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Standardization Of Explant Sterilization For *In Vitro* Propagation Of *Pterocarpus Santalinus* L.F.- An Endemic, Endangered Multipurpose Medicinal Tree Taxon.

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ABSTRACT

Plant tissue culture is the finest method to propagate in large scale and to protect the rare, endemic, endangered and an important tree species. It is in urgent to concentrate on conservation of threatened endemic medicinal and commercially important plant species. The oozing of secondary metabolites in media from explants nullify the growth and development of callus/multiple shoots which is a common phenomenon in woody plants. The standard procedure for sterilization of explants is not developed so far. Hence the present study was taken to reduce the secondary metabolites from explants before the inoculation. Therefore, in the current study we have selected axillary buds; nodal segments and shoot tips as explants for the direct organogenesis in woody plant, B5 and MS media. We have analysed the effect of 0.1% mercuric chloride ($HgCl_2$), 1% of ascorbic acid and 1% of PVP on surface sterilization of the explants at different time intervals (1-5mins) and various concentrations of activated charcoal was also used in the media to reduce the effect of phenols for the micropropagation of *Pterocarpus santalinus*. The results indicate that, longer the duration of treatment, lower was the bacterial contamination (80% contamination at 5 min duration of $HgCl_2$ treatment) but the survival percentage of the culture decreased as exposure time increased (35% at 5 min). 3 min of exposure gave the best result for both bacterial contamination (18%) and survival percentage (68%) among other time durations and concentrations. The present study has shown a high frequency of sprouting from axillary bud explants (80%) in MS medium.

KEYWORDS

Red sanders, Explants, Shoot tip, Sterilization, Standardization, Micropropagation.

INTRODUCTION

Plant tissue culture is an important tool to propagate the plants in large scale through the eminent way in the short period. Culture of various parts and plant in the aseptic condition with the concept of totipotency [1]. Cell totipotentiality and cellular plasticity is the major physiological principle behind the plant tissue culture. Cell plasticity responses for the division and differentiation capacity of the culture cells [2]. The ability of the single cell to transform into a whole plant alike as the mother plant [3]. The propagation method should be under the controlled environment, hence there is absence of seasonal effect. Though the plants are cultivating in the largescale, plants will be genetically uniform, true to type [4]. *In-vitro* organ culture offers an alternative source for the conservation of endangered genotypes [5], which can be achieved using slow growth (short- and medium-term conservation) procedure or cryopreservation (long-term conservation) [6]. Cytokinins generally promote cell division and induce shoot formation and axillary shoot proliferation. High cytokinin to auxin ratio promotes shoot proliferation while high auxin to cytokinins ratio results in root formation [7]. Shoot-tip culture is used for the multiplication of plants that are already freed from known diseases. It should be emphasized that it does not free the plant from viruses. In fact, it favours propagation of viruses and increases virus concentration in the daughter plants. (8). Multiplication from shoot-tip and axillary bud culture is the better choice, because meristem culture may remove the virus, and the ornamental variegation is lost (9). Surface sterilization is the main aspect of tissue culture to prevent it from contamination, to obtain sterile plant material is difficult because in the process of sterilization living materials should not lose their biological activity (10). Mercuric chloride (HgCl2) has been effective in decontaminating pre conditioned mature *Uapaca kirkiana* stock plants where sodium hypo chlorite (NaClO) and calcium hypo chloride ($Ca(OCl_2)2$) have not been effective in decontaminating the stock plant (11). Therefore, here in this experiment we are using $HgCl_2$ as sterilizing agent and optimizing its perfect time of explant exposure to it. The successful culture of plant tissue needs to provide aseptic condition, selection of plant tissue free from microbes, proper sterilization, appropriate nutrition in tissue culture media, taking right measures in the browning and soma clonal variation.

The selected medicinal plant *P.santalinus* L.f. (Fabaceae) **(Fig 1)** is commonly known as Red sanders and an endemic medicinal tree in India and considered globally endangered [12]. Red sander has fallen back into the endangered category in the IUCN Red list -2022.It was classified as near threatened in 2018. The Union Ministry of Environment, Forest and climate change (MoEFCC) 2022 jan 8^{th} assessed as endangered. The slow growth of species and continued harvesting of trees has no time for the species to recover naturally. *P.santalinus* is a large deciduous tree; bark exudes blood red juice on incision, Leaves 3 foliate, leaflet very rarely 2 pairs, coriaceous, entire, obtuse. Flower yellow in auxiliary racemes panicles. Standard ovate petal, stamens 10, pods obliquely orbicular, becoming narrowed into short stalk, narrowly winged [13]. The colour and fragrance of *P. santalinus* heartwood are derived from santalins while the pleasant aroma is caused by the presence of terpenoids [14]. A dye prepared from the heartwood *of P. santalinus* is used as a stain in light microscopy [15], as a colouring agent in pharmaceutical preparations, in food, leather and textile industries [16], and as a textile dye [17]. The texture and colour differentiate

good quality of trees, with ‘‘wavy grain wood texture with intense red color’’ in the former and ‘‘straight grain wood texture with light red colour’’ in the latter and it is the superior quality of *P. santalinus* that makes it popular in the furniture industry [18]. In Japan, *P. santalinus* is used to make carvings and musical instruments, shamisen and koto [19], as well as name seals or hankos. In Buddhism, *P. santalinus* is considered to be a symbol of holiness, and is thus used for carved statues, as a constituent of incense [20], and for cremation [21]. In China, *P. santalinus* wood has a long history of use in furniture and other valuable wood products [22].

In the traditional system of medicine, the decoction prepared from the heartwood is attributed various medicinal properties. It has been used in inducing vomiting and treating eye diseases, mental aberrations and ulcers. The heartwood of red sanders is known to have antipyretic, anti-inflammatory, anthelmintic, tonic, hemorrhage, dysentery, aphrodisiac, diaphoretic activities, and cooling agent. Ethanol extract of stem bark was reported to possess anti-hyperglycemic activity. The wood in combination with other drugs is also prescribed for snake bites and scorpion stings [12]. *P. santalinus* is a highly impressive indigenous medicinal tree species [23], thus researchers during the past two decades have shown a renewed interest [24-25]. The heartwood is rubbed with water, honey, ghee, and oil, applied as collyrium to alleviate defects of vision. It is also used for treating skin diseases, bone fracture, leprosy, spider poisoning, hiccough, ulcers, general debility, and mental aberrations [26]. A natural dye, santalin, extracted from red sanders wood is used for colouring pharmaceutical preparations, food stuffs etc. Extracts of wood and fruit find extensive applications as astringents, diaphoretics, external applications for inflammations, headache, skin diseases, bilious infections, and chronic dysentery [27].

A) B) C)

Fig.1 Morphology of *Pterocarpus santalinus* A) Habit B) Shoot tip C) Axillary bud

MATERIALS AND METHODS

The experiment was performed at the Plant Tissue Culture Laboratory of the Botany Department, Sri Venkateshwara University, Tirupati. Healthy Shoot tips and axillary buds of Red sanders were collected growing from BIOTRIM Biotechnology centre of Tirupati, Andhra Pradesh, India. The excised shoot tips and axillary buds were washed thoroughly under running tap water for 30 min and then with 5% teepol for 8 - 10 min and rinsed 2-3 times in sterile distilled water. Then explant treated with 1% carbendazim to avoid microbial contamination after that washed 2-3 times to remove the traces of carbendazim and washed with distilled water.

Thereafter, the explants were pre-soaked with 1% ascorbic acid for 2-6 min followed by 1% PVP for 2-6 min and Surface sterilized with 0.1% $HgCl_2$ solution for 0- 5 min followed by thorough washing with sterile distilled water. The sterilized explants were inoculated on MS medium supplemented with various concentrations of Activated charcoal for shoot initiation. The pH of the media was adjusted between

5.6 and 5.8 before autoclaving at 15 lbs/cm2 at 121^{0}C for 20 min. Cultures were incubated at 25 ± 2 C and 65 - 70% relative humidity with photoperiod of 16/8 h at 3000 lux intensity by florescent tubes.

RESULTS AND DISCUSSION

To optimize the efficient surface sterilization protocol, various sterilant were used for various time durations on the different red sanders explant types such as shoot tip, axillary bud, segment using MS medium as a basal medium. The present findings of ***P.santalinus*** demonstrate the possibility for mass propagation of red sanders through axillary buds and shoot tip culture. For successful micro propagation axillary buds and shoot tip cultures are preferred as pre-existing meristem easily develop into shoots while maintaining clonal fidelity.

Table-1 Standardization of surface sterilisation of Axillary buds as explants of *Pterocarpus santalinus*. Each treatment consisted of 12 explants. Observations were made after 7 days of culture.

Sterilant/detergent used-Treatment duration (min)			Number of explants (%)		Number of explants responded. (%)	Mortality (%)
0.1% $HgCl_2$	1% Ascorbic acid	1% PVP	Infection free	With Infection		
0	0	0	03(25.00)	9(75.00)	3(25.00)	0(0.00)
1	0	2	07(58.33)	5(41.67)	6(50.00)	1(8.33)
2	2	4	08(66.67)	4(33.33)	6(50.00)	2(16.67)
3	4	6	12(100.0)	0(00.00)	11(91.67)	1(8.33)
4	6	8	10(83.33)	2(16.67)	6(50.00)	4(33.33)

Table-2 Standardization of surface sterilization of shoot tip as explants from *Pterocarpus santalinus*.

Sterilant/detergent used-Treatment duration (min)			Number of explants (%)		Number of explants responded. (%)	Mortality (%)
0.1% $HgCl_2$	1% Ascorbic acid	1% PVP	Infection free	With Infection		
0	0	0	01(8.333)	11(91.67)	0(0.00)	1(8.33)
1	0	2	03(25.00)	9(75.00)	2(16.66)	1(8.33)
2	2	4	05(41.67)	7(58.33)	3(25.00)	2(16.67)
3	4	6	07(58.33)	5(41.67)	6(50.00)	1(8.33)
4	6	8	08(66.67)	4(33.33)	4(33.33)	4(33.33)

Table-3 Standardization of activated charcoal on Axillary buds as explants from *Pterocarpus santalinus*.

Sterilant used	Number of explants (%)		Number of explants responded. (%)	Mortality (%)
Activated charcoal used in blank MS medium(100ml)	Infection free	With Infection		
0.1 gm	0	12(100)	0(0.00)	0
0.5 gm	01(8.33)	11(91.67)	0(0.00)	1(8.33)
1.0 gm	03(25.00)	9(75.00)	2(16.66)	1(8.33)
1.5 gm	08(66.67)	4(33.33)	4(33.33)	4(33.33)
2 .0gm	10(83.33)	2(16.67)	8(66.67)	2(16.66)

From the above results **Table 1** describes that the axillary buds were treated with 1% ascorbic acid for 2-6 min followed by presoaking in PVP at different time intervals of 2-8 min and then the sterilant $HgCl_2$ 0.1% was treated during the surface sterilization at different time intervals of 0-4 min. They have shown 100% infection free explants at the time interval of 4 min with 1% ascorbic acid followed by 1% of PVP at 6 min and 0.1% $HgCl_2$ for 3min of duration with 90% of response. Therefore, the axillary buds were selected for micropropagation. The explants at 4 min of 0.1% $HgCl_2$ is showing 83% of infection free plants but the response is only 50%. **Table 2** describes that the shoot tips as explants were treated with 1% of ascorbic acid for 2-6 min followed by presoaking in PVP at different time intervals of 2-8 min and then sterilant HgCl2 0.1% was treated during the surface sterilization at different time intervals of 0-4 min. To remove the phenolic exudates from explants, 0.5% of ascorbic acid (an antioxidant) was used during collection of explants and further treatment. Antioxidants and adsorbents on removal of phenolic exudates from plants of *Tectona grandis.* They treated the explants with 0.1% (w/v) solution of inorganic compounds and adsorbents viz. ascorbic acid, citric acid, glutamine, polyvinylpyrrolidone, boric acid and activated charcoal for 18 hours prior to their surface sterilization with 0.1% (w/v) mercuric chloride solution. Among these boric acid and ascorbic acid was proved to be the most effective resulting in 50-60% establishment of nodal segments on culture media [28].

Mercuric chloride (HgCl2) is stronger than sodium hypochlorite (NaClO), which is the likely reason for its effectiveness in combating fungi, bacteria and endogenous microbial species [11]. Shoot initiation and proliferation was found maximum, when the callus of black pepper was shifted to medium supplemented with BA at the concentration of 0.5 mg/l [29]. Plants grown under greenhouse conditions give rise to better results than the ones grown in field conditions [30]. There are huge variations regarding tissue culture response in explants excised from plants grown in field condition depending on weather conditions during the year [31]. However, the best results are obtained from explants excised from *in vitro* grown seedlings [32]. The mortality of the cultures may be higher due to damage caused by stronger disinfectants, as reported from *Calophylulum apetalum* [33]. When considering length of exposure to the disinfectant

agent, the differences between the results of bacterial contamination were significant, indicating that the longer the exposure to HgCl2, the lower the rate of bacterial contamination [34]. similar type of result was recorded in *Cochlospermum religiosum* [35], *Clinacanthus siamensis* [36].

From **Table 3** describes that the 100ml of full-strength MS media has been added with the activated charcoal at different concentrations of 0.1gm,0.5gm,1.0gm,1.5gm and 2.0gm. The sterilized axillary buds were inoculated in the media with these different concentrations. Initiation was occurred in better levels at 2 gm of charcoal in 100ml of full-strength MS media. It was observed that contamination was reduced, and infection free plants were obtained by 83% and the response was up to 66%.

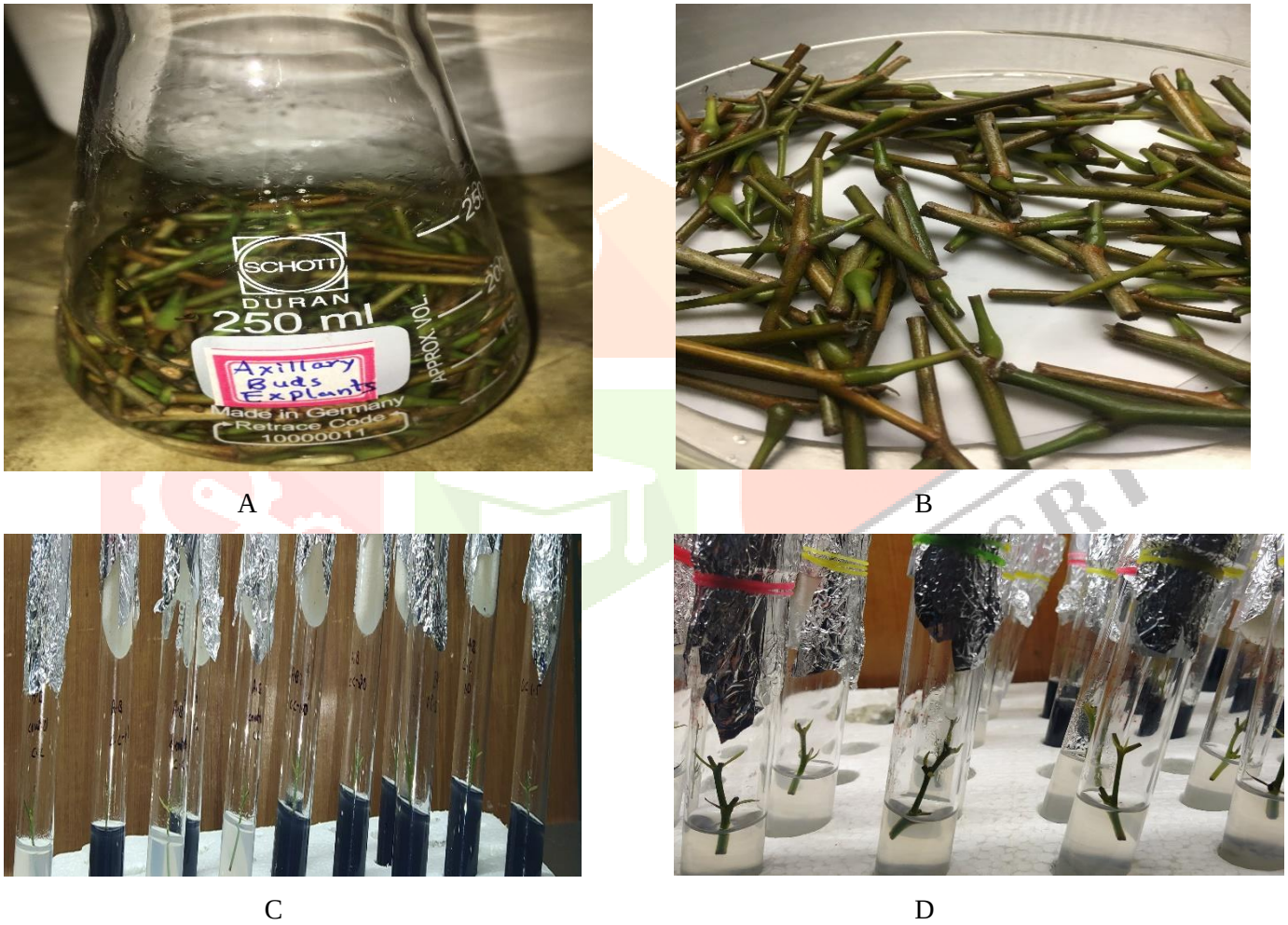

Fig.2 sterilized and inoculated explants during *In vitro* culture of *P.santlinus*

A) Axillary buds treating with 0.1% $HgCl_2$.
B) Completely sterilized explants
C) Inoculated axillary buds
D) Inoculated shoot tips

A B C

Fig.3 Contamination during invitro culture

A) & B) Fungal contamination C) Bacterial

A B C

Fig.4 Invitro Shoot initiation from axillary buds of *P.santalinus*

A) Shoot Initiation B) Shoot Multiplication C) Formation of fine leaves.

CONCLUSION

The present study was conducted to develop the effective disinfection protocol for the *in vitro* micropropagation of red sanders with the use of shoot tips and axillary bud segments as explants. The explants used in this study were surface sterilized using 0.1% $HgCl_2$, Ascorbic acid, fungicides and other sterilant at different time intervals. There is scanty of literature available in micropropagation of red sanders and therefore there is no standard method of sterilization of red sanders plant. The ultimate aim of the investigation was to optimize the effect of 0.1% $HgCl_2$ surface sterilization on in-vitro propagation of ***P.santlinus,*** a king to overcome the problem of fungal and bacterial contamination. In the present study we have succeeded in controlling bacterial contamination whereas the fungal infection was controlled by using carbendazim. The explants of shoot tips have not shown the effective result when compared to axillary bud segments.

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REFERENCES

[1] Tazeb, A., (2017): Plant tissue culture technique as a novel toolin plant breeding: A review article. Am.-*Eurasian J. Agric. Environ. Sci.,*: 111-118.

[2] Garcia-Gonzales, R., K. Quiroz, B. Carrasco and P. Caligari, (2010): Plant tissue culture: Current status, opportunities andchallenges. Ciencia Investigacion Agraria, 37: 5-30.

[3] Bhoite, H.A. and G.S. Palshikar, (2014): Plant tissue culture:A review. *World J. Pharm. Sci.,* 2: 565-572.

[4] Abass, M.H., (2013): Microbial contaminants of date palm (*Phoenix dactylifera* L.) in Iraqi tissue culture laboratories.*Emirates J. Food Agric.,* 25: 875-882.**[5] Sengar, R.S., Chaudhary, R., Tyagi, S.K. (2010):** Present status and scope of floriculture developed through different biological tools. *Res J. of Agri. Sci.,* 1(4): 306-314.

[6] Scherwinski-Pereira, J.E., Costa, F.H.S., Camillo, J., Silva, D.B., Alves, R.B.N., Vieira, R.F. (2010): Tissue culture storage of Brazilian medicinal plants germplasm. *Acta Horticulturae,* p. 211-214.

[7] Rout, G.R. (2004): Effect of cytokinins and auxin on micropropagation of *Clitoria ternatea L. Biol. Lett.* 41(1): 21-26.

[8] Lee, E.K, Cho, D.Y. and Soh, W.Y. (2001) : Enhanced production and germination of somatic embryos by temporary starvation in tissue cultures of *Daucus carota*. *Plant Cell Rep*. 20: 408-415.

[9] Cassells, A.C., Minas, G. and Long, R. 1980. Culture of *Pelargonium* hybrids from meristems and explants: Chimeral and beneficially-infected varieties. In: Tissue Culture Methods for Plant Pathologists Ingram, D.S. and Helgeson, J.P. (Eds.) *Blackwell Scientific Pub. Oxford.* Pp.125-130.

[10] Razdan MK(2012): Aseptic culture techniques. Introduction to Plant Tissue Culture. 2012:36-37.

[11] Mng'Omba SA, Sileshi G, Dutoit ES, Akinninfesi FK(2012): Efficacy and utilization of fungicides and other antibiotics for aseptic plant cultures. In:D. Dhanasekaran, N. Thajuddin and A. Pannerselvan. Fungicides for plant and animal diseases. Croatia: In Tech. 2012: 245-254.

[12] Kodithuwakku Kankanange Indika Upali Arunakumara, Buddhi Charana Walpola, Siripala Subasinghe and Min-Ho Yoon (2011): *Pterocarpus santalinus* Linn. f. (Rath handun): A Review of Its Botany, Uses, Phytochemistry and Pharmacology *J. Korean Soc. Appl. Biol. Chem.* 54(4), 495-500

[13] Madhava chetty K, Sivaji K and Tulasi Rao K (2015): Flowering Plants of Chittoor District Andhra Pradesh, India

14] Kumar N, Ravindranath B, Seshadri TR (1974): Terpenoids of *Pterocarpus santalinus* heartwood. *Phytochemistry* 13:633–636.

[15] Banerjee A, Mukherjee AK (1981): Chemical aspects of santalin as a histological stain. *Stain Technol* 56(2):83–85

[16] Ankalaiah C, Mastan T, Reddy MS (2017): A study on the density, population structure and regeneration of red sanders *Pterocarpus santalinus* (Fabales: Fabaceae) in a protected natural habitat— Sri Lankamalleswara Wildlife Sanctuary, Andhra Pradesh, India. *J Threat Taxa* 9(9):10669–10674

[17] Gulrajani ML, Bhaumik S, Oppermann W, Hardtmann G (2002): Kinetic and thermodynamic studies on red sandalwood. *Indian J Fibre Text Res* 27(1):91–94.

[18] Prakash E, Sha Valli Khan PS, Sreenivasa Rao TJV, Meru ES (2006): Micropropagation of red sanders (*Pterocarpus santalinus* L.) using mature nodal explants. *J For Res* 11:329–335

[19] Kukrety S, Jose S, Alavalapati JRR (2013): Exploring stakeholders' perceptions with analytic hierarchy process—a case study of red sanders (*Pterocarpus santalinus* L.) restoration in India. *Restor Ecol* 21(6):777–784

[20] Wu SF, Chang FR, Wang SY, Hwang TL, Lee CL, Chen SL, Wu CC, Wu YC (2011): Anti-inflammatory and cytotoxic neoflavonoids and benzofurans from *Pterocarpus santalinus. J Nat Prod* 74:989–996

[21] Ramakrishna A (1962): The red sanders and its future. Indian For 88:202–206

[22] Berliner N (1996): Beyond the screen: Chinese furniture of the 16th and 17th centuries. Mus Fine Arts, Boston.

[23] Shoba N, Rethinam SD, Vani G, Chennam S, and Shyamala D (2007): Effect of *Pterocarpus santalinus* extract on the gastric pathology elicited by a hypertensive dru in Wistar Rats. *Pharm Biol* 45, 468-474.

[24] Gupta PP, Srimal RC, and Tandon JS (1998): Antiallergic activity of some traditional Indian medicinal plants. *Indian J Pharmacol* 31, 15-18.

[25] Kwon HJ, Hong YK, Kim KH, Han CH, Cho SH, Choi JS, and Kim BW (2006): Methanolic extract of *Pterocarpus santalinus* induces apoptosis in Hela cells. *J Ethnopharmacol* 105, 229-234.

[26] Arokiyaraj S, Martin S, Perinbam K, Marie Arockianathan P, and Beatrice V (2008): Free radical scavenging activity and HPTLC fingerprint *Pterocarpus santalinus* L.-an in vitro study. Indian *J Sci Technol* 1, 1-3.

[27] Anonymous (1969): *Pterocarpus* in the wealth of india raw material vol.7 CSIR Publications, New Delhi.

[28] Shirin F, Sarkar AK(2003): Removal of phenolic exudates from explants of *Tectona grandis.* Teak net. 2003; 30:4-6.

[29] Hussain, A., Naz, S., Nazir, H., Shinwari, Z.K. (2011): Tissue culture of black pepper (*Piper nigrum* L.) *Pakistan. Pak. J. Bot.* 43(2): 1069-1078.

[30] Gürel E, Türker AU (2001): Organogenesis. In: Babaoğlu M, Gürel E, Özcan S (eds.) Plant Biotechnology, Tissue Culture and Applications. Selcuk University Publications, Konya, Turkey; 2001. P36-70.

[31] Pierik RLM (1987): *In vitro* Culture of Higher Plants. Martinus Nijhoff Publishers, Dordrect;.

[32] Yıldız M, Ozcan S, Er C (2002): The effect of different explant sources on adventitious shoot regeneration in flax (*Linum usitatissimum* L.). *Turkish Journal of Biology* 26: 37-40.

[33] Nair LG, Seeni S. (2003): In vitro multiplication of *Calophyllum apetalum* (Clusiaceae), an endemic medicinal tree of the Western Ghats. Plant Cell, Tissue and Organ Culture. 78(2):169-174.

[34] Silveria SS, Silva RC, Goldbach JD, Quoirin M. (2016): Micropropagation of *Calophyllum brasiliense* (Cambess.) from nodal segments. *Braz.J.Biol.*; 76(3):656- 663.

[35] Sasikala A and Savithramma N (2012): In vitro plant regeneration of *Cochlospermum religiosum* through shoot tip culture *Research journal of biotechnology* Vol 7(4) 1-3.

[36] Beena Prabha and Savithramma N (2015): In vitro Propagation of *Clinacanthus siamensis* A medicinal taxon of western ghats, India *International journal of pharma and biosciences* 6(1) 160-167.