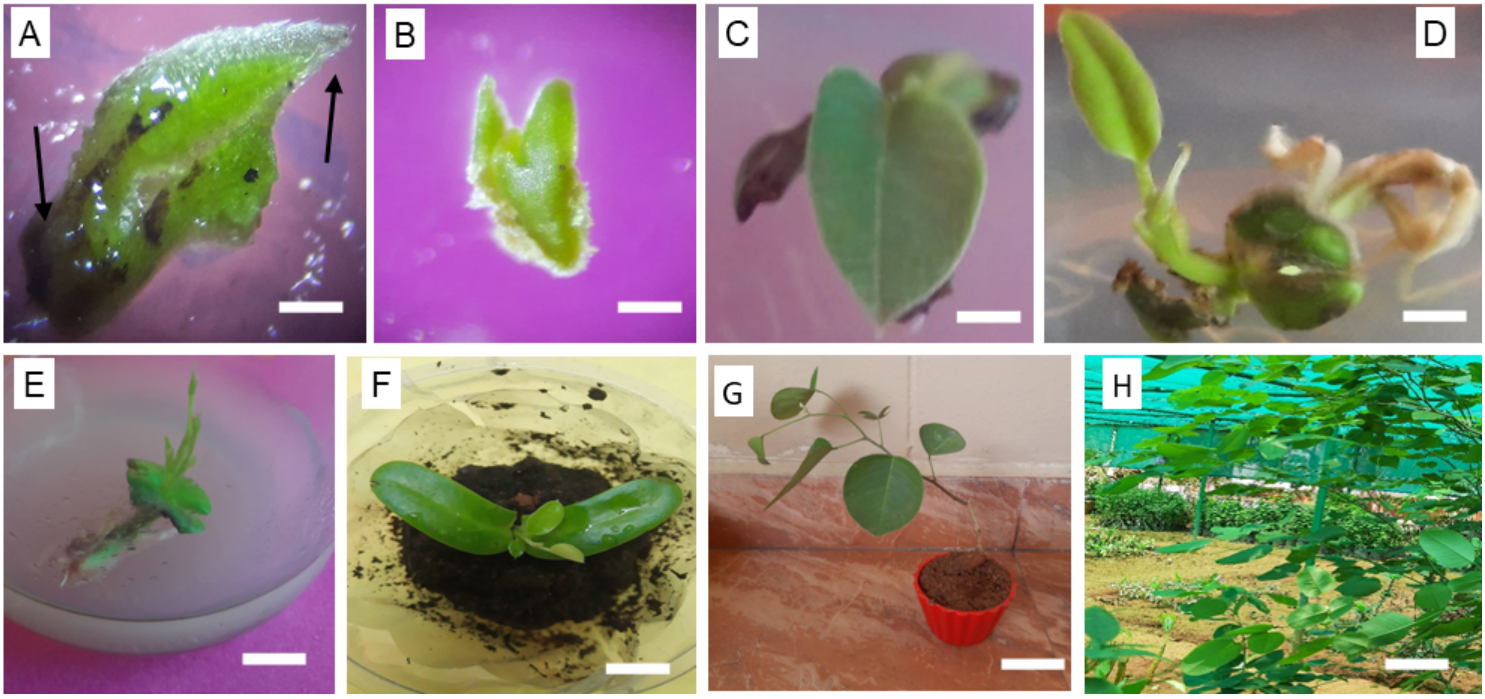

Figure 5

Somatic embryo conversion and plant formation. (A) Cotyledonary embryo showing shoot and root apex (arrows) (bar = 1 mm), (B) Isolated singular cotyledonary-staged somatic embryo (bar = 0.50 mm), (C) New leaves obtained from conversion of a somatic embryo (bar = 0.58 cm), (D and E) Plantlet formation of *P. santalinus*, with concurrent development of shoot and root, obtained by somatic embryogenesis after 15 weeks ofculture initiation ((D) bar = 0.35 cm and (E) bar = 0.25 cm), (F) Plantlet under acclimatization after 18 weeks of culture initiation (bar = 1.45 cm), (G) Development of plantlet under laboratory conditions after 20 weeks of culture initiation (bar = 9.50 cm), (H) Plant under greenhouse condition (bar = 9.09 cm).

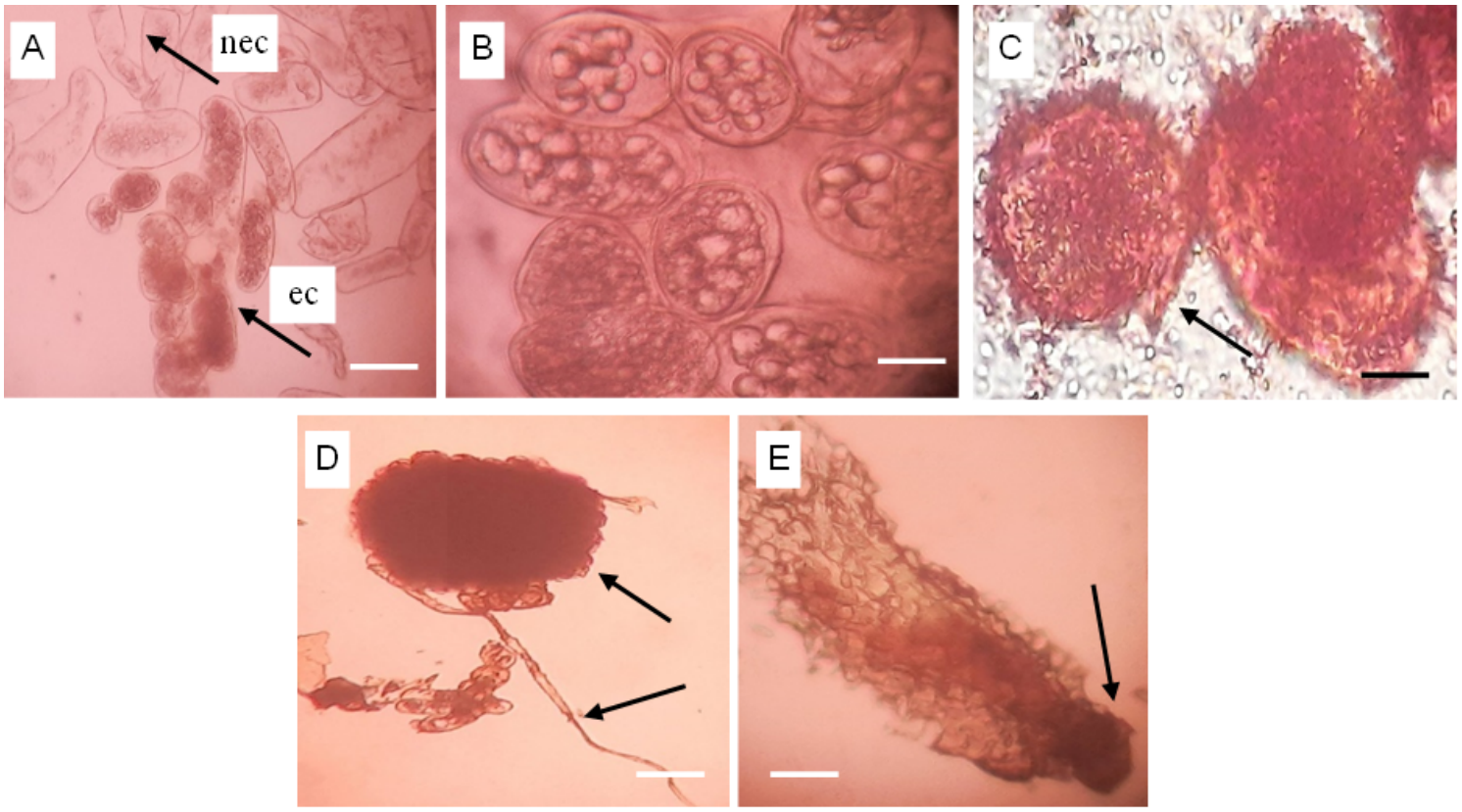

Figure 6

Histological analyses of somatic embryo differentiation. (A) Dense cytoplasm of embryonic cell (ec, arrow) surrounded by non-embryonic cells (nec, arrow) (bar = 20 µm), (B) Typical characteristic of embryonic cell having protoderm and accumulated meristematic cells (bar = 10 µm), (C) Globular embryo under microscope (arrow) (bar = 10 µm), (D) Globular to heart-stage embryo transition with attached suspensor (arrows) (bar = 8 µm), (E) Polar movement of embryogenic cells (arrows) (bar = 10 µm).