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Effect of Nanoparticles in Development of Novel Protocol for Micropropagation of *Pterocarps santalinus* L.

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Abstract: *Pterocarpus santalinus* L. a medicinal and highly commercial timber yielding tree taxon of Fabaceae and is endemic to deccan region and included in critically endangered plant list. Number of attempts had been carried out by researchers to develop protocol for *in vitro* propagation of this plant for production of plants, as the seeds have dormancy. In the current attempt, nanoparticle mediated/included medium was developed for multiple shoot production from explants and also identification of suitable sterilization techniques to nullify the effects of secondary metabolites extruded from explants in to the medium used for micro propagation of *Pterocarpus santalinus*. Axillary buds were selected after screening several explants like nodal segments, shoot tips and leaves based on the response of shoot initiation. MS medium better than Woody plant and B5 media. Surface sterilization of explant with various concentrations and time initials of ascorbic acid PVP and $HgCl_2$ 35% of $HgCl_2$ at 3 min was best among the tested chemicals to remove microbial contamination. Maximum number of secondary metabolites were extracted with methanol than aqueous, acetone, chloroform and diethyl ether from axillary buds to synthesize the nanoparticles.

Keywords: *Pterocarpus santalinus,* Mcropropagation, Nanoparticles

Silver nanoparticles (AgNPs) are one of the most widely fascinating area and applicable particles whose application is enhanced in the nano world circadian. There is growing awareness in correlating the phytochemical constituents of a medicinal plant with its pharmacological activity. A red sander has fallen back into the endangered category in the IUCN Red list-2022. The seeds of *Pterocarpus santalinus* possess dormancy due to its hard seed coat. Moreover the growth is also slow. Hence in vitro cultivation is one of the methods to propagate. Number of authors tried with embryonic axis, cotyledons and leaves (Rajeswari and Paliwal 2008, Padmalatha and Prasad 2008). The explants oozed out phenols into the medium is another obstacle for micropropagation of *P. santalinus*. Hence sterilization and removal of secondary metabolites from explants is the prime step for *in vitro* propagation. In the present study an attempt has been made to *in vitro* propagation of *P. santalinus* with AgNP after removing the secondary metabolites from explant.

MATERIAL AND METHODS

Selection of candidate plus trees: Candidate plus tree was selected in the natural population of Seshachalam hills based on girth of the trunk and collected explants for experimentation.

Collection of explants: Healthy shoot tips and axillary buds of red sanders were collected from the tree. 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 was 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 explant was 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. /cm^2 at 121°C for 20 min. Cultures were incubated at 25 ± 2 C and 65 - 70% relative humidity with a photoperiod of 16/8 h at 3000 lux intensity by fluorescent tubes.

Phytochemical analysis: Explants were collected and carried out qualitative analysis of secondary metabolites.

Preliminary Phytochemical Screening: The above obtained Soxhlet extracts were used for preliminary phytochemical testing nearly 13 components namely flavonoids, steroids, tannins, glycosides, saponins, alkaloids, phenols, anthraquinones, anthocyanins, coumarins, lignins, proteins and triterpenoids was done by the standard procedures (Harborne 1984 , Kokate et al 1991).

Synthesis and characterization of nanoparticles: Five ml of aqueous bark extract were taken into 250 ml conical flask and titrated with 50 ml of silver nitrate with heating between 60-80oC for 60 min. Color change from light brown to deep brown indicated formations of silver nanoparticles. Then were centrifuged at 20000 rpm for 20 min to remove the presence of biological admixture, and were used for characterization and as well as antibacterial, antioxidant activities.

Characterization: UV-Vis absorption spectrum of SNPs was used with nanodrop 800 nm spectrophotometer. Fourier Transform Infra-Red (FT-IR) spectra of synthesized SNPs were analyzed in the range of 4000 to 500 cm1 with an ALPHA interferometer (ECO-ART), Bruker, Ettlingen, Karlsruhe, Germany by KBr pellet method. Crystalline nature of metallic silver nanoparticles were monitored using an X-ray diffract meter (XRD) from Shimadzu, XRD-6000 equipped with Cu Ka radiation source using Ni as filter at a setting of 30 kV/30 mA. Scanning electron microscopy (SEM) and percentage of silver ions in synthesized samples was done by using FEI Quanta 200 FEG HR-SEM machine equipped with EDAX instrument. Transmission electron microscopy (TEM) analysis was performed with the using HF-3300 advanced 300 kV TEM from Hitachi.

RESULTS AND DISCUSSION

The axillary buds from candidate plus tree were treated with 1% ascorbic acid for 2-6 min followed by in PVP at different time intervals of 2-8 min and then the sterilant HgCl2 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% HgCl2 for 3min of duration with 90% of response. Therefore, the axillary buds were selected for micro propagation. The explants at 4 min of 0.1% HgCl2 is showing 83% of infection free plants but the response is only 50%.

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 (MngOmba et al 2012). The effect of 0.1% $HgCl_2$ surface sterilization on *in-vitro* propagation of *P. santlinus*, to overcome the problem of fungal and bacterial contamination.

There are huge variations regarding tissue culture response in explants excised from plants grown in field conditions depending on weather conditions. The mortality of the cultures may be higher due to damage caused by stronger disinfectants, as reported from *Calophylulum apetalum* (Nair and Seeni 2003).

Aqueous extract showed the presence of flavonoids, glycosides, saponins, alkaloids, phenols, coumarins and lignins followed by Acetone and diethyl ether extracts showed the presence of steroids, glycosides, saponins, alkaloids and phenols. In chloroform and diethyl ether extracts only flavonoids, glycosides, saponins, phenols and steroids were present. Among all the solvents, methanol is the best suitable solvent for extracting *P. santalinus* bioactive compounds. Similar results were observed in *Boswellia ovalifoliolata*, *Shorea tumbuggaia, Cochlospermum religiosum, Syzygium alternifolium, Terminalia pallida*. The quantitative estimation of secondary metabolites of *P. santalinus* axillary buds were rich in alkaloids, flavonoids, phenols, saponins, tannins, proteins and carbohydrates. Highest number of alkaloids 0.617 mg/g d.wt are present in the shoot tip. Alkaloids are beneficial chemicals to plants with predators and parasite repelling and physical state. Number alkaloids are isolated from dicots and using efficient drugs. The alkaloids are one of the most diverse groups of secondary metabolites found in living organisms and have an array of structure types, biosynthetic pathways and pharmacological activities. The presence of alkaloids contained in plants are used in medicine as aesthetic agents. Axillary buds are rich in phenols 1.893 mg/g dw and flavonoid 1.713 mg/g dw. When the 1 mM Ag(NO3)2 solution was added to aqueous axillary buds extract of *P. santalinus*, the color changed from light brown to deep brown which is the primary method to confirm that the synthesized nanoparticles are silver (Fig.1). The color change is because of the reduction of silver ions with the help of bio active molecules

Table 1. Standardization of surface sterilization of axillary buds as explants of *Pterocarpus santalinus*

Treatment duration (min)			Number of explants (%)		Number of explants responded (%)	Mortality (%)
$HgCl_2$0 (1%)	Ascorbic acid (1%)	PVP (1%)	Infection free	With Infection		
0	0	0	25.00	75.00	25.00	0.00
1	0	2	58.33	41.67	50.00	8.33
2	2	4	66.67	33.33	50.00	16.67
3	4	6	100.0	00.00	91.67	8.33
4	6	8	83.33	16.67	50.00	33.33

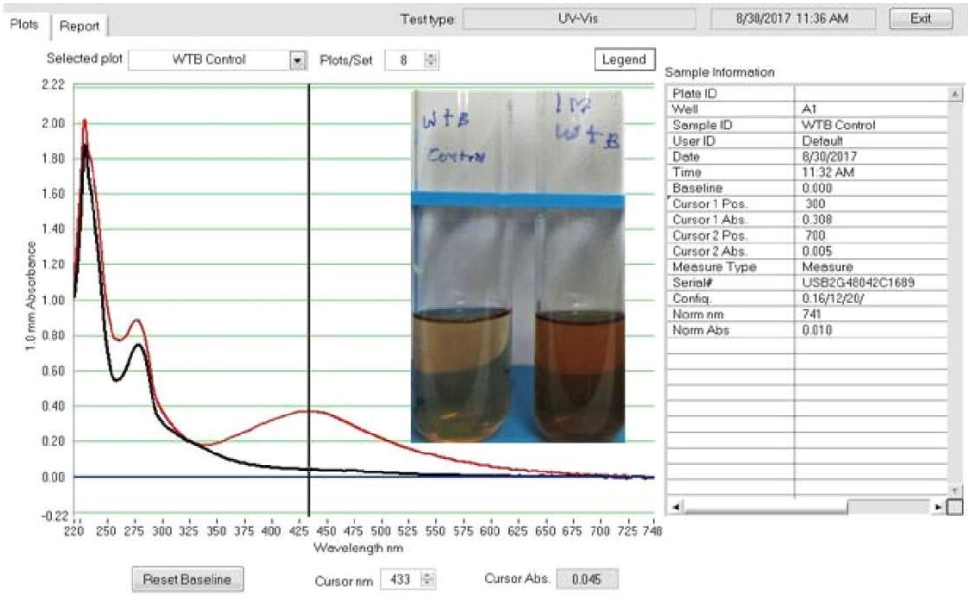
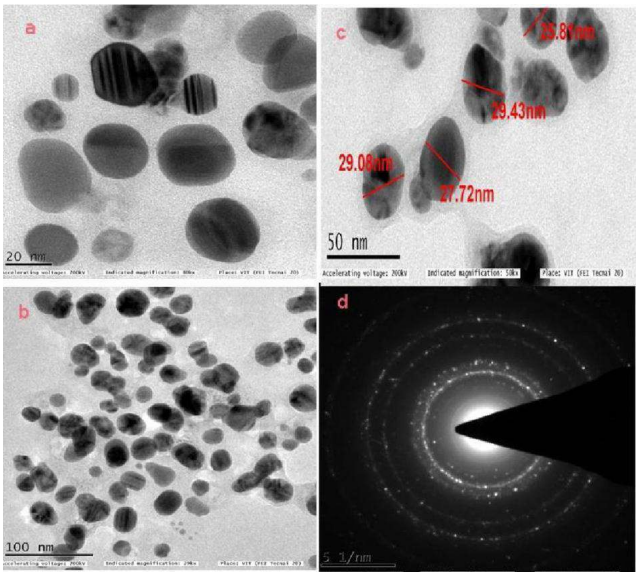


Fig. 1. Visible colour change of SNPs with UV Visible absorbance peak at 433 nm



a) Spherical shape of SNPs at 20 nm
b) Dispersion of the nanoparticles at 100 nm
c) Average size of AgNPs at 50 nm 28.01nm
d) Imaging at 51 nm

Fig. 2. TEM Images of synthesized nanoparticles

Control AgNP treated

Fig. 3. Growth of axillary buds on MS medium supplemented with silver nanoparticles

present in the sample. NAD and ascorbic acid present in plant parts at higher levels act as strong reducing agents by donating electrons to Ag^{+} to form Ag^{0} nanoparticles. This may be the main reason behind the reduction and color change pattern of silver nanoparticles.

TEM: Morphological structure and distribution of synthesized silver nanoparticles monitored at high magnifications (20nm) were done by TEM. TEM micrographs show that the synthesized nanoparticles are poly dispersed, predominantly spherical in shape, owing 25.81-29.43 nm size and no physical contact with each other i.e. no agglomeration of nanoparticles were seen. For TEM analysis the SNPs are coated on copper grids and analyzed by Hitachi HF3300 advanced with 300kV (Fig. 2). AgNPs due to its nano size enter into the tissue and allow the nutrients to utilize optimum level by growing tissue; hence the shoot growth is enhanced in AgNP treated medium when compared to that of control (Fig. 3). The initial finding of the present study paves the way to further research to find out fast growth of shoots and roots in micropropagation of woody plants.

Table 2. 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	100	0.00	0
0.5 gm	8.33	91.67	0.00	8.33
1.0 gm	25.00	75.00	16.66	8.33
1.5 gm	66.67	33.33	33.33	33.33
2.0 gm	83.33	16.67	66.67	16.66

Table 3. Qualitative analysis of bio metabolites from axillary buds of *P. santalinus*

Tests	Aqueous	Acetone	Chloroform	Diethyl ether	Methanol
Flavonoids	+	-	+	+	+
Steroids	-	+	-	+	+
Tannins	-	-	-	-	+
Saponins	+	+	-	+	+
Alkaloids	+	+	-	-	+
Phenols	+	+	-	+	+
Triterpenoids	-	-	-	-	+
Anthraquinones	-	-	-	+	+
Anthocyanins	-	-	-	-	+
Coumarins	+	-	-	-	-
Lignins	+	-	-	-	+
Proteins	-	-	-	-	-
Glycosides	+	+	+	-	+
Total	07	05	02	05	11

+ indicate presence & - indicates absence

Table 4. Quantitative analysis of bio metabolites from different parts of *P. santalinus*

Phytochemicals	Quantity mg/g D.W.
Alkaloids	0.114±0.088
Flavonoids	0.333±0.088
Phenols	0.371±0.057
saponins	0.362±0.088
Tannins	0.052±0.057
proteins	0.212±0.021
Carbohydrates	0.075±0.057

Values are average ±, SE

CONCLUSION

The present study concluded that axillary bud is the best explant for micropropagation of *Pterocarpus santalinus* and for effective disinfection optimum concentration of 0.1% $HgCl_2$ for surface sterilization of explant. The presence of high phenols, flavonoids, tannins and alkaloids exudation from explant into culture medium affects the growth of shoot induction. Silver nanoparticles in the medium reverse the effect of secondary metabolites in the MS culture medium for *in vitro* propagation of *Pterocarpus santalinus.*

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