PLANT SCIENCE TODAY
ISSN 2348-1900 (online)
Vol x(x): xx–xx
https://doi.org/10.14719/pst.1474

HORIZON
e-Publishing Group
HePG

RESEARCH ARTICLE

Histological and *in vitro* seed culture studies on *Biancaea sappan* (L.) Tod. (Caesalpiniaceae)

M.G. Ebbie[1], K. A. S. Grace[2], S. Ravi Shankar[2] & M. Venkatesan[3*]

[1]Department of Botany, Presidency College (Autonomous), Chennai– 600 005, Tamil Nadu, India
[2]Department of Botany, Madras Christian College (Autonomous), Tambaram– 600 059, Tamil Nadu, India
[3]Department of Botany, Sourashtra College (Autonomous), Madurai– 625 004, Tamil Nadu, India

*Email: swertia@gmail.com

OPEN ACCESS

ARTICLE HISTORY
Received: 06 September 2021
Accepted: 20 March 2022

Available online
Version 1.0 (Early Access): 30 April 2022

Check for updates

Additional information

Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

Reprints & permissions information is available at https://horizonepublishing.com/journals/index.php/PST/open_access_policy

Publisher's Note: Horizon e-Publishing Group remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Indexing: Plant Science Today, published by Horizon e-Publishing Group, is covered by Scopus, Web of Science, BIOSIS Previews, Clarivate Analytics, etc. See https://horizonepublishing.com/journals/index.php/PST/indexing_abstracting

CITE THIS ARTICLE

Ebbie M G, Grace K A S, Shankar S R, Venkatesan M. Histological and *in vitro* seed culture studies on *Biancaea sappan* (L.) Tod. (Caesalpiniaceae). Plant Science Today (Early Access). https://doi.org/10.14719/pst.1474

Abstract

Establishment of a tissue culture protocol for clonal regeneration is an essential prerequisite for the potential and economically important plant species *Biancaea sappan* (L.) Tod. (syn. *Caesalpinia sappan* L.; Caesalpiniaceae). This plant is a valuable dye-yielding legume shrub native to Indomalayan and is used in India by the local people of Kerala and Tamil Nadu States for its biological potential. The present study evaluated seed development both *in vivo* and *in vitro* through histological analysis. The mature seed was used as explants and cultured on modified Murashige and Skoog (MS) medium supplemented with 2, 4-D, BAP, GA_3 and IBA. The cultures showed direct regeneration of shoots from nodular structures in the shoot apical meristem (SAM) region. During *in vitro* regeneration, the callus cells exhibited ballooning of cells, and the morphogenetic seed developed parenchymatous, polygonal cells filled with polyphenols. Histological studies indicated that *in vitro* plant regeneration involved the organogenic pathway that leads to *de novo* shoot primordia development.

Keywords

Biancaea sappan, Callus, Histology, Nodular structure, Shoot apical meristem (SAM), Shoot primordia.

Introduction

Natural products have bestowed a variety of lead structures, which serve as templates for developing new drugs (1). Many plant species have shown promising results in drug discovery, textile making and other vital fields. Global understanding of natural products is on a surge. Nature has gifted us with more than 500 colour yielding plants used in the colouring of textiles, drugs, cosmetics etc (2). Indians have been considered as forerunners in the art of natural dyeing (3). *Biancaea sappan* (L.) Tod. (syn. *Caesalpinia sappan* L.) is an important medicinal and dye yielding plant (1). It is a medium-sized thorny tree that grows up to a height of 6-9 m (4). The plant is distributed and cultivated in Southeast Asia, Africa and America (5).

The heartwood of this plant is traditionally used in Ayurveda for the vitiated conditions of pitta, burning sensation, wounds, ulcers, leprosy, skin diseases, diarrhoea, dysentery, epilepsy, menorrhagia, leucorrhoea and diabetes (6-7). Dry wood chips from the bark of sappan tree were boiled in water in a ratio of 6:1. During large scale preparation they were immersed in huge reactors of about 150 l capacity. The wood chips are boiled at 70 °C, 80 °C, 90 °C and 100 °C for 3 hrs in order to get various shades (8).

It has been reported that *B. sappan* contains phytoconstituents such

as aromatic compounds (9), homoisoflavonoids (10) phenolic compounds (11), protosappanins (12) and diterpenoids (13). Biological studies such as anticancer (14), antibacterial (15), analgesic (16), immunomodulatory (17) antioxidant, anthelmintic properties (18) and toxicological studies (19) have been already documented.

Conventional breeding methods blended with *in vitro* culture method serve as an excellent tool for crop improvement. *In vitro* culture of seeds or embryos results in the development of somatic embryos (20). The mode of callus regeneration could be better understood with the help of histological studies. Hence, the present study was envisaged to understand direct organogenesis of *B. sappan* through histological analysis.

Materials and Methods

Collection of Plant Materials

The seeds of *B. sappan* were collected from Sri Paramakalyani Centre for Environmental Sciences (SPKCES) campus, Manonmanium Sundaranar University, Alwarkuruchi, during the month of December, 2015. The plant was authenticated and a voucher specimen (Accession Number: 9875) has been deposited in the herbarium of the Madras Christian College, Tamil Nadu.

All the chemicals were procured from the Himedia laboratories, Mumbai, India. Macro and micro photographs were performed using DSLR Nikon D7000 camera and DSLR Canon EOS450D camera fitted to the Carl Zeiss Axio vision microscope respectively.

Methods

Isolation and Sterilization of Plant Tissues

Fully ripe fruits of sappan (*Biancaea sappan* (L.) Tod.) collected and maintained in the laboratory. The seed coat was removed using sterile blade and fully mature, healthy and disease-free seeds were selected and used for present studies. Seeds were surface sterilized by treating with a mixture of 1:1 absolute ethanol: hydrogen peroxide 30 % (v/v) for 15 minutes. in the laminar airflow cabinet under aseptic conditions. Later the seeds were sterilized using 0.005% of mercuric chloride. Seeds were cut into small segments and were used as explants.

Explant and Growth Conditions

In the present study, two different media compositions were used *i.e.* MS medium supplemented with 2, 4 – D 1 mg/l and BAP 1 mg/l and GA3 1 mg/l and IBA 1 mg/l (21). The explants were cultured in the MS medium supplemented with 2, 4 – D 1 mg/l and BAP 1 mg/l on culture vials for callus induction. The callus cultures were aseptically transferred to MS media supplemented with GA_3 1 mg/l and IBA 1 mg/l for the development of shoot primordia.

Histology

Histological analysis was carried out in the seed and the callus of *in vitro* cultured seed. Hand sections were made in seed and callus of *in vitro* cultured seed to understand the formation of shoots. Thin sections were identified and stained with I_2KI, Toluene blue O and Sudan IV (22-24) observed under a compound microscope and photographed for visualizing various types of cells.

Results

The plant *B. sappan* bears 3-4 seeds inside a pod. The pods are woody, compressed and re-curved at the apex. The seed coat is hard, brown and protects the embryo until germination. The seed surface is rough in texture (Fig. 1a). Seeds absorb water and get hydrated when soaked in water overnight. This is the first step in breaking seed dormancy. The seeds bulge and cracks develop on the surface

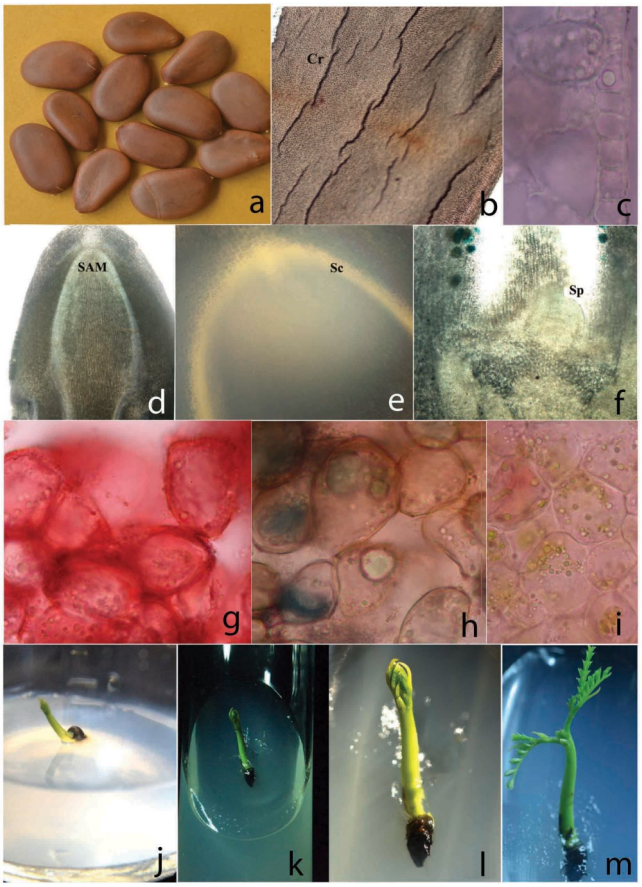

Fig 1. **a** Seeds of Sappan, **b** Water imbibed seeds with cracks on the seed coat, **c**.Exotesta of the seed, **d**. LS of seed showing SAM in the distal end, **e.** Mature seed showing scutellum found in between embryo and endosperm, **f.** Development of nodular appearance in shoot apical meristem (SAM), **g.** Rapid proliferation of parenchymatous cells, **h.** Elongation of highly meristamatic cells filled with polyphenols, **i.** Zone of transition of cells with starch accumulation, **j.** Seed explant response in induction media 2,4 – D 1 mg/l and BAP 1 mg/l after a period of 2 weeks, **k.** Shoot regeneration from seed explant in a sub cultured media rich in GA3 1 mg/l and IBA 1 mg/l (3 weeks old), **l.** Regenerated shoot exhibiting leaf primordia development in 3 weeks time, **m.** Shoot regeneration showing leaf expansion in a period of 4 weeks GA3 1 mg/l + BAP 1 mg/l.

of the seed coat (Fig. 1b). The seed coat, when removed looks plumpy cream in colour. The seed coat is made out of hard sclerenchymatous cells and constitutes the exotesta. The exotesta remains distinctly visible and is seen as a single layer of cell (Fig. 1c).

The L.S. of water soaked seeds were observed for the subsequent developmental variations. The cotyledons expand due to the deposition of reserve food material. The longitudinal section of the seed shows a proximal and dis-

tal end. The proximal end of the seed is oval in morphology and consists of the chalaza. The distal end is a little sharp at the apical region and constitutes the micropyle. Shoot apical meristem (SAM) starts to initiate from the chalazal end (Fig. 1d).

The cotyledon is found in the inner region and the endosperm in the outer region. A prominent palisade cell layer is observed in between the cotyledon and the endosperm (Fig. 1e). The nodular cell appears to be spherical which develops into SAM (Fig. 1f). The SAM develops rapidly dividing cells, instead of forming a bipolar structure.

The cultured callus cells were histologically analyzed. Callus cells reveal the presence of parenchymatous cells. These cells are round in structure and visualized with the help of safranin (Fig. 1g). During development, the cells become loosely arranged, elongated and are spotted as non-embryogenic callus cells with a nucleus. The cells also contain polyphenols (Fig. 1h). This region is the transition zone. The cells encompass large reserve food and start to develop into shoot primordia (Fig. 1i). The sequential development of shoot primordia is well understood over days (Fig. 1j-l). Initial shoot primordia development has been observed after four weeks of incubation (Fig. 1m).

Discussion

In the present study, an attempt has been made to understand the seed germination potential of *B. sappan*. Critical seed moisture enhances the percentage of seed germination. Water soaking overnight helps the seeds overcome the state of quiescence (25). The epidermal cells of the exotesta develop small gaps for the entry of water during soaking (26). Due to osmotic pressure, turgor pressure inside the seed increases. During this physiological process irregular cracks develop on the surface of the seed. In *Ipomoea lacunose* (L.), breaking of seed dormancy by the development of cracks were reported. A crack in the hilum pad appears to be the possible route of water entry (27). Irregular cracks in the seed coat may also be responsible for the occurrence of an innately permeable seed fraction (28). The peculiar feature in the longitudinal section of seed shows scutellum development, a common feature in monocots and a rare feature in dicots (29). On the other hand, the *in vitro* developed callus undergoes various morphogenetic changes, including the development of polygonal cells. Most of these cells were parenchymatous and isodiametric in shape. In contrary, the seeds of *B. sappan* form nodular structures. The cells in the nodular structure undergo rapid cell division and form a small mass of undifferentiated callus followed by the regeneration of shoot primordia. Pro-meristem were found to develop from shoot primordia. The polyphenols encompassed in the cells are associated with the defense against pathogens during seed germination (30).

A striking change found between the development of pro-meristem and shoot primordia is the development of transition cells. Transition cells are those cells in the phase of transition by gaining a large quantity of starch as a food reserve. Morphogenetic changes in the cell structure transform the callus cells into putative initials of vascular strands. This clear transition of cell study corroborates with the earlier studies (31). The formation of vascular connections has been studied in the developing callus of Rosa hybrid (32). In the present study, the formation of shoot primordia is through direct organogenesis. The possible pathway of organogenesis is either directly from the initial explant or indirectly from callus (33-36). The reason, attributed to direct organogenesis is the auxin to cytokinin ratio. The auxin to cytokinin ratio also plays a vital role in morphogenetic differentiation (37).

Conclusion

The seed during growth represents a remarkable transition from starch reserve metabolism to the development of photosynthetic organs. Histological studies reveal the formation of shoot apical meristem (SAM) by the formation of nodular structures in the distal end. This is in contrary to the formation of somatic embryos in many plants. The steps involved in direct shoot regeneration and histology of seed helps us to understand its development in *B. sappan*. From the histological analysis, we conclude that the developing shoots are predominantly organogenic in origin.

Acknowledgements

The authors are thankful to late Dr. GAI Ebenezer, (Former Head of Botany Department) Madras Christian College, Chennai, Tamil Nadu, India for providing necessary laboratory facilities.

Authors contributions

MGE has worked in conceiving the idea. He has also worked on the histology, plant tissue culture studies and wrote the entire manuscript. KASG has conducted the experiments in the plant tissue culture laboratory. MV and SR have contributed towards designing the work, addition of information and reading of manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

References

1. Badami S, Moorkoth S, Suresh B. *Caesalpinia sappan*: a medicinal and dye yielding plant, Natural Products Radiance. 2004;3 (2):75-82.
2. Mahanta D, Subhash T. Natural dye yielding plants and indigenous knowledge on dye preparation in Arunachal Pradesh, Northeast India. Current Science. 2004;88:1474-80.
3. Siva R. Status of natural dyes and dye-yielding plants in India. Current Science. 2007;92(7):916-25.
4. Pawar CR, Landge AD, Surana SJ. Phytochemical and Pharmacological Aspects of *Caesalpinia sappan*. Journal of Pharmacy Research. 2008;1(2):131-38.
5. Uddin GM, Kim CY, Chung D, Kim KA, Jung SH. One-step isolation of sappanol and brazilin from *Caesalpinia sappan* and their

effects on oxidative stress-induced retinal death. BMB Reports. 2015;48(5):289-94. https://doi.org/10.5483/BMBRep.2015.48.5.189

6. The Wealth of India: A Dictionary of Indian Raw Materials and Industrial Products-Raw Materials Series, Vol. II, Publication and Information Directorate, CSIR, New Delhi, 1988;14-16.
7. Warrier PK, Nambiar VPK, Ramankutty C, Varriers VPS. Indian Medicinal Plants, A compendium of 500 species, Orient Longman Ltd., Chennai. 1993;Vol.1: 291-94.
8. Tharaka KPD, Anandawansha WAKTM, Dayananda PNA, Dilani PVD, Sampath UWR, Yahampath DIM. Developing a dye using *Caesalpinia sappan* (Pathangi) wood extract. In: Proceedings of the Higher Education for the Twenty first Century 2015, Faculty of Science, University of Colombo, Sri Lanka.
9. Shimokawa T, Kinjo JE, Yamahara J, Yamasaki M, Nohara T. Two novel aromatic compounds from *Caesalpinia sappan* L. Chemical and Pharmaceutical Bulletin. 1985;33:3545-47. https://doi.org/10.1248/cpb.33.3545
10. Namikoshi M, Nakata H, Saitoh T. Homoisoflavonoids from *Caesalpinia sappan*, Phytochemistry. 1987;26:1831-33.https://doi.org/10.1016/S0031-9422(00)82298-0
11. Tu PF, Zhao MB, Li J, Shi SP, Cai CQ, Tang L, Zeng KW, Jiang Y. Two new phenolic compounds from the heartwood of *Caesalpinia sappan* L. Journal of Molecules. 2014;19:1-8. https://doi.org/10.3390/molecules19010001
12. WangZ**,** Sun JB, Qu W, Guan FQ, Li LZ, Liang JY. *Caesappin A* and *B*, two novel protosappanins from *Caesalpinia sappan* L. Fitoterapia. 2014;92:280-84. https://doi.org/10.1016/j.fitote.2013.12.004
13. Wang DS, Nie W, Jiang TT, Ding LF, Song LD, Wu XD, Zhao QS. Caesalpanins A–C, Three dimeric cassane diterpenoids from the seeds of *Caesalpinia sappan* L. Chemistry and Biodiversity. 2020;17:1-9. https://doi.org/10.1002/cbdv.202000103
14. Lee JS, Kim YG, Kim JH. Studies on anticancer effects of extract *Caesalpinia sappan* on oral carcinoma and osteosarcoma cells. Journal of the Korean Association of Oral and Maxillofacial Surgeons. 2001;27(4):281-88.
15. Xu HX, Lee SF. The antibacterial principle of *Caesalpina sappan*. Phytotherapy Research. 2004;18(8):647-51. https://doi.org/10.1002/ptr.1524
16. Hemalatha K, Kiran AS, Bannappa U, Satynarayana D. Analgesic Activity of *Caesalpinia sappan* Heartwood. Pharmaceutical Biology. 2007;45:360-62. https://doi.org/10.1080/13880200701213005
17. Sunitha VS, Sunil MA, Radhakrishnan EK, Jyothis M. Immunomodulatory activity of *Caesalpinia sappan* L. extracts on peritoneal macrophage of albino mice. International Journal of Science and Research. 2015;4(12):2319-7064. https://doi.org/10.21275/v4i12.NOV151953
18. Harjit K, Amini MH, Suttee A. Evaluation of antioxidant and anthelmintic properties of *Caesalpinia sappan* L. leaves. International Journal of Pharmacognosy and Phytochemical Research. 2016;8(2):362-68.
19. Athinarayanana G, Ranjitsingh AJA, Usha Raja Nanthini A, Padmalatha C. Toxicological studies of *Caesalpinia sappan* wood derived dye in Wisteralbino rats. Food Science and Human Wellness. 2017; 6: 34-38. https://doi.org/10.1016/j.fshw.2016.10.004
20. Vinodhini Jochebed V, Fathima Benazir J, Mathi Thumilan B, Ravichandran P. *In vitro* regeneration of *Caesalpinia sappan* L.–A rare and natural dye yielding medicinal plant. In: Proceedings of the National Seminar on Biotechnology: Hitherto and Henceforth, The American College, Madurai, APT-7. 1999; 35.
21. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiologia Plantarum. 1962;15:473-97. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x
22. Krishnamurthy KV. Methods in Plant Histochemistry. S. Viswanathan Printers and Publishers Private Limited. Madras, India. 1988.
23. Dixon RA, Gonzales RA. Plant Cell Biology: A Practical Approach. In: Harris N, Oparka KJ, Editors. 1994. Oxford University Press, New York.
24. Gomori G. Microscopic Histochemistry: Principles and Practice, University of Chicago Press, Chicago. 1952. https://doi.org/10.5962/bhl.title.6273
25. Bareke T. Biology of seed development and germination physiology. Advances in Plants and Agriculture Research. 2018;8(4):336-46.https://doi.org/10.15406/apar.2018.08.00335
26. Muhl QE, Du Toit ES, Steyn JM, Robbertse PJ. The embryo, endosperm and seed coat structure of developing *Moringa oleifera* seed. South African Journal of Botany. 2016;106:60–66. https://doi.org/10.1016/j.sajb.2016.05.009
27. Jayasuriya KMGG, Baskin, JM, Geneve RI, Baskin CC. Morphology and anatomy of physical dormancy in *Ipomoea lacunosa*: Identification of the water gap in seeds of Convolvulaceae (Solanales). Annals of Botany. 2007;100:13–22.https://doi.org/10.1093/aob/mcm070
28. Ma F, Cholewa E, Mohomad T, Peterson CA, Gigen M. 2004. Cracks in the palisade cuticle of soybean seeds correlate with their permeablity to water. Annals of Botany. 94:213-28.https://doi.org/10.1093/aob/mch133
29. Damayanti F, Suharsono, Tjahjoleksono A, Mariska I. Regeneration and histological study of somatic embryogenesis of sugarcane (*Saccharum officinarum* L.) cultivar PS 864. Journal of Biological Researches. 2018;24(1):53-57. http://dx.doi.org/10.23869/bphjbr.22.2.20178
30. GripenbergS, RotaJ, Kim J, Wright SJ, Garwood NC, Fricke EC, Zalamea PC, Salminen JP. Seed polyphenols in a diverse tropical plant community. Journal of Ecology. 2018;106:87–100. https://doi.org/10.1111/1365-2745.12814
31. Konieczny R, Sliwinska E, Pilarska M, Tuleja M. Morphohistological and flow cytometric analyses of somatic embryogenesis in *Trifolium nigrescens* Viv. Plant cell tissue and organ culture. 2012;109:131-41. https://doi.org/10.1007/s11240-011-0081-x
32. Rout GR, Samantary S, Mottley J, Yokoya K, Mandegaran Z, Sarasan V, Kandasamy K, Roberts AV, Das P. 1998. Histology of *In vitro* organogenesis and somatic embryogenesis in callus cultures of Rosa. Biologia Bratislava. 1998;53(1):121-26.
33. Williams EG, Maheswaran G. Somatic embryogenesis: factors influencing coordinated behaviour of cells as an embryogenic group. Annals of Botany. 1986;57(4):443-62. https://doi.org/10.1093/oxfordjournals.aob.a087127
34. Kuo HL, Chen JT, Chang WC. Efficient plant regeneration through direct somatic embryogenesis from leaf explants of Phalaenopsis 'Little Steve'. *In vitro* Cellular and Developmental Biology. 2005;41(4):453-56. https://www.jstor.org/stable/4293883. https://doi.org/10.1079/IVP2005644
35. Jasrai YT, Thaker KN, D'Souza MC. *In vitro* propagation of *Euphorbia pulcherrima* Willd. through somatic embryogenesis. Plant Tissue Culture. 2003;13(1):31-36.
36. Huang X, Chen J, Bao Y, Liu L, Jiang H, An X, Dai L, Wang B, Peng D. Transcript profiling reveals auxin and cytokinin signaling pathways and transcription regulation during *In Vitro* organogenesis of ramie (*Boehmeria nivea* L. Gaud). PLoS ONE.2014;9:1–24. https://doi.org/10.1371/journal.pone.0113768
37. Ebida AIA, Hu Cy. *In vitro* morphogenetic responses and plant regeneration from pepper (*Capsicum annuum* L. cv. Early California Wonder) seedling explants. Plant Cell Reports**.** 1993;13:107-10. https://doi.org/10.1007/BF00235301

§§§