



Analysis of regeneration protocols for micropropagation of *Pterocarpus santalinus*

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Abstract

A micropropagation is a powerful tool in the era of the biotechnology revolution. It has a broad range of potentiality as compared to conventional vegetative propagation attracting researchers, industrialists, governmental and nongovernmental organizations at the national and international level. The potential methods of organogenesis and somatic embryogenesis, primarily through callogenesis, allow the production of genotypically identical and pharmacologically conserved disease-free healthy stocks in shorter times. *Pterocarpus santalinus*, the pride of Andhra Pradesh, has become endangered due to medicinal and commercial overexploitation. The micropropagation of *P. santalinus* poses many cultural challenges due to limited regeneration potential through callogenesis, organogenesis, and somatic embryogenesis. The lack of proper explant treatment and the effect of plant growth regulators limit the application of published protocols to reproduce the results. The challenge, such as heavy contamination of mature explants with endophytic fungi, forced us to explore the potential of immature tissues for regeneration through induction of somatic embryogenesis. We observed that immature tissues (zygotic embryo, petal, ovary, and anther) are better responsive than mature tissues with the scantiest contamination and phenolic release. The present study analyzed, evaluated, and interpreted the different parameters applied in the micropropagation of *P. santalinus*. The aim is to solve the discrepancies of existing protocols to present complete insight for future needs in the successful regeneration of the species. The review also compared various treatments to overcome dormancy and promote germination. It also discussed the possibilities of induction of somatic embryogenesis for future research.

Keywords *Pterocarpus santalinus* · Red sandalwood · Organogenesis · Callogenesis · In vitro germination · Shoot regeneration

Introduction

Pterocarpus is a tree of the family Fabaceae, has 15 species, among which five are found in India, namely, *P. dalbergioides*, *P. indicus*, *P. macrocarpus*, *P. marsupium*, and *P. santalinus* (Anonymous 2012; Pullaiah and Anuradha 2019). The *P. santalinus*, commonly called ‘Red Sandalwood’ or ‘Red Sanders’, is an endangered endemic species of the southeastern part of the Indian peninsula (Soundararajan and Joshi 2012; Bhagyaraj and Ramana 2013; Ramabrahmam and Sujatha 2016; Barstow 2018). The species is highly valued, nationally, and internationally, for its exceptionally distinctive timber quality with a wavy grain texture and suffused with 16% of a red color dye santalin (Rao and Raju 2002; Teixeira da Silva et al. 2019). The wood of *P. santalinus* costs approximately Rs 30-100 lakhs/tonne among importers, such as China, Japan, and Myanmar, where it has been used to make high-quality furniture and

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musical instruments (Soundarajan et al. 2016; Bhagyaraj 2017). Hence, it has been smuggled over the years resulting in reduced population. Further, the difficulty in propagating naturally due to hard seed coat, low fruit set, narrow niche, lengthened dormancy, and poor seed viability made its existence questionable (Rao and Raju 2002; Hegde et al. 2012; Arunkumar and Joshi 2014). Since 1995, the species has been listed in Appendix II of CITES to prevent illegal international trade. The RST (review of significant trade) prioritized the plant to inform seven Asian medicinal plant species at the 15th and the 21st meeting of the plant committee (UNEP-WCMC 2017; Barstow 2018). The plant contains a lot of medicinally important compounds (Fig. 1), making it a high potent herbal remedy for hepatitis (Manjunatha et al. 2010; Bulle et al. 2016), diabetes (Arunakumara et al. 2011), cancer (Azamthulla et al. 2015), wound healing (Bhagyaraj 2017), skin problems (Arunakumara et al. 2011; Reddy 2018), blood diseases (Keshavamurthy et al. 2018), diabetic neuropathy (Halim and Misra 2011; Shree et al. 2019), insect stings (Rao et al. 2019), bilious infections (Shree et al. 2019), and many more. Given its immense potential, the plant is considered ‘The Pride of Eastern Ghats’ (Anonymous 2012). Owing to its high market demand and diminishing numbers, it is imperative to maintain its population.

The micropropagation mastered the drawback of conventional vegetative propagation (Prakash et al. 2006; Teixeira da Silva et al. 2019). The technique was first applied in 1960 when George Morel attempted to commercialize

orchids (Bhatia and Sharma 2015). Since then, it has opened a new way of vegetative propagation with much elaborate efficiency in many species with promises in red sanders (Vipranarayana et al. 2012; Warakagoda and Subasinghe 2013; Teixeira da Silva et al. 2019). In *P. santalinus*, complete plant regeneration at a large scale was reported through organogenesis and, to some extent, by callogenesis. Since the first report on plantlet production from the shoot tip culture of the species (Lakshmisita et al. 1992), many researchers have taken advantage of micropropagation to enhance regeneration potential using various explants.

Micropropagation of *P. santalinus* is superior to conventional propagation technique by having the potential to regenerate many plants in less time (Prakash et al. 2006; Teixeira da Silva et al. 2019). However, many applications of plant biotechnology, such as germplasm conservation, synthetic seed production, and cell and protoplast culture for secondary metabolite production, could not be possible due to lack of somatic embryogenesis (Bhatia and Sharma 2015; Akhtar 2013, 2018; Pullaiah and Anuradha 2019). The process is in progress with the authors of the present review. The development of somatic embryogenesis follows a pathway similar to zygotic embryogenesis. In dicotyledonary plants, the process passes through the globular, heart, torpedo, and cotyledonary phase, and in monocotyledonary plants, it passes through globular, scutellar, and coleoptile phases (Mordhorst et al. 1997). Principally in vitro plantlet production is achieved either indirectly via a callus or directly from the explant’s surface. When

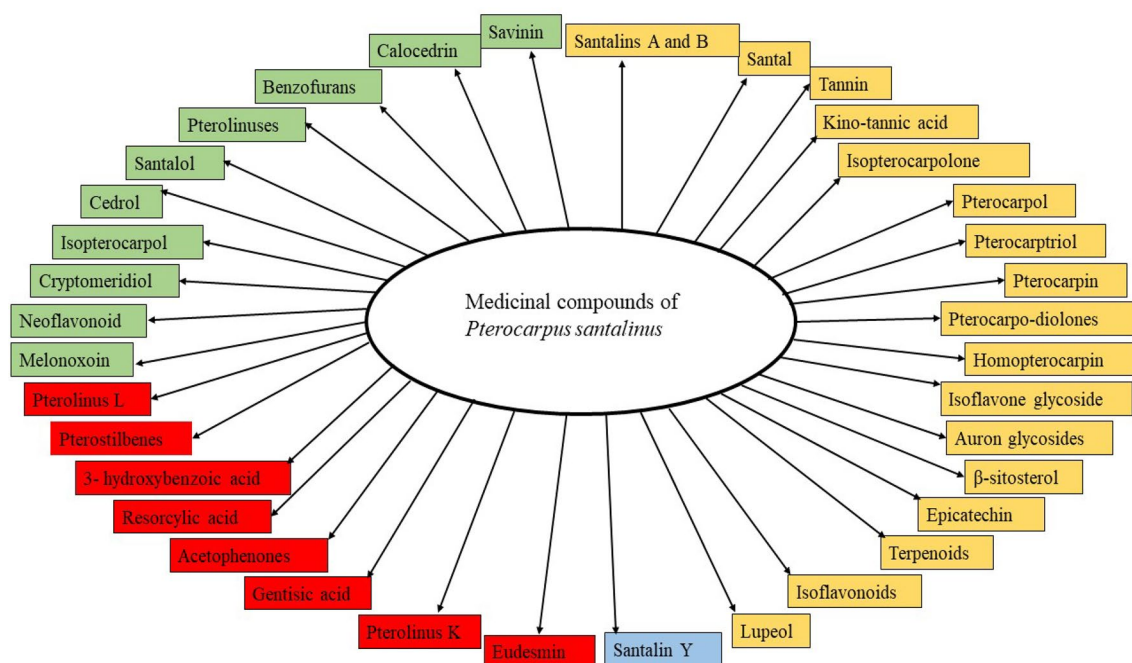


Fig. 1 Illustrative representation of important phytochemicals of *Pterocarpus santalinus*. The phytochemicals in yellow box were mentioned by Arunkumara et al. (2011), blue box by Kinjo et al. (1995), red box by Bulle et al. (2016), and green box by Navada (2014)

regeneration occurs via an intervening callus mass, the process is indirect organogenesis or indirect somatic embryogenesis. The callus production from nodal cuttings, shoot tips, and leaf discs have been possible in red sanders (De Silva et al. 2005). The regenerated shoots are rooted in the second hormonal treatment referred to as organogenesis, demonstrated in red sanders (Prakash et al. 2006; Padmalatha and Prasad 2008; Warakagoda and Subasinghe 2013; ChengXian et al. 2019). In contrast, somatic embryos are germinated mostly without any hormonal treatment, not yet reported in *P. santalinus*. The remarkable advantages of somatic embryogenesis over organogenesis and callogenesis are the single-cell origination, reduced or no chimera formation, and synthetic seed production. The process is crucial for woody plants that are arduous to propagate through conventional methods (Guan et al. 2016; Isah 2016). Further, this method is used to protect and preserve many valuable and endangered plant species throughout the world, as evident from a massive number of research publications emphasizing their role and application. Secondary somatic embryogenesis is beneficial for woody plants because its embryogenic property can be maintained over the years. It is also helpful when we need to commercialize the process by automated bioreactors that help to generate a massive number of somatic embryos (Guan et al. 2016). Hence, micropropagation, primarily through somatic embryogenesis, is emerging as a potent tool for transforming and proliferating a wide range of plant species every passing year (Jain and Gupta 2018).

Although a review paper on micropropagation of *P. santalinus* is already present, the paper was detailed with biology, importance, propagation, and micropropagation of red sandalwood (Teixeira da Silva et al. 2019). An essential element of the present review study is to highlight the importance of every parameter and chemical used for micropropagation. This review rationalized the trends in mother plant and explant selection, the effect of explant orientation, the importance of growth regulators, agar and sucrose concentration, the effect of subculture and sterilization. The contradictory results were highlighted to get complete insight knowledge for further manipulation. This review also discussed the in vitro germination methods to overcome lower natural regeneration problems. The success in *P. santalinus* regeneration is summarized in Table 1 and discussed after critical analysis in the following sections.

In vitro germination in *P. santalinus*

The complication of germinating plantlets from the seed is its hard seed coat, which makes it difficult to absorb water and nutrients (Renganayaki et al. 2020). The lack of these two essential components halts the normal process of growing because it stops the biosynthesis of proteins inside the cells (Patel et al. 2018). Proteins are the

vital elements for growth since it helps form new cells and repair damaged cells, the scarcity of which does not allow proper development of tissues. In vitro germination methods can overcome this drawback of the natural budding process.

The initial target was to overcome the above problem by making the hard seed coat permeable to absorb nutrients that help the embryo grow and germinate. Gibberellic acid (GA_3) and water are the most used medium to overcome seed dormancy. Treatment, duration, and concentration of GA_3 and water play a pivotal role in breaking dormancy (Naidu 2001). GA_3 enhanced seed germination at 800 ppm by 83% and 80% for 50 h and 40 h, respectively (Naidu 2001). Although GA_3 applied for 24 h at 250 ppm showed 63.33% and at 500 ppm by 66.67% increased seed germination (Patel et al. 2018). The response of Renganayaki et al. (2020) contradicts the earlier work with GA_3 and reported 88% germination at 500 ppm for 12 h. Previously GA_3 applied at any concentration was found ineffective as no seed germination was achieved by Padmalatha and Prasad (2007). The rationale could be that a hard seed coat may not be the sole factor responsible for dormancy. Using water to presoak the seeds also helps soften the seed coat and allows nutrients to plunge, increasing germination by 50–60% (Padmalatha and Prasad 2007; Vijayalakshmi and Renganayaki 2017; Karthikeyan and Arunprasad 2019; Renganayaki et al. 2020). Comparing the two, GA_3 and water, it is observable that GA_3 overcome the dormancy better because it also helps in new protein synthesis, ethylene synthesis, α -amylase formation, etc. (Patel et al. 2018).

Germination can also be enhanced by culturing the seed in various media. Anderson (1980) media increased germination by 92%, whereas the Vitis media (Chee and Pool 1987) with charcoal enhanced the hypocotyl length (Chaturani et al. 2006). Woody plant media (WPM) (Lloyd and McCown 1980) with activated charcoal was also beneficial to enhance germination (Chaturani et al. 2006; Warakagoda and Subasinghe 2013). Activated charcoal (AC) helps absorb phenolic compounds and, therefore, was beneficial for germination, which is otherwise a factor for reduced sprouting. A schematic representation of factors responsible for seed dormancy and their respective treatments with mechanism is depicted in Fig. 2.

Other parameters that create trouble for developing plantlets are the pod size, storage time, etc. Researchers worked on these parameters and proved their effect to overcome the problem of germination (Chaturani et al. 2006; Renganayaki et al. 2020). Strong consideration of all the bounds is required to overcome the aforementioned problems. Domain-like differences in self and cross-pollinated seeds (Rao and Raju 2002), abortion of embryo, and the absence of embryo in seed might also be the factors not considered in these reports.

Table 1 Summary of micropropagation work on *Pterocarpus santalinus*

Explant details (a) explant, (b) explant size	Explant source	Media used (a) shooting, (b) rooting	Treatments for (a) shooting, (b) rooting	Multiplication rate	Mean no. of (a) shoots, (b) roots	Mean (a) shoot length (cm), (b) root length (cm)	Acclimatization	Final survival rate (%)	Report of contamination (%)	References
(a) Embryo (b) –	–	(a) MS (b) –	(a) 3 mg/l BA + 0.3 mg/l IBA (b) –	–	(a) – (b) –	(a) – (b) –	–	–	–	Chen et al. (2019) (Abstract) Chenxian et al. (2019) (Abstract)
(a) Seed (b) –	–	(a) – (b) –	(a) 3 mg/l BA + 0.3 mg/l IBA (b) 1 mg/l IBA + 0.25 mg/l IAA + 0.05 mg/l BA	2.8	(a) – (b) –	(a) – (b) –	–	–	–	
a) Leaves b) –	1–3 years old mother plant	(a) MS (b) –	(a) 0.5 mg/l 2,4-D + 2.5 mg/l BA + 0.5 mg/l NAA (b) –	83.33% callus	(a) – (b) –	(a) – (b) –	–	7.14	–	Ashrafee et al. (2014)
(a) Cotyledon- ary node (b) –	Laboratory- grown plantlet	(a) B5 (b) ½ MS after pulse treat- ment	(a) 2 mg/l BA + 0.4 mg/l NAA (b) 2500 ppm IBA for 12 h then to 0.5 µM IBA	–	(a) 4.95 ± 0.22 (b) 4.10 ± 0.00	(a) 3.62 ± 0.22 (b) 2.67 ± 0.71	Sand + coir dust (1:1)	80	–	Warakogoda and Subasin- ghe (2013)
(a) Cotyledon- ary node (b) –	Laboratory- grown plantlet	(a) MS (b) ½ MS after pulse treat- ment	(a) 1 mg/l BA + 0.5 mg/l NAA (b) 1500 mg/l IBA for 5 min	–	(a) 8.80 ± 0.76 (b) 4.6 ± 0.12	(a) 5.16 ± 0.21 (b) –	1500 mg/l IBA for 5 min then trans- ferred to sterile soilrite and irri- gated with ½ MS for 1 month and 2 weeks on every 3 rd day. Humidity was reduced with time	85.4	–	Viprinarayan et al. (2012)
(a) Apical meristem (b) –	Laboratory- grown plantlet	(a) MS (b) MS	(a) 1 mg/l BAP + 0.1 mg/l TDZ (b) 0.1 mg/l IBA	–	(a) 11.12 ± 0.42 (b) 3.21 ± 0.23	(a) 4.18 ± 0.09 (b) 5.13 ± 0.42	Autoclaved organic manure + sand and transferred to garden soil after 30 days in green house	73.33	–	Balaraju et al. (2011)

Table 1 (continued)

Explant details (a) explant, (b) explant size	Explant source	Media used (a) shooting, (b) rooting	Treatments for (a) shooting, (b) rooting	Multiplication rate	Mean no. of (a) shoots, (b) roots	Mean (a) shoot length (cm), (b) root length (cm)	Acclimatization	Final survival rate (%)	Report of contamination (%)	References
(a) Seed (b) –	Laboratory-grown plantlet	(a) MS (b) MS	(a) 2 mg/l Kn + 1 mg/l BA (b) –	–	(a) 17 ± 2.36 (b) –	(a) 5.04 ± 1.13 (b) –	Sterile soilrite in green house irrigated with MS basal every alternate days for 15 days and thereafter with water	20	–	Padmalatha and Prasad (2008)
(a) Cotyledonary node (b) 1.5 cm	Laboratory-grown plantlet	(a) MS (b) –	(a) 2.5 µM BA + 2 µM 2-iP (b) 5 µM IAA + 1 µM IBA	2.75 ± 0.02	(a) 4.40 ± 0.50 (b) 2.81 ± 0.19	(a) 4.15 ± 0.05 (b) 3.82 ± 0.18	Coarse sand + clay + farmyard manure (1:1:1)	95	–	Rajeswari and Paliwal (2008)
(a) Nodal segment (b) 1 cm	Laboratory-grown plantlet	(a) MS (b) ½ MS	(a) 4.4 µM BA + 2.2 µM TDZ (b) 4.9 µM IBA	3.5	(a) 2.9 ± 0.1 (b) 1	(a) 5.6 ± 0.5 (b) 3.9 ± 0.5	Autoclaved vermiculite + acid-washed sand (1:1) and then to soil + farmyard (4:1) for 4 weeks in a shady place	70	20	Prakash et al. (2006)
(a) Nodal cutting (b) –	(a) MS (b) –	(a) 0.2 mg/l Kn + 0.1 mg/l NAA + 0.5 mg/l BA + 0.3 mg/l IBA + 0.1 mg/l calcium panthotenate (b) –	Callus obtained	(a) – (b) –	(a) – (b) –	–	–	–	–	Silva et al. (2005) (Abstract)
(a) Cotyledons (b) 1.5 cm	Laboratory-grown plantlet	(a) MS (b) ¼ MS	(a) 1 mg/l BA + 1 mg/l Kn + 0.1 mg/l NAA (b) 1 mg/l IAA	–	(a) 10.4 ± 1.50 (b) 1.73 ± 0.05	(a) – (b) –	Sterile garden soil + sand (1:1). First week plantlets were irrigated with ¼ MS + 1 mg/l IAA and then with tap water after 15 days transferred to soil and irrigated with Hoagland solution (Hoagland and Arnon 1950)	90	–	Arockiasamy et al. (2000)
(a) Mesocotyl (b) –	Laboratory-grown plantlet	(a) B5 (b) ½ MS	(a) 3 mg/l BA + 1 mg/l NAA (b) 1 mg/l IAA + IBA + NAA	–	(a) 10–15 (b) 1	(a) 2.50 ± 0.024 (b) –	–	–	50	Anuradha and Pulliah (1999)

Table 1 (continued)

Explant details (a) explant, (b) explant size	Explant source	Media used (a) shooting, (b) rooting	Treatments for (a) shooting, (b) rooting	Multiplication rate	Mean no. of (a) shoots, (b) roots	Mean (a) shoot length (cm), (b) root length (cm)	Acclimatization	Final survival rate (%)	Report of contamination (%)	References
(a) Shoot tip (b) 5–8 mm	Laboratory-grown plantlet	(a) B5 (b) MS	(a) 1 mg/l BA + 1 mg/l Kn (b) 5 mg/l IAA	–	(a) 8 (b) 1	(a) 3–5 (b) –	Soil + sand (1:1) irrigated with sterile MS for 2 weeks. Then transferred finally to pot	60		Lakshmisita et al. (1992)

MS: Murashige and Skoog; B5: Gamborg media; BA: 6-benzylaminopurine; 2,4-D: 2,4-dichlorophenoxyacetic acid; Kn: Kinetin; NAA: Naphthalene acetic acid; IAA: Indole-3-acetic acid; IBA: Indole-3-butyric acid; TDZ: Thidiazuron; 2-IP: 2-isopentyl adenine

Micropropagation challenges in *P. santalinus*

The micropropagation of *P. santalinus* faces many more challenges than other trees and crop plants, proven by its limited literature. However, critique of various factors such as the source of the mother plant, type of the explants, the combination of hormones, the effect of nutrients, and a few physical characteristics is needed for successful proliferation and regeneration of plantlets.

This review section discusses the factors responsible to standardize micropropagation protocol for *Pterocarpus santalinus*. Owing to the lack of proper evaluation and analysis, the success rate is still not satisfactory and less in number. Although working on the regeneration of *P. santalinus* species, the authors' group encountered heavy culture loss due to endophytic fungi. Unfortunately, the problem has not been mentioned in any published reports, though few papers reported the contamination problem (Anuradha and Pullaiah 1999; Prakash et al. 2006; Ashrafee et al. 2014). At the same time, the fungal endophyte was isolated from *P. santalinus* to demonstrate its antimicrobial and antioxidant activity from field-grown trees (Venkateswarlu et al. 2015). They have identified the endophyte as *Xylaria* spp. with no morphological details. Regeneration of this species is only possible if one can overcome the contagion of endophytic fungi. Hence, we need to critically analyze, evaluate, and interpret the regeneration features on micropropagation of *P. santalinus*. The details of micropropagation steps are depicted in Fig. 3.

Effect of plant source and explant type on micropropagation

The first criteria that limit micropropagation are to select an appropriate mother plant and an explant source. The age and place of the mother plant likely to have a significant effect on its growth characteristics and efficiency in laboratory conditions. When comparing the regeneration potential, the mother plant from Tirupati and Cuddapah district of Andhra Pradesh, India, showed maximum proliferation of 10–15 and 19–20 shoots, respectively (Anuradha and Pullaiah 1999; Padmalatha and Prasad 2008). The response indicates that the choice of the habitat of the growing mother plant is necessary, as environmental conditions affect the behaviors of plant genotype and its successful establishment in culture. The age of the mother plant also influences the response of explants during in vitro culture. As the mother plant ages, it secretes more phenolic compounds and has high contamination rate, creating culture difficulty (Anuradha and Pullaiah 1999). We have also encountered this problem while establishing the explants from mature trees of 15–30 years old. Even earlier reports using explants from 10 to 25 years old

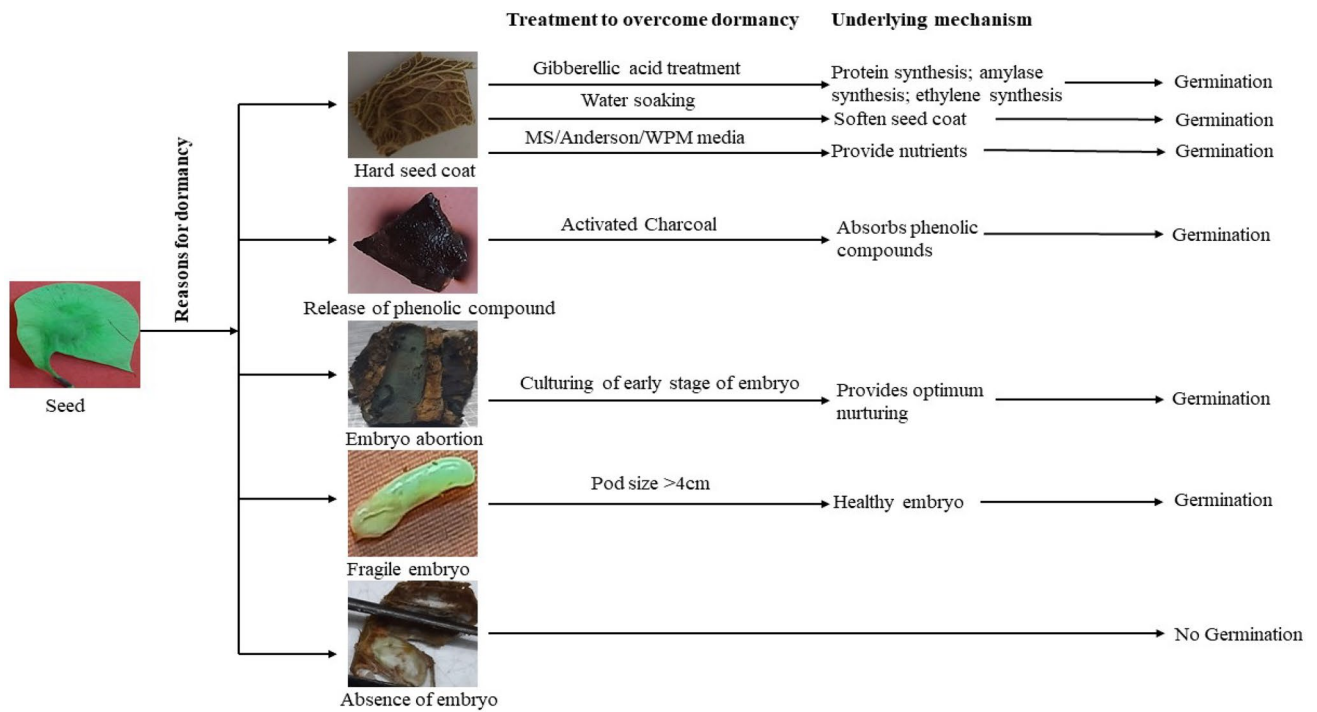


Fig. 2 A schematic representation of factors responsible for seed dormancy and their respective treatments with mechanism

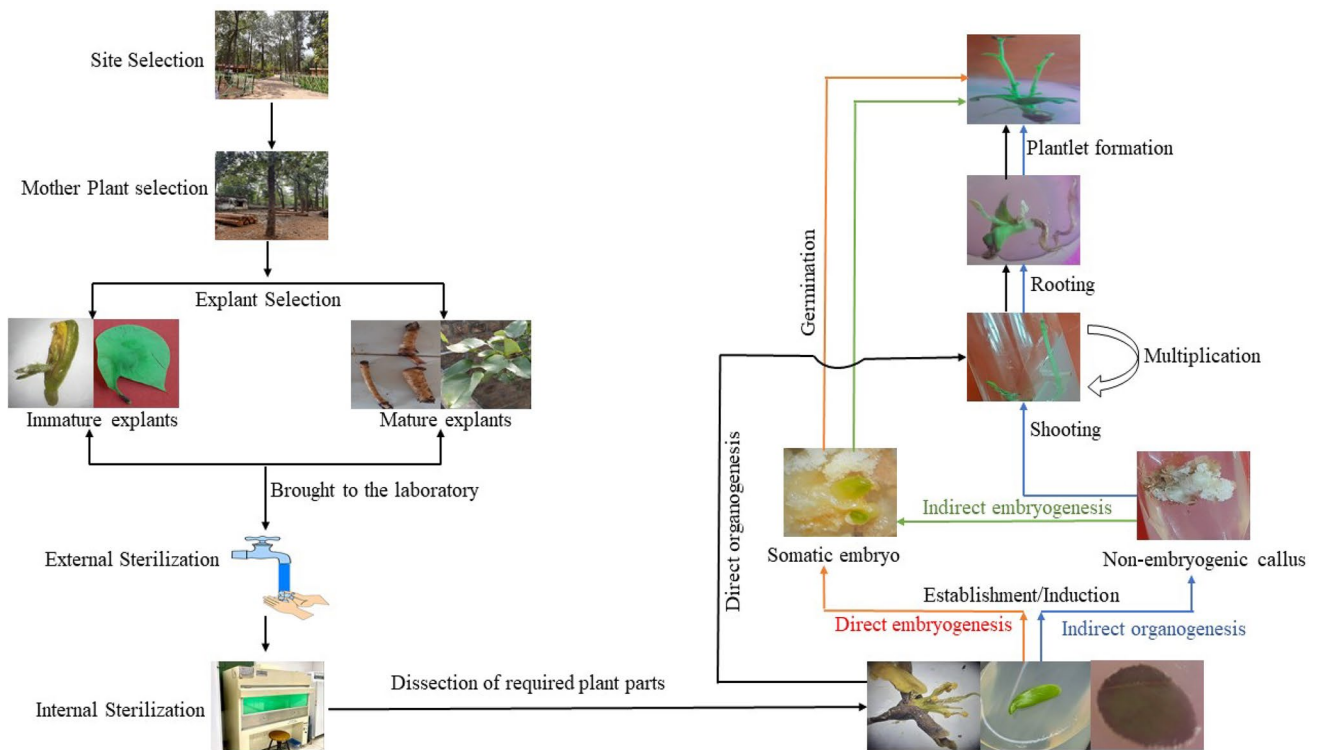


Fig. 3 A schematic representation of the details of micropropagation steps

plants for organogenesis have not reported the problem with endophytic fungi (Prakash et al. 2006; Warakagoda and Subasinghe 2013). Similarly, in other reports on in vitro regeneration of this species, there was no mention of contaminating endophytes or the age of the mother plant (Table 1).

The next factor that affects the successful establishment of the culture in the laboratory is the choice of explants. The explants should consist of meristematic cells because of having a general tendency of cell division and growth and are easy to manipulate in culture. A wide variation exists to select explants for micropropagation of *P. santalinus*. The divergence is from hard to soft and mature to immature tissues. The mature explants are recalcitrant, release more phenolic compounds and has high contaminant load. Conversely, the immature explants are responsive, release less or no phenolic compounds, and contain fewer contaminants. Therefore, using immature tissues from the field-grown plants to establish and proliferate the species will lead to more success, yet to be explored. The authors' group is working to regenerate *P. santalinus* through immature tissues. Many reports used seeds as the explants from the mother plant to germinate it in the laboratory. The seedling obtained was employed to procure secondary explants like mesocotyl, hypocotyl, cotyledonary nodes, internodes, and shoot tips. In the first report on this plant, shoot tip explants from germinating seeds were applied for single and multiple shoots regeneration. In the subsequent cycle, nodal segments were used from the single shoot regenerants. (Lakshmisita et al. 1992). The latest work used well-grown seed embryos as explants to achieve enhanced germination and seedling recovery (Chen et al. 2019). The explants from in vitro germinated plantlets are the primary choice compared to explants from the mother plant, as it does not require elaborated sterilization (Padmalatha and Prasad 2008).

The most favorable explants are the cotyledonary nodes and mesocotyl from an in vitro grown seedling. However, the use of explants from a seedling is not considered ideal because it can cause variation in genotype (Padmalatha and Prasad 2008). The size of the explants is another factor to consider because it affects the absorption of nutrients and the contamination load. Small-sized explants are preferred more because of better nutrient absorption and less chance of contamination. Most of the reports have selected explants size as 0.5–1.5 cm (Lakshmisita et al. 1992; Arockiasamy et al. 2000; Prakash et al. 2006; Rajeswari and Paliwal 2008). We found smaller explants from the mature trees necrosed and became dead due to sterilization treatment; while the explants even less than 0.5 cm size from in vitro regenerated plantlets responded for callogenesis and somatic embryo production.

Explant sterilization and culture establishment

The successful regeneration in tissue culture technique is dependent on a balanced sterilization procedure. As the species is associated with endophytic fungi, a proper sterilization protocol is needed. The general sterilization procedure includes surface disinfection by washing explants under running tap water for 10–15 min to remove all the extra outer specks of dust. Then, treating the explants with a mild anti-septic and a detergent removes outer germs. Depending on the fungal population, which relies on the source of explants, an antifungal agent can make the explants free from fungal contamination during the surface disinfection process. In the next step, aseptic treatment is performed inside a laminar airflow chamber (LAF) by giving a short rinse with 70% ethanol followed by 15–20 min rigorous shaking in 0.05–0.1% HgCl₂ to make the explants microbial free. Finally, explants are washed 4–5 times with sterile double-distilled water to remove traces of HgCl₂. This generalized protocol needs optimization for the complete aseptic preparation of different types of explants. Amongst the various practices, leaves were treated for 3 min and internode for 7 min with 0.1% HgCl₂ (Ashrafee et al. 2014). However, the success in leaves establishment was made in 12 min using 0.1% HgCl₂ (Padmalatha and Prasad 2008). A 10-min treatment of seeds with 0.1% HgCl₂ was sufficient for in vitro seedling growth (Rajeswari and Paliwal 2008). In comparison, treating pods with 0.05% HgCl₂ for 5–10 min was adequate in another case (Vipranarayana et al. 2012). Being cytotoxic, a balance between concentration and treatment period for HgCl₂ is crucial. The tissues become dead either in high concentration or by treating for extended periods. Conversely, if used in low concentration or less time, it may not give an acceptable result. Another factor that needs consideration is the nature of the explant; hard and mature tissue needs more vigorous treatment than soft and immature tissue. Chlorox is used for different time intervals to avoid the toxic effect of HgCl₂, as it is mild than HgCl₂ (Warakagoda and Subasinghe 2013). In conclusion, when the contamination load is heavy, various sterilizing agents need to be optimized to succeed in culture establishment.

Organogenic regeneration

Effect of plant growth regulators (PGR) on shoot regeneration

Cellular functions like cell division, elongation, differentiation, etc., are essential for proper plant growth and development and are regulated by the plant growth regulators (PGRs). A judicious combination and optimum concentration of PGR is the deciding factor for shoot initiation and proliferation (Skoog and Miller 1957). Among the PGRs,

6-benzylaminopurine (BAP) is the most widely used singly or with other cytokinin or auxin for in vitro shoot regeneration. It is evident from Table 1 that BAP gives a better response in the range of 0.5–3 mg/l. When applied in combination, the concentration of BAP was kept higher because it can overcome dormancy and help to develop shoot. The statement is supported by the response of Balaraju et al. (2011), who obtained 11 shoots by treating apical meristem with BAP (1 mg/l) and TDZ (Thidiazuron, 0.1 mg/l), and Anuradha and Pullaiah (1999), attained 10–15 shoots from mesocotyl explants when treated with BAP (3 mg/l) and NAA (α -naphthalene acetic acid, 1 mg/l). The contradictory response was collected by Prakash et al. (2006), who achieved only 2–3 shoots by treating nodal segment with BAP (4.4 μ M) and TDZ (2.2 μ M), and Warakagoda and Subasinghe (2013), gained only 4–5 shoots by treating cotyledonary node with BAP (2 mg/l) and NAA (0.4 mg/l). A lower concentration of BAP (1 mg/l) used in combinations with Kn (2 mg/l) induced a much-enhanced number of shoots (19–20) from seed as explants (Padmalatha and Prasad 2008). Hence, the concentration and combination of plant growth regulators depend on the explant type. Extensive research is needed to empirically optimize the regeneration process concerning PGR concentrations, explants type, age, the genotype of the mother plant, and the environmental condition for the species.

Effect of subculture

Shoot multiplication requires subculturing of explants either in the same media (Lakshmisita et al. 1992; Rajeswari and Paliwal 2008; Warakagoda and Subasinghe 2013) or in a different media or to the same media with reduced concentration (Balaraju et al. 2011). The proliferation potential generally decreases after a certain number of subculturing, as Lakshmisita et al. (1992) reported. A decline in multiplication frequency from 95 to 85% was observed after 2nd subculture (Rajeswari and Paliwal 2008). In contrast to these reports, an increase in multiplication coefficient was reported from the first (5.4 shoots) to sixth (8.3 shoots) subculture by Prakash et al. (2006). Hence, the regeneration potential of particular explants for the shoot multiplication ratio during the subsequent subculture cycle needs further investigation.

Effects of sucrose concentration on plantlet growth

Sucrose, a carbon source, helps the plant to grow by providing energy. Sucrose at 2–3% concentrations is optimal for plant growth. A lower sucrose concentration can lead to stunted growth because of less energy supply, and higher sucrose concentration can also lead to reduced growth due to the secretion of phenolic compounds (Lakshmisita

and Raghavaswamy 1993; Padmalatha and Prasad 2008). Higher sucrose levels are found stimulatory for somatic embryogenesis (Akhtar 2013, 2018). While optimizing concentration, almost 100% response, 8–9 shoots, and 9.71 cm shoot length were obtained with 3% sucrose, whereas 2% sucrose also gave 100% response but with 5–8 shoots and 3.66 cm shoot length (Anuradha and Pullaiah 1999). A similar experiment reported the best result in 3% sucrose, but data was not demonstrated separately by the author (Padmalatha and Prasad 2008). Apart from being an energy source, it affects the osmotic potential of the medium. Therefore, carbon source and its concentration are crucial to influence morphogenesis (Neto and Otoni 2003).

Agar concentration, media type, and orientation of explant

Agar does not have much effect on the growth of explants. The most widely used agar concentration is 0.8%. Optimization of agar concentration showed better results at 0.6%, but the author does not demonstrate the effect with growth parameters (Padmalatha and Prasad 2008). Higher agar concentration makes the media tight so that nutrient mobilization may become difficult for explants, and lesser agar concentration makes the media loose and not desirable if the experiment needs a semisolid medium. As the concentration of agar increases, it lowers the water potential of the medium. The water potential lower than -0.5 MPa does not support plant growth. The macroelements present in gelling agents also affect the osmotic potential of media (Buah et al. 1999). Therefore, the choice of gelling agent and its concentration can also be responsible for establishing culture.

The media type can also affect plant growth because media vary in their salt concentration. The best-known media for micropropagation is MS (Murashige and Skoog 1962) media. Almost similar results were obtained using B5 (Gamborg et al. 1968) media in many reports (Lakshmisita et al. 1992; Anuradha and Pullaiah 1999; Warakagoda and Subasinghe 2013). Shoot induction generally works well in full-strength media. For rooting, some authors preferred half or quarter-strength media (Anuradha and Pullaiah 1999; Arockiasamy et al. 2000; Prakash et al. 2006; Vipranarayana et al. 2012; Warakagoda and Subasinghe 2013). As the solute potential of soil is less than the tissue culture media, reducing media strength prepares the root for the soil transfer that otherwise will collapse during hardening and acclimatization. Orientation of explant can also be a factor in regeneration. The result indicated a better response of horizontal orientation because of having a large surface area to absorb more nutrients (Padmalatha and Prasad 2008).

Effect of PGRs on rooting

Phytohormones, especially auxins, regulate rooting, which is vital to plant sustainability. The maximum positive result obtained to date is with IBA (3-indole butyric acid) followed by IAA (3-indole acetic acid). The concentration ranges between 0.5 and 2 mg/l, but some authors worked beyond this limit and received positive results (Table 1). Initially, IBA is described as the synthetic auxin; however, genetic studies identified enzymes most likely involved in converting IBA to free IAA that contributes to the regulation of root development (Overvoorde et al. 2010). Since the endogenous concentration of IAA is inversely proportional to the growth rate, it is the key to understand the action of auxins on roots (Tanimoto 2005). Both IBA and IAA work well for root development. The former links internal IAA with an amino acid that help to initiate the process and later activates gene for the same (Tanimoto 2005; Overvoorde et al. 2010). IBA helps to elongate roots by breaking cellulose microfibrils' bond (hydrogen) and de-differentiating cambium tissues (Cosgrove 2000; Kumar et al. 2015; OuYang et al. 2015; Shiri et al. 2019). But we need to optimize because excess concentration can have an inhibitory effect depending upon the species. The inhibitory effect of IBA at high concentrations may be because of potassium ion toxicity, which harms the epidermis of the nearby cells (Shiri et al. 2019). Very recently, about 65.2% of success in rooting was achieved with a combination of IBA, IAA, and BAP (1 mg/l, 0.25 mg/l, 0.05 mg/l, respectively) (ChengXian et al. 2019). A 60% rooting with 3.21 ± 0.23 average roots/shoots was achieved by applying 0.1 mg/l IBA (Balaraju et al. 2011). On the other hand, only 47.2% rooting with a single thick root of 3.9 ± 0.5 cm was reported on the application of 4.9 μ M IBA (Prakash et al. 2006). In comparison, a single thick root with 5 mg/l of IAA and an adventitious root with 1–2 mg/l of IAA was reported by Lakshmisita et al. (1992). About 82.5% success in rooting along with 80.5% of root hair development and 2.81 ± 0.19 mean number of roots was achieved by treating shoots with IAA (5 μ M) and IBA (1 μ M) in combination (Rajeswari and Paliwal 2008). While rooting was described in the MS basal medium, the response percentage and other root parameters are not mentioned (Padmalatha and Prasad 2008). Regarding the subculture cycle, an increase in rooting from the first subculture (47.2% rooting) to the sixth (66.6% rooting) subculture is reported by Prakash et al. (2006).

Some authors reported better rooting with pulse treatment of auxins at a particular concentration and for a specified period. A quite big difference is evident concerning the treatment protocol and the corresponding results reported by different authors. A cent percent rooting response with 4.10 ± 0.00 roots/shoot was reported by pulse treatment with 2500 ppm IBA for 12 h (Warakagoda and Subasinghe 2013). In comparison, slightly less rooting was reported with 85%

response and 4.6 ± 0.12 roots/shoot by applying pulse treatment of IBA at a concentration of 1500 ppm for only 5 min (Vipranarayana et al. 2012). Only 50% rooting was achieved by treating the shoots with IBA, IAA, NAA, each with only 0.1 mg/l for 48 h (Anuradha and Pullaiah 1999). The results of these authors supported the fact that pulse treatment enhances the frequency of rooting and the number of roots formed per shoot, pointing towards the inductive effects of auxins on rooting at lower concentrations.

Callogenesis and regeneration

The callus is the unorganized mass of cells that develop in complete plants either by shoot organogenesis or somatic embryogenesis. The multiplication process is much higher when micropropagation proceeds via callus formation. Its morphogenetic potential can be increased through repeated cycles by breaking into smaller pieces to get more calluses simultaneously. Additionally, individual cells of callus can be separated in suspension culture to generate more microcalli in a shorter time, each of which can be induced to give rise to a separate plantlet. Characteristics of callus vary considerably for their regeneration potential to express either as embryogenic or nonembryogenic. Embryogenic callus proceeds through the embryonic pathway, and a nonembryogenic callus may not differentiate or can be induced to give rise to a shoot that can be rooted to get complete plantlets.

Regeneration of shoot from nonembryogenic callus

In *P. santalinus*, the most successful callogenesis was obtained with 100% response from cotyledons, 90% in hypocotyl segments, 80% in leaf, and 70% from internode explants (Arockiasamy et al. 2000). They used BAP and Kn at a concentration of 1 mg/l and obtained 10 shoots/explant in 88% explanted tissues for regeneration. They obtained organogenesis and callogenesis simultaneously on to the same responsive explants. On the other hand, only 10% of established culture showed callus formation in BAP (0.5 mg/l) when used in combination with Kn (0.2 mg/l), NAA (0.1 mg/l), IBA (0.3 mg/l), and calcium pantothenate (0.1 mg/l), further development not mentioned (De Silva et al. 2005). Recently, callogenesis was also reported in BAP with 2.5 mg/l along with 2,4-D (2,4-dichloro phenoxy acetic acid) and NAA (Ashrafee et al. 2014). Development of roots is common in tissue culture in the presence of auxins in the medium. Root induction was reported only in 50% of calli in the presence of 0.5, 1, 1.5 mg/l IBA (Arockiasamy et al. 2000). However, the authors did not mention any characteristic features of the roots. Although callus formation is common in most regeneration reports, induction of roots or shoots indirectly from the callus is not identified. The parameters must

therefore be empirically tested to root organogenesis from the callus and the developing shoots.

Somatic embryogenesis and plantlet regeneration

Somatic embryogenesis is a promising technique for large-scale clonal propagation of plant species that are at high risk of becoming extinct (Vogel 2005; Loyola-Vargas et al. 2008; Nic-Can et al. 2013). It helps understand the regeneration process at the molecular and genetic levels (Nic-Can et al. 2015). It is applied in germplasm conservation and improves the genetic makeup of woody plants (Merkle and Dean 2000; Chiancone and Germanà 2013; Ozudogru and Lambardi 2016). One of the advantages of somatic embryogenesis is its single-cell origin, making it suitable for clonal propagation from genetically transformed cells. It reduces chimeras' formation and can be applied for synthetic seeds (Giri et al. 2004; Normah et al. 2013). It is an efficient process as it reduces the step of rooting needed in organogenesis due to the bipolar nature of somatic embryos (Von Arnold et al. 2002). Somatic embryogenesis was reported in some woody, horticultural, and conifers plants (Akhtar et al. 2000; Guan et al. 2016; Akhtar 2013, 2018). To date, somatic embryogenesis is not explored to proliferate *P. santalinus* for its commercialization. The author's group is working hard to proliferate the economically significant species through somatic embryogenesis using immature explants.

Acclimatization of regenerated plantlets

Hardening and acclimatization are essential to establish the laboratory-grown plantlets successfully in fields. The most widely used materials for acclimatization are mixtures of sand (coarse) and soil (garden) in different proportions. Farmyard manure and vermiculites are also equally prevalent. These are used in combinations in mostly 1:1 or 1:2 ratios. During acclimatization, the relative humidity should maintain high at the beginning and gradually progresses towards low. The *P. santalinus* plantlets were acclimatized by maintaining the relative humidity from 85 to 65% (Prakash et al. 2006). A maximum of 95% of plantlets survival was observed in treatment with coarse sand, clay, and farmyard manure in a 1:1:1 ratio (Rajeswari and Paliwal 2008). These observations indicate that once plantlets are established successfully in culture, they can be transplanted and acclimatized well in the soil.

Current research progress for regeneration of *P. santalinus*

To evaluate the regeneration capacity, various explants were established under in vitro conditions. The mature explants selected were a leaf, axillary node, nodal segment, pulvinus,

and pods. As a novel approach, the immature explants selected were gynoeceum, androeceum, zygotic embryo, and petals. Gynoeceum, androeceum, and petals were dissected from closed flower buds, whereas zygotic embryo was dissected from immature green pods after sterilization inside laminar airflow. The explants were sterilized with running tap water, then with 2% savlon and 10–20 drops of Tween 20 with shaking, and finally under tap water. This procedure was followed by rinsing with 70% ethanol for 2 min and 0.1% HgCl₂ for 15 min inside the laminar airflow hood. The mature explants were vigorously sterilized with HgCl₂ (0.1% and 0.01%), methylene blue (0.1%), Bevastin (1% and 2%), and Neem oil (5, 10, 15, 20%), singly and in combination. The explants were then established under the aseptic condition to induction medium. The medium used was MS supplemented with a wide range (0.01–2 mg/l) of growth regulators (BAP and 2,4-D). Although working with *P. santalinus*, we encountered the problems mentioned by previous researchers (hard seed coat and release of phenolic compounds) regarding low seed germination. In addition, embryo abortion, fragile embryo, and absence of embryo were also found to be responsible for failure of seedling formation. The reasons were noticed during the dissection of immature pods for the zygotic embryo.

The callogenic potential of aforementioned explants were evaluated for the micropropagation of the plant species. It was observed that immature explants were responded better compared to mature explants. The major problem encountered with mature explants was contamination due to endophytic fungi, later isolated and identified. The success rate with mature explants was scarce. The results for callogenic frequency of mature (leaf and axillary node) and immature explants were presented in Table 2. Even the vigorous sterilization treatments mentioned above didn't work efficiently for establishment. The temporal treatments with these chemicals were also proved to be inefficient. The explants surpassed the contamination problem remained recalcitrant either due to the release of phenolic compounds or the nature of the explants itself not allowing them to respond in vitro. Zygotic embryos showed 100% callogenic frequency in all the tested treatments. Immature explants like petals, anthers, and ovaries also responded with 80–100% callogenic potential. Therefore, immature explants could be the better choice for micropropagation of the species. A full-length research paper related to organogenesis and somatic embryogenesis is in process. Further experimentation for the generation of large-scale propagules is under investigation.

Conclusions

The recalcitrant nature of *P. santalinus* to in vitro regeneration is the bottleneck for its large-scale production and hence, the conservation. Identifying key factors in

Table 2 Evaluation of callogenic potential of mature and immature explants of *P. santalinus* observed after 16 and 8 weeks, respectively

Sl. no.	PGR		Average callogenic frequency (%)					
	BAP	2,4-D	Mature explants		Immature explants			
			Leaf	Axillary node	Zygotic embryo	Petals	Ovary	Anther
1	0.00	0.00	0.00 ± 0.00 ^C	0.00 ± 0.00 ^C	0.00 ± 0.00 ^B	0.00 ± 0.00 ^G	0.00 ± 0.00 ^G	0.00 ± 0.00 ^F
2	0.01	0.00	0.00 ± 0.00 ^C	0.00 ± 0.00 ^C	100.00 ± 0.00 ^A	0.00 ± 0.00 ^G	0.00 ± 0.00 ^G	0.00 ± 0.00 ^F
3	0.10	0.00	0.00 ± 0.00 ^C	0.00 ± 0.00 ^C	100.00 ± 0.00 ^A	0.00 ± 0.00 ^G	66.66 ± 4.16 ^{BCD}	0.00 ± 0.00 ^F
4	1.00	0.00	0.00 ± 0.00 ^C	0.00 ± 0.00 ^C	100.00 ± 0.00 ^A	87.50 ± 0.00 ^{BCD}	75.00 ± 0.00 ^B	100.00 ± 0.00 ^A
5	2.00	0.00	0.00 ± 0.00 ^C	20.83 ± 4.16 ^A	100.00 ± 0.00 ^A	100.00 ± 0.00 ^A	58.33 ± 4.16 ^{DEF}	100.00 ± 0.00 ^A
6	0.00	0.01	0.00 ± 0.00 ^C	0.00 ± 0.00 ^C	100.00 ± 0.00 ^A	0.00 ± 0.00 ^G	0.00 ± 0.00 ^G	0.00 ± 0.00 ^F
7	0.00	0.10	0.00 ± 0.00 ^C	0.00 ± 0.00 ^C	100.00 ± 0.00 ^A	0.00 ± 0.00 ^G	0.00 ± 0.00 ^G	0.00 ± 0.00 ^F
8	0.00	1.00	0.00 ± 0.00 ^C	0.00 ± 0.00 ^C	100.00 ± 0.00 ^A	79.16 ± 4.16 ^{DE}	58.33 ± 4.16 ^{DEF}	58.33 ± 4.16 ^E
9	0.00	2.00	0.00 ± 0.00 ^C	20.83 ± 4.16 ^A	100.00 ± 0.00 ^A	83.33 ± 4.16 ^{CD}	0.00 ± 0.00 ^G	66.66 ± 4.16 ^{DE}
10	1.00	0.01	12.50 ± 0.00 ^B	0.00 ± 0.00 ^C	100.00 ± 0.00 ^A	95.83 ± 4.16 ^{AB}	62.50 ± 0.00 ^{CDE}	79.16 ± 4.16 ^{BC}
11	1.00	0.10	16.66 ± 4.16 ^{AB}	0.00 ± 0.00 ^C	100.00 ± 0.00 ^A	91.66 ± 4.16 ^{ABC}	70.83 ± 4.16 ^{BC}	75.00 ± 0.00 ^{CD}
12	2.00	0.01	20.83 ± 4.16 ^A	12.50 ± 0.00 ^B	100.00 ± 0.00 ^A	95.83 ± 4.16 ^{AB}	75.00 ± 0.00 ^B	83.33 ± 4.16 ^{BC}
13	2.00	0.10	12.50 ± 0.00 ^B	12.50 ± 0.00 ^B	100.00 ± 0.00 ^A	83.33 ± 4.16 ^{CD}	91.66 ± 4.16 ^A	87.50 ± 0.00 ^B
14	0.01	1.00	12.50 ± 0.00 ^B	0.00 ± 0.00 ^C	100.00 ± 0.00 ^A	70.83 ± 4.16 ^{EF}	95.83 ± 4.16 ^A	66.66 ± 4.16 ^{DE}
15	0.10	1.00	12.50 ± 0.00 ^B	0.00 ± 0.00 ^C	100.00 ± 0.00 ^A	66.66 ± 4.16 ^F	66.66 ± 4.16 ^{BCD}	66.66 ± 4.16 ^{DE}
16	0.01	2.00	16.66 ± 4.16 ^{AB}	16.66 ± 4.16 ^{AB}	100.00 ± 0.00 ^A	87.50 ± 4.16 ^{BCD}	54.16 ± 4.16 ^{EF}	58.33 ± 4.16 ^E
17	0.10	2.00	16.66 ± 4.16 ^{AB}	12.50 ± 0.00 ^B	100.00 ± 0.00 ^A	79.16 ± 4.16 ^{DE}	50.00 ± 0.00 ^F	79.16 ± 4.16 ^{BC}

Values represent mean ± SE of three independent experiments with 8 explants per treatment. Data having the same letter in a column were not significantly differed by Duncan's multiple comparison test ($P < 0.05$)

regeneration, optimization, and standardization of micro-propagation techniques are the prime steps to conserve and protect this endangered plant species. Lacking standardized and optimized methods for *P. santalinus* proliferation is the key stimulus and inspiration for researchers working for this plant. Many researchers attained success by developing multiple shoots mainly through organogenesis but failed to apply the procedure for mass multiplication of the species. In conclusion, we say that immature tissues from the seedlings or the in vitro established cultures are the best responsive explant tissues for shoot organogenesis. Although a few reports mentioned callus production and shoots, none of the researcher showed organogenesis from callus. Since callogenesis is a successful strategy, it promises high-frequency regeneration through organogenesis or somatic embryogenesis. It is also concluded that a combination of two cytokinins (BAP and Kn) or a cytokinin and an auxin, each at a concentration up to 1 mg/l, is better for high-frequency shoot regeneration. However, a better understanding of the missing links with the application of callogenesis and somatic embryogenesis can prove to be an essential turning point for large-scale production to conserve and exploit its pharmacological property. The work on somatic embryogenesis will also help in reducing labor costs because of its automation potential. The majority of the published reports claimed a high percentage of regeneration but did not mention the sample size for the ratios of explanted tissues to the hardened

plantlets. While working on the regeneration of the plant, the author's group encountered heavy contamination after 6–14 days of culture establishment by endophytic fungus. The endophytic interaction seems to be mutualistic for the production of natural dye santalin. The report of contamination by endophytic fungi in *Pterocarpus* is scarce. Further and more substantial efforts in these respects are in progress by the authors' group to induce somatic embryogenesis.

Future prospects

To overcome the endangered status of *P. santalinus*, we need to explore different explant types having high-efficiency of regeneration potential. Experiments applying various means to control the endophytic fungus in mature explants are highly desirable for future research to achieve productive output. It is the utmost requirement of the future to apply the organogenic mode of regeneration process to maintain the clonal property and meet its high international demand. For the high-volume production of propagules, an optimized propagation method through somatic embryogenesis is needed. The somatic embryo fluidics system, which is already used for large-scale somatic embryo production in conifers, can also be explored to multiply woody plants (Egertsdotter et al. 2019). The success in optimizing somatic embryogenesis can find application in synthetic

seed technology, cryopreservation of germplasm, secondary metabolite production using bioreactor, and possible genetic modification for its natural ‘santalin’ production, are the aspects to be explored for future research.

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Declarations

Conflict of interest All the authors declared that there is no conflict of interest with regard to any part of the manuscript.

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