Int.J.Curr.Microbiol.App.Sci (2020) Special Issue-11: 2108-2116

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Special Issue-11 pp. 2108-2116**
Journal homepage: http://www.ijcmas.com

Original Research Article

Organogenic Responses in Dried Seed and Nodal Explants of Teak (*Tectona grandis*) and their Phytochemical Analysis

Pushpendra Singh[1*], Yesh Pal Singh[2], Anant Kumar[3] and Sumit Sharma[4]

[1]Helix Biogenesis, Noida, India
[2]KVK, Ujhani, Budaun, SVPUA &T, Meerut, U.P., India
[3]KVK, Ghaziabad, SVPUA &T, Meerut, U.P., India
[4]Department of Biotechnology, Bennett University, Greater Noida, India
**Corresponding author*

A B S T R A C T

Teak is a very high values and demanded timber plant. It is versatile in nature and longer in durability. Teak is used in making furniture based items, sculptures, etc. Due to higher demand this plant is needed more in quantity, but its low germination potential and slow growth made it limited in supply. We, therefore, have developed a fast growing tissue culture based approach using mature seed, nodal and intermodal explants. In vitro tissue culture clones were developed from embryos, nodes, and internodes with different concentrations of plant growth regulators. Seed coat softening by 1N KOH (Potassium hydroxide) and phenolic extract removal using ascorbic acid and citric acid before inoculation was implemented as accelerating measures for explants preparation for this hard wood species. The callus formation from seed embryo and nodes were observed at the concentration of 8.88 µM and 13.32 µM BAP (Benzyl aminopurine), respectively. The shoot of upto 4 cm were also developed from embryo and callus at 8.88 µM BAP and 11.10 µM BAP + 0.22µM IAA respectively. High concentration of auxin was also found to promote callus formation. For photochemical analysis, aqueous extract of leaves of *Tectona grandis* was analyzed which indicates the presence of flavonoids, steroids, saponins, alkaloids, inulin and reducing compounds. However, tannin, naphthoquinone, glycosides, phlabtomines were found to be absent.

Keywords

Tectona grandis, Seed coat softening, Phenolics removal, Callus formation and regeneration

Introduction

Teak (*Tectona grandis*) is a high quality and one of the most valuable timbers in the world (Vyas *et al.*, 2019). *Tectona grandis* is a large natural tree from Southeast Asia and grows upto 50 m in height. During the second half of the 19th century, the increasing demand for teak wood led to the implantation of the agroforestry system for teak in Indonesia and other countries (Macias *et al.*, 2008). Teak is one of the most valuable timber in international trade and an important species for tropical forestry (Sousa *et al.*, 2012). Traditionally, this species is globally used to relieve fever, diabetes, lipid disorders, ulcers, inflammation, bronchitis, cancer and tuberculosis (Rajuri *et al.*, 2010; Warrier, P.S., 1994).In the Northern part of Cameroon, leaves of teak are used for their laxative

properties while in the Western Region; it is used for the treatment of skin diseases. It is locally known as teak, commonly known as sagwan in Hindi and Saka in Sanskrit and belongs to Verbenaceae family (Giri and Varma, 2015). It is grown largely in India (Madhya Pradesh and Kerala) and Burma regions.

It is a slow growing hardwood tree, which takes around 7-8 years for thinning and around 15-20 years to get high quality of timber (Dotaniya *et al.,* 2013). The fruits of tree are very hard each bearing 1-4 seeds enclosed in thick, hairy exocarp with stony endocarp of the fruit (Palanisamy *et al.,* 2009). It is, therefore, very difficult to grow plants from seed in natural as well as artificial environment, so, the fast growth alternatives like tissue culture are always a need for it. The micropropagation of woody plants is often problematic because of genotypic variation in the regeneration responses and the process of aging. Shoot cultures are commonly used in the micropropagation of economically important woody plants (Skirvin 1986). Most of the studies in tissue culture on *Tectona grandis* were also done using nodal explants to obtain faster results (Tiwari *et al.,* 2002). The tissue culture using explants such as nodes and internodes from mature plants is still problematic, because of its poor growth response and higher phenolic exudations (Kozgarand Shahzad, 2012). Teak is so rich in phenolic and aromatic compounds that under in vitro condition; it leaches out secondary metabolites to the medium resulting in browning of medium and reduction in the nutrient transport. Previous phytochemical investigation of Tectona species have led to the isolation of triterpenoids, flavonoïds (Ragasa *et al.,* 2008a), chromomoric acid derivatives (Ragasa *et al.,* 2008b), anthraquinones (Sumthong *et al.,* 2006; Sumthong *et al.,* 2008), naphthoquinones (Pradeep and Pahub 2004; Lacret *et al.,* 2011), anthraquinone-naphthoquinones (Lacret *et al.,* 2011; Aguinaldo *et al.,* 1993), apocarotenoids (Macias *et al.,* 2008) and lignans (Lacret *et al.,* 2012). Some of these metabolites particularly the quinines showed anti-mycobacterial, antifungal and allopathic activities (Lacret *et al.,* 2011; Aguinaldo *et al.,* 1993; Pradeep and Pahub2004; Macias *et al.,* 2008). Due to its high timber properties and other uses, it is important to propagate and conserve its high quality germplasm available at selected sites across world.

Materials and Methods

The present study was conducted at Helix Biogenesis, Noida, to develop a standardized protocol from mature tree. In this study we have focused on softening of seed coat, pre-extraction of phenolics and callus & shoot development in context to reduce difficulties in regeneration of hardwood teak plant. We have used nodal, internodal and embryo excised from mature seeds for micropropagation of teak comprising anti-oxidant treatment of the explants before culturing into the medium.

Collection of plant material and media preparation

Plant material used in the present study such as mature seeds, twigs having nodes and internodes were collected from 5-6 years old teak plants located on the service lane near the Yamuna Expressway, Greater Noida, India. Cut ends of twigs were immediately dipped in distilled water to avoid oxidation at cut ends and to avoid drying of material during transportation.

The MS (Murashige and Skoog, 1962) medium was prepared by using stocks of 20X macronutrients, 200X micronutrients and

freshly prepared Fe-EDTA (10X) with 3% sucrose; pH was adjusted to 5.8 and agar was used at a final concentration of 1.5% (Kumar and Loh, 2012, Gupta *et al.,* 1980). MS media was supplemented with various concentrations / combinations of BAP (2.22 to 17.76 µM) and IAA (0.11 to 0.44 µM) for growth initiation and culture establishment. 250 ml glass bottles having 60 ml media each and autoclaved were used for culturing and subculturing purpose.

Explants preparation and culturing

There were three kinds of explants used, mature embryo dissected from seeds, nodes and internodes. Seed coat softening and phenolic removal was done before explants sterilization.

Softening of seed coat and sterilization of seeds

Mature seeds were soaked in 1 N KOH solution and kept for 16 hours for softening of the hard seed coat so that the seed embryo can be recovered easily with the help of nutcracker. After 16 hours of soaking, seeds were removed from the KOH solution and transferred to sterile double distilled water for washing (repeated 3 times). Seeds were later soaked in a 0.5% Tween-20 solution for two minutes. After that, two cycle of wash was given with ADW (autoclaved distilled water) for two minutes. Seeds were then treated with 7:3 (ethanol: ADW); for three minutes (repeated 3 times), followed by treatment with mercuric chloride (1 g/l) for 5 min with continuous shaking. Final washing was done for three times with ADW for 3 minutes each. Sterilized seed explants were cracked open by nut cracker to remove seed coat and seed embryos were carefully recovered to avoid any damage to the embryo. Each embryo was then inoculated in growth medium in duplicates. These cultures were incubated in plant tissue culture room, having 25±2℃ temperature along with 16:8 hours light cycle.

Phenolics removal and sterilization of nodes and internodes

Healthy twigs collected from 5-6 year mature tree were cut into the nodes & internodes and were kept in 0.2% ascorbic acid and 0.2% citric acid solutions for overnight to remove the phenolic compound from the explants as these phenolic compounds can prevent the initiation or growth of explants in the medium. The explants were than surface sterilized with two cycle of distilled water for 5 minutes each, followed by washing in 0.5% Tween-20 solution for 5 minutes. Explants were then treated with ethanol:ADW (7:3) for 5 minutes; washed 3 times with ADW for two minutes followed by treatment with mercuric chloride (1g/l) for 5 minutes with continuous shaking and washing with ADW three times for 2 minutes each. Explants were also treated with antifungal solution of Keratenazole for 5 minutes, washed two times with autoclaved double distilled water for 2 minutes each. Explants were then exposed to H_2O_2:ADW (1:1) for 5 minutes. Finally, explants were washed thrice with autoclaved double distilled water for 2 minutes each. All the surface sterilized nodes and internodes were cut 2-3 mm from both ends to expose the fresh meristematic cells. Then, explants were inoculated in MS media supplemented with various growth regulators. The cultures were then kept in culture room at 25±2℃. All the sample jars were incubated at 16hrs.light :8 hrs.dark photoperiod.

Establishment of Cultures

Explants thus cultured started showing growth approximately after 10-15 days from inoculation. Those explants showing growth were subcultured in similar medium for

further growth and shoot or callus development. Undesirable or brownish plant parts were removed from the explants, afterward; callus and germinating embryos were transferred aseptically to the fresh culture medium.

Phytochemical screening

The aqueous extract of mature leaves from 5-6 year old teak plant were subjected for phytochemical screening as per the methods suggested by Eluyode and Alabi, 2007. Leaf powder (0.1 g) was mixed with 10 ml of water and boiled for 10 minutes. The boiled samples were further filtered through whatman filter and the filtered extract was used for further analysis.

Results and Discussion

Seed coat softening and embryo germination

The first step in the explant preparation was to remove seed coat, which was very hard and could not be easily broken down. If it was directly tried to break with nutcracker, the embryo was certainly getting damaged and even sometimes embryo was completely crushed. We, therefore, used KOH for treating seed coat for softening it. KOH treated seeds were broken down easily by nutcracker and mature embryos were recovered without any damage. Figure- 1 showed the seed treatment, embryo removal and inoculation. The germination of embryo and regeneration of plantlets at different hormonal concentration was studied. Plantlet regeneration was observed to be maximum in 8.88 µM BAP concentration (alone) and 4.44 µM BAP + 0.22 µM IAA (in combination). Different stages of growth of plantlet regeneration from embryo are shown in Figure- 2 and 3. Plantlets were subcultured at 21 days interval and grown for upto 60 days in media before acclimatization. The cotyledonary leaves and initial leaves of the plant were sub-cultured for callus induction and shoot development. The callus was developed from embryo and leaves in approximately 30-40 days (Figure 4).

Callus regeneration and shoot development

The callus obtained from embryo and cotyledonary leaves was subcultured aseptically and further transferred to the shoot and root development medium. The shoot development was observed in MS media supplemented with 11.10 µM BAP and 0.22 µM IAA concentrations (Figure 5), which represents that higher concentration of growth regulators is required for shoot formation.

Tiwari *et al.,* 2002, also found similar observation with higher concentration of 22.2 µM BAP and 0.57 µM IAA for shoot development from teak plants. The callus was compact as observed during cutting, and it took longer time to regenerate into shoots. It took more than three months (110 days) for shoot to develop (4 cm in length) from this callus (Figure 5).

Callus formation from node and internodes

The ascorbic acid and citric acid treated nodes and internodes were inoculated in the medium and callus formation was observed. The amount of callus was insignificant in the nodes and internodes in comparison to the callus developed from embryo and leaves. In case of ascorbic acid treated explants callus induction was observed after 30 days of incubation. Release of phenolic components in medium was also analyzed to study the effect of ascorbic acid and citric acid treatment.

Table.1 Identification of compounds in *Tectona grandis*by phytochemical analysis

S. No.	Compounds name	Results
1.	Alkaloids	++
2.	Flavonoids	++
3.	Glycosides	-
4.	Inulin	++
5.	Naphthoquinone	-
6.	Phlabtomines	+
7.	Protein (by ninhydrin test)	-
8.	Reducing compounds	+
9.	Saponins	++
10.	Starch	-
11.	Steroids	+++
12.	Tannin	-

+++ Highly Positive, ++, Moderately Positive, + Less Positive, - Negative

Fig.1 Seed coat treatment, embryo removal and inoculated embryos

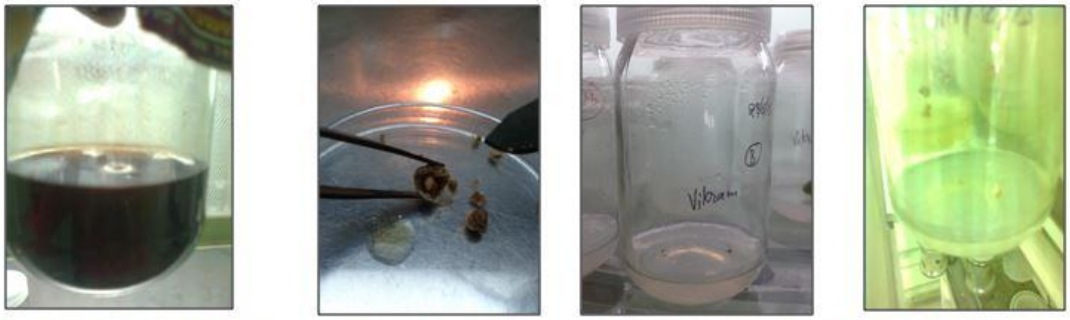


Fig.2 Teak seed embryo development in MS medium supplemented with 8.88 µM BAP

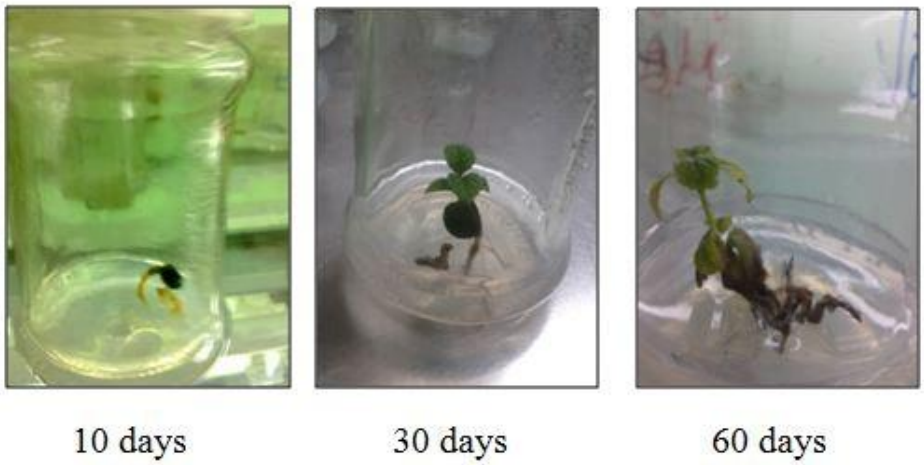


Fig.3 Teak's seed embryo germination development in MS + 4.44 µMBAP + 0.22µM IAA concentration

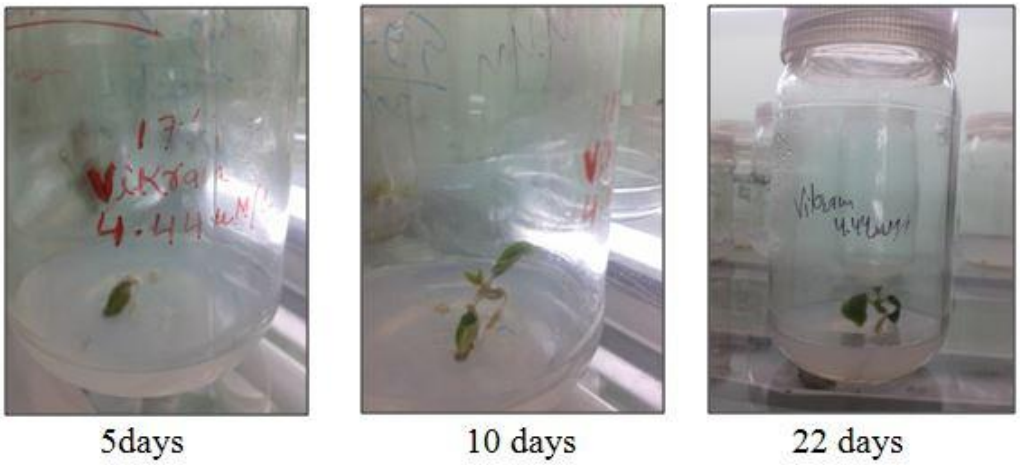


Fig.4 Callus induced from embryo, cotyledon and leaf explants of teak

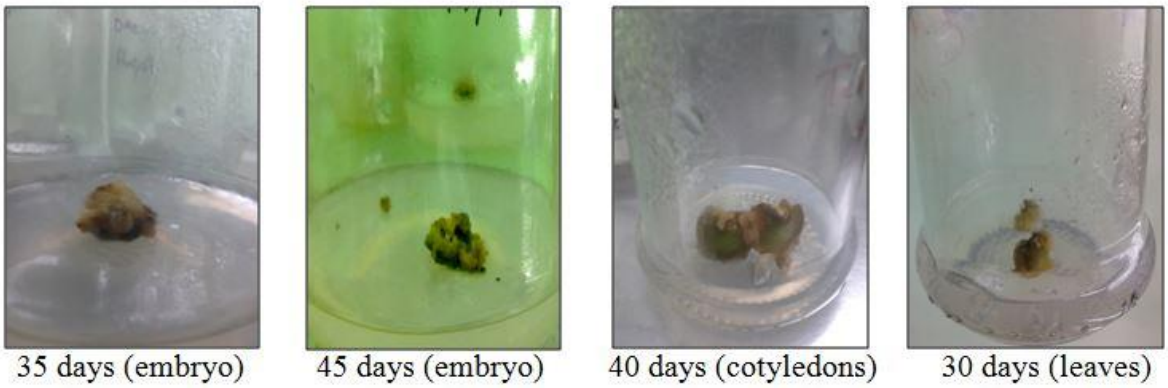


Fig.5 Plantlet regeneration from cotyledonary callus at 11.10µM BAP and 0.22 µM IAA concentration

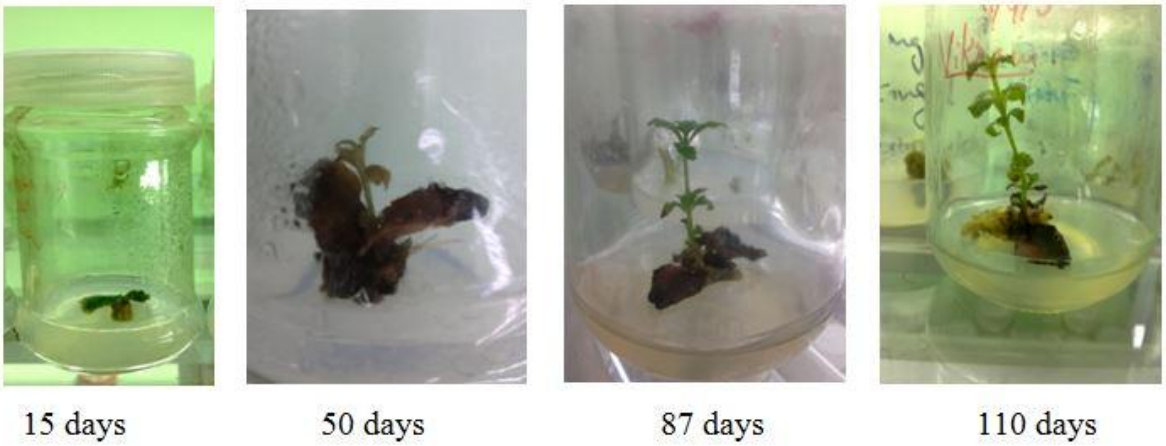
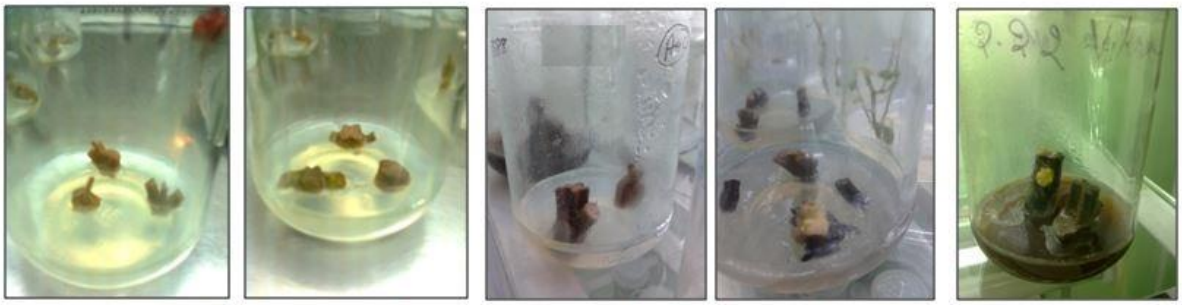


Fig.6 No or very less phenolics released in ascorbic acid (0.2%) and citric acid (0.2%) treated explants, while control shows phenolics release in the medium (brown colour)



Teak explants release phenolic components in the medium, effect of which were minimized by treating with 0.2% v/v of ascorbic acid or citric acid. Figure 6, shows the effect of pre-phenolic extraction and treatment with organic acids.

The medium was semitransparent in both organic acid treated explants even after two weeks, while, browning of medium was observed in control (without treatment) due to release and oxidation of phenolic compounds. The callus formations from node, internodes were studied to be from 8.88 µM to 17.76 µM BAP in combination with 0.22 µM IAA. Best callus formation was observed in 17.76 µM BAP concentration with 0.22 µM IAA.

Phytochemical studies on *Tectona grandis* leaves extract

The aqueous extract from teak plant was analyzed for its phytochemical content. Leaves of *Tectona grandis* contains the presence of inulin, saponins, reducing compounds, flavonoids, steroids, alkaloids, however, tannin, starch, naphthaquinone, glycosides and protein were not observed however, phlobatannins were present in trace amount in the aqueous extracts - (Table 1). Similar observations were reported by other researchers such as Eluyode and Alabi, 2007. He reported that tannin, phlobatannins, alkaloids and saponins were present while, glycosides, sterol, flavonoids and Carbohydrate were absent from solvent extract.

The present study identifies important, as an integral approach for tree improvement using latest genetic and molecular tools. In this study protocol for KOH as softening agent for teak seed coat was established and this resulted in establishing tissue culture from mature embryo. The dried fruit coat softening for seed removal and phenolics removal from nodes and internodes by using mild organic acid are better advancements implemented in this study. The development of callus and shoots from embryo, nodes and internodes showed promising results on working with mature teak plant. Phenolic components such as phenolic acids, tannins flavonoids, etc. are considered the most substantial phytochemical components produced by plants (Farag *et al.,* 2020). These compounds exist in various parts of the plant and their quantities significantly depends on the kind of the plant organ, climate, variety, location, etc. (Sifaoui *et al.,* 2017). The phytochemical study of aqueous extract of leaves of *Techtona grandis* also showed presence of various phenolic compounds which could be associated for its high medicinal value.

Acknowledgements

Authors are thankful to Helix Biogenesis Pvt. Ltd. for providing all the necessary inputs during the course of investigation including their lab facility. They are also a privilege to acknowledge their pleasure to laboratory staff and Mr. Vikram Chauhan (summer intern) for helping intakes care of study samples.

Conflict of interest

Authors are reporting no potential conflict of interest in this study.

References

Aguinaldo AM, Ocampo OPM, Bowden BF, Gray AI, Peter G. (1993). Tectograndone, an anthraquinone-naphthoquinone pigment from the leaves of *Tectona grandis*. Phytochemistry; 33(4): 933–935.

Dotaniya ML, Meena VD, Lata M and Meena HP (2013). Teak plantation-a potential source of income generation. Popular Kheti, 1(3): 61-63.

Eluyode OS and Alabi OS (2007). Preliminary phytochemical screening of crude extracts of gliricidiasepium, tectonegrandis and heveabracilliensi trees. Continental J. Agricultural Science 1: 22 – 27.

Farag, R. S., Abdel-Latif, M. S., Abd El Baky, H. H., & Tawfeek, L. S. (2020). Phytochemical screening and antioxidant activity of some medicinal plants' crude juices. Biotechnology reports (Amsterdam, Netherlands), 28, e00536.

Giri, S. P. and S. B. Varma, (2015). Analgesic and anti-inflammatory activity of *Tectona grandis* Linn. stem extract. Journal of basic and clinical physiology and pharmacology, 26(5), 479–484.

Gupta PK, Nadgir AL, Mascarenhas AF and Jagannathan V. (1980). Tissue culture of forest trees: clonal multiplication of *Tectona grandis* L. (teak) by tissue culture. Plant Science Letters, 17(3): 259-268.

Kozgar MI and Shahzad A (2012). An improved protocol for micropropagation of teak tree (*Tectona grandis* L.). Rendiconti Lincei, 23(2): 195-202.

Kumar PP and Loh CS (2012). Plant tissue culture for biotechnology. In Plant Biotechnology and agriculture, Academic Press.P; 131-138.

Lacret R, Varela RM, Molinillo JMG, Nogueiras C, Macias FA. (2011). Anthratectone and naphthotectone, two quinones from bioactive extracts of *Tectona grandis.* J Chem Ecol. 37:1341–1348.

Lacret R, Varela RM, Molinillo JMG, Nogueiras C, Macias FA. (2012). Tecto-noelins, new norlignans from bioactive extract of *Tectona grandis.* Phytochem Lett.; 5:382– 386.

Macias, F. A., Lacret, R., Varela, R. M., Nogueiras, C., & Molinillo, J. M. (2008). Bioactive apocarotenoids from *Tectona grandis*. Phytochemistry, 69(15): 2708–2715.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15:473–497.

Palanisamy K, Hegde M and Yi JS (2009). Teak (*Tectona grandis* Linn. f.): A renowned commercial timber species. Journal of forest and environmental science, 25(1):1-24.

Pradeep KG, Pahup S (2004). A naphthoquinone derivative from *Tectona grandis* (Linn). J Asian Nat Prod Res.; 6: 237–240.

Ragasa, C. Y., Lapina, M. C., Lee, J. J., Mandia, E. H. and Rideout, J. A.

(2008a). Secondary metabolites from *Tectona philippinensis*, Natural Product Research, 22(9): 820–824.

Ragasa CY, Tepora MM, Espinelli DH, Mandia EH, Rideout JA (2008b). Chromomoric acid derivatives from *Tectona philippinensis*. J Nat Prod.; 71: 701–705.

Rajuri A, Rao KNV, Banji D, Chaithanya RK(2010). A review on *Tectona grandis* L.: chemistry and medicinal uses (family: Verbenaceae). Herbal Tech.In..; 1: 6–9.

Sifaoui, I., Lopez-Arencibia, A., Martin-Navarro, C. M., Reyes-Batlle, M., Wagner, C., Chiboub, O., Mejri, M., Valladares, B., Abderrabba, M., Piñero, J. E., & Lorenzo-Morales, J. (2017). Programmed cell death in *Acanthamoeba castellanii* Neff induced by several molecules present in olive leaf extracts. PloSOne, 12(8), e0183795.

Skirvin R.M. (1986). Propagation of temperate fruits and nuts. In: Conger BV (ed) Cloning of agricultural plants via in vitro techniques. CRC Press, Boca Raton, pp

Sousa, Vicelina B., Cardoso, Sofia, Quilhó, Teresa, and Pereira, Helena. (2012). Growth rate and ring width variability of teak, *Tectona grandis* (Verbenaceae) in an unmanaged forest in East Timor. Revista de Biologia Tropical, 60(1), 483-494.

Sumthong P, Damveld MC, Lee JJ, Mandia EH, Rideout JA (2008). Secondary metabolites from *Tectona philippinensis*. Nat Prod Res. a; 9:820–824.

Sumthong P, Romero-Gonzalez RR, Verpoorte R (2008). Identification of anti-wood rot compounds in Teak (*Tectona grandis* L. f) Sawdust extract. J Wood Chem Technol.; 28: 247–260.

Sumthong, P., Damveld, R. A., Choi, Y. H., Arentshorst, M., Ram, A. F., van den Hondel, C. A., and Verpoorte, R. (2006). Activity of quinones from teak (*Tectona grandis*) on fungal cell wall stress. Plantamedica, 72(10), 943–944..

Tiwari, S. K., K. P. Tiwari, and E. A. Siril (2002). An improved micropropagation protocol for teak. Plant Cell, Tissue and Organ Culture 71(1): 1-6.

Vyas P, Yadav DK, Khandelwal P (2019). *Tectona grandis* (teak) - A review on its phytochemical and therapeutic potential. Nat Prod Res.; 33(16):2338-2354.

Warrier, P.S., (1994).Indian Medicinal Plants, 1st ed. Orient Longman Pvt. Ltd., New Delhi, India, pp. 245-248.