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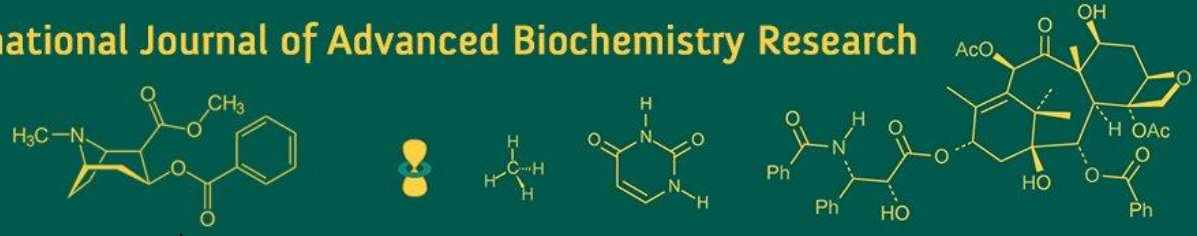

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Development of *in vitro* protocol for *Khaya senegalensis*

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Abstract
Studies were carried out during 2023, for the development of *in vitro* protocol for *Khaya senegalensis*. Among the media of MS and MS media with growth regulators, the medium of MS with kinetin was found to be optimal and ideal compared to other media with 22.05% Morphogenic response (%). Among the explants collected, explants from Nodal segments showed greater response compared to the explants from shoot tips and axillary buds. But the explants rom shoot tips showed good response at earlier stages while the response became slower later. Compared to MS basal medium, treatment with MS +5.0 mg/l Kinetin was found to be best treatment. The best treatments for obtaining shoot induction was MS medium supplemented with MS + 7.0 mg/l BAP recorded highest per cent shoot induction (27.50%), number of shoots per explants (3.00) and average shoot length (1.92 cm). Among the various concentration of kinetin investigated MS + 5.0 mg /l Kinetin showed highest per cent shoot induction with (32.50%) number of shoots per ex-plant (4.00) and highest average shoot length (2.90cm). The shoots subcultured in MS medium supplemented with auxins such as IBA and IAA did not induce rhizogenesis.

Keywords: *Khaya senegalensis, in vitro*, auxin, cytokinin, sterilization technique

Introduction
Khaya senegalensis is a species of tree in the Meliaceae family that is native to Africa. Common names include African mahogany, dry zone mahogany, Gambia mahogany, khaya wood, Senegal mahogany, callcedrat, acajou, djalla, and bois rouge Conservation status of this species is vulnerable.

Native distribution of African mahogany is tropical west Africa to Uganda. Its natural habitat is riverine forests and savannah woodlands in tropical Africa. It can also be found at higher elevations in moister areas.

It is used for furniture and cabinetry, flooring, construction and boat building. The bark contains components of tannin and is sometimes used for tanning leather. It is widely used on a commercial scale, particularly in West Africa. The wood density ranges from 0.6 to 0.85, depending on locality. The sapwood is pinkish-tan in colour and the heartwood an attractive dark red-brown. It is moderately resistant to fungi, insects and termites.

Natural regeneration from seed is poor, but fresh seed germinates readily when sown in a sand and peat mix in flat trays Germination commences after about 10 to 14 days and high germination rates, usually around 90%, can be expected when fresh seed is used. Seedlings can be picked out into deep containers and grown in the nursery until they reach 25 to 40 cm in height.

Since natural regeneration from seed is poor conventional methods of asexual propagation like grafting, budding, layering etc. for many of the plants and trees are often too slow or fail completely. Tissue culture allows much greater control and manipulation of the development of tissues within the culture tube than conventional methods. Tissue culture techniques help to mass multiply the trees species and the techniques have already revolutionized mass propagation of many hardwoods and softwoods (Bajaj. 1986; Tewari, 1994) [1]. Tissue culture is the method of choice for production of huge number of genetically identical plants or cloning of superior genotypes in shorter time span (Chelak and Rogers, 1990) [6].

In-vitro tissue culture techniques offer a promising avenue for mass propagation, genetic improvement, and conservation of economically important plants. By providing a controlled environment and precise nutrient formulations, in-vitro protocols allow for the rapid multiplication of plantlets from small explants, such as shoot tips or axillary buds. Moreover, tissue culture techniques can be employed for the production of disease-free and genetically uniform plant material, which is essential for establishing plantations and germplasm banks.
The development of an efficient *in-vitro* protocol for Khaya senegalensis presents several challenges and research opportunities. The species is recalcitrant to conventional propagation methods, such as seed germination and cutting techniques. Additionally, *Khaya senegalensis* exhibits slow growth rates and exhibits high susceptibility to microbial contamination and tissue browning during in-vitro culture. Overcoming these obstacles is crucial for the successful establishment of clonal plantations and the conservation of this valuable tree species.

Materials and Methods

Materials

The experimental materials for this study consisted of shoot tip and node l segments from Forest College and Research Institute, Tamil Nadu Agricultural University, Mettupalayam The explants *viz*., nodal segments and shoot tips were collected from the clonal multiplication area of *Khaya senegalensis*. The *in vitro* propagation study was carried at the tissue culture laboratory of Forest College and Research Institute, Mettupalayam.

In vitro propagation techniques

Explant selection

The first step is to select suitable plant parts (explant) for initiating the tissue culture. Expants can be taken from shoot tips, axillary buds, hypocotyls, cotyledons or leaves. But the explants that is taken for this project is from shoot tips and axillary buds and nodal segments.

Surface sterilization

To prevent contamination, the explants need to be surface sterilized. This involves rinsing the explants in a disinfectant solution (e.g., sodium hypochlorite or Mercuric chloride or ethanol) followed by several rinses with sterile distilled water. The sterilization procedure helps eliminate any microorganisms present on the surface of the explants. Surface sterilization is a critical step in the *in-vitro* protocol of *Khaya senegalensis*, or any other plant tissue culture protocol. It involves removing and killing surface contaminants such as bacteria, fungi, and other microorganisms that may be present on the plant material.

Prepare a sterilization solution

Typically, a combination of disinfectants is used, such as a 5-10% sodium hypochlorite solution or alcohol-based solutions like ethanol or isopropyl alcohol or 0.1% Mercuric chloride solution. The exact concentration and composition of the sterilization solution may vary depending on the specific protocol or laboratory preferences. In case of *Khaya senegalensis* 5% sodium hypochlorite solution and ethanol gave good results, while he Mercuric chloride does not.

Wash the plant material

Rinse the plant material thoroughly under running tap water to remove any visible dirt, debris, or dust particles.

Immerse in a detergent solution

Place the plant material in a container with a mild detergent solution and gently agitate for a few minutes. This step helps in removing any waxy coating or contaminants present on the surface. Usually tween 20 is used.

Rinse with sterile distilled water

Rinse the plant material several times with sterile distilled water to remove the detergent residue.

Surface sterilization

Transfer the plant material to a sterile container and submerge it in the sterilization solution prepared in step 1. The duration of sterilization can vary, but commonly it ranges from 1 to 30 minutes, depending on the plant species and tissue type. For this species it is done for 5 minutes. Rinse with sterile distilled water: After the sterilization period, rinse the plant material thoroughly with sterile distilled water to remove any traces of the sterilization solution.

Macronutrients (10X concentration)

Each salt was weighed exactly and dissolved separately to the last particle in a small amount of distilled water. Finally all the salt solutions were pooled together and the volume made up with distilled water. Each chemical was weighed exactly, dissolved separately, mixed together and finally the volume was made up with distilled water. Macro nutrients is prepared for 500 ml and the volume taken for 1 l of media is 50 ml.

Micronutrients (100X concentration)

Each micronutrients are weighed separately and dissolved in distilled water of small amount. Later the volume is made up with distilled water. Micronutrients are made up to 250 ml and the volume taken for 1l of media is 2.5 ml.

Minor nutrients (100X concentration)

Minor nutrients are prepared for 100 ml and the volume taken or 1 l of media is 1 ml.

Iron stock (50 X concentration)

EDTA and ferrous sulphate are weighed and disolved seperately in 100 ml distilled water and heated. Then EDTA is poured in ferrous sulphate to avoid precipitation. Iron stock is made up to 250 ml and the volume taken for 1l of solution is 5 ml.

KI (100 X concentration)

KI is made up to 250 ml and the volume taken for 1 l media is 2.5 ml

MS vitamins (100 X concentration)

MS vitamins are made for 100 ml and the volume taken for 1 l media is 1 ml.

Agar and sucrose

8 g of agar is used for 1litre of media and 30 g of sucrose is used for 1 l of media.

Table 1: Preparation of stock

Ingredients	Composition mg/l	Stock solution(W/V) (g)	Volume of stock prepared	Volume of stock taken per litre of medium
Macro nutrients (10X)				
NH_4NO_3	1690	16.90	500 ml	50 ml
KNO_3	1900	19.00		
$CaCl_2.2H_2O$	440	4.40		
$MgSO_4.7H_2O$	370	3.70		
KH_2PO_4	170	1.70		
Minor nutrients (100X)				
$MnSO_4.4H_2O$	22.3	2.23	250 ml	2.5 ml
$ZnSO_4.4H_2O$	8.6	0.86		
H_3BO_3	6.2	0.62		
Micro nutrients (100X)				
$Na_2MoO_4.2H_2O$	0.25	0.025	100 ml	1 ml
$CuSO_4.5H_2O$	0.025	0.0025		
$CoCl_2.6H_2O$	0.025	0.0025		
Iron stock (50X)				
Na_2EDTA	37.25	1.863	250 ml	5 ml
$FeSO_4.7H_2O$	27.85	1.393		
KI (100X):				
KI	0.83	0.083	250 ml	2.5 ml
MS Vitamins (100X)				
Nicotinic acid	0.5	0.05	100 ml	1 ml
Pyridoxine. HCl	0.5	0.05		
Thiamine. HCl	0.1	0.01		
Glycine	2.0	0.2		

Growth regulators

In tissue culture of *Khaya senegalensis*, various growth regulators can be used to manipulate the growth and development of the plant material. The choice of growth regulators and their concentrations depends on the specific objectives of the tissue culture protocol. Here are some commonly used growth regulators for tissue culture of *Khaya senegalensis*.

Cytokinins

Cytokinins are plant hormones that promote cell division and shoot proliferation. They are often used in tissue culture to induce multiple shoots from explants. Common cytokinins used in tissue culture include:

1. Benzyladenine (BA)
2. Kinetin
3. Zeatin

Auxins

Auxins are plant hormones that play a crucial role in root formation and initiation. They are used to induce rooting in tissue culture. Commonly used auxins include:

1. Indole-3-butyric acid [IBA]
2. Indole-3-acetic acid [IAA]
3. Naphthalene acetic acid [NAA]

Media preparation

Appropriate quantities of various stock solutions including growth regulators were pipetted out and added to required volume of distilled water. Required quantity of sucrose (2%) was weighed and dissolved in the above solution. The final volume was made up with distilled water and pH was checked. The appropriate pH for the media is 5.6 to 5.8. This can be adjusted by adding two to three drops HCl or NaOH. Then agar (0.8%) was added after boiling the solution. About 15-20 ml of medium was dispensed into each of the 25 x 150 mm culture tubes. The culture tubes were plugged with non absorbent cotton and autoclaved at 121 °C for 20 min. Subsequently the medium was allowed to cool at room temperature and stored inside culture room.

Inoculation

Transfer the surface-sterilized explants to the prepared culture medium using sterile techniques. You can use a laminar flow hood or a clean workspace to maintain a sterile environment. Place the explants onto the medium in culture vessels like Petri dishes or test tubes.

Incubation

Seal the culture vessels with sterile closures (e.g., parafilm or cotton plugs) and place them in a controlled environment, such as a growth chamber or a culture room. Maintain the appropriate temperature, light conditions (e.g., 16-hour photoperiod), and humidity levels for the growth of *Khaya senegalensis*. This typically involves maintaining the culture at a temperature of around 25-28 °C and providing light at an intensity of around 50-100 µmol m^2s^1.

Subculture

After a few weeks, the explants will start to produce callus or develop shoots. At this stage, you can initiate subculturing by transferring the developed shoots or callus onto fresh media with the same composition as the initial medium. Regular subculturing will help maintain the cultures and promote further growth and development. For shoot multiplication add cytokinin and for root development add auxin.

Results and Discussion

Growth of *Khaya senegalensis*

Growth of *Khaya senegalensis* under *in-vitro* condition is observed to be very slow. The shoot growth was started after the 2 weeks of inoculation and the growth tends to be very slow but having positive response.

Screening basal medium and basal with kinetin and BAP

Culturing of plants is done in MS media. Plants are cultured in MS media, basal media + BAP and basal media + kinetin. Among these MS media + kinetin showed greater results compared to Basal media and basal media + BAP. The Highest response was found in Nodal explants in MS + kinetin media with 38.60% response and the contamination was observed less. The lowest was observed in MS media with 20.25% and the media of basal with BAP as 22.30%. Lowest response was found in shoot tip explant where in MS media the response is 17.5%, MS+ BAP the response is 12.50% and MS +Kinetin is 19.00%. The explants also differed significantly for their per cent morphogenic response. The highest morphogenic response was recorded by nodal segment explants (29.05%) which differed significantly followed by shoot tip explants (16.33%).

Effect of Kinetin on shoot induction

The treatments with Kinetin treated cultures recorded higher values for percent cultures with shoot induction and the maximum value was recorded by the treatment MS+5.0 mg/l Kin (32.5%). Considering average number of shoots per explant five treatments *viz*., MS + 5.0 mg/l Kin(4.00), MS+10.0 mg/l Kin (3.02), MS+ 9.0 mg/l Kin (2.6)), MS+4.0 mg/l kin (2.4), MS+8.0 mg/l (2.3) recorded significantly increased values compared to MS basal medium (1.75). Besides the average shoot length also significant difference among the treatments, the treatments barring MS +5.0 mg/l Kin (2.50cm) and MS + 7.0 mg/l Kin(2.65 cm) showed superior value compared to others. Among Kinetin added treatments, MS +10 mg/l Kin showed greater response to multiple shoot induction. Considering the percent cultures with shoot induction and average number of shoots per explant the treatment MS+ 5 mg/l Kin and MS + 10 mg /l Kin was found to be superior. Percent culture with shoot induction value ranges from 20.00 to 32.5

Effect of BAP on shoot induction

Compared to MS basal medium, the treatment with BAP expressed increased shoot Induction which ranged between 15.00 and 27.50%. However, treatments with MS +7.0 mg/l BAP (27.50%), recorded significantly higher values over that of MS basal medium (11.25%) followed by MS +9.0 mg l' BAP (23.75%) and MS + 5.0 mg l BAP (20.00%).However, considering the number of shoots per explant, three treatments MS+7.0 mg/l BAP (2.75), MS+ 6.0 mg l BAP (2.5)) and MS+ 10.0 mg BAP (2.2) significantly higher values over MS basal medium (1.75). With regarding average shoot length no significant difference obtained but the treatment barring MS +7.0 mg/l BAP (2.04 cm) recorded higher value compared than other treatments. All other treatments ranges from 0.43 to 1.42.

Subculturing of explants

Three subcultures were done in different media. First subculture was done for the explants grown in media containing MS+3 mg/l BAP. Multiplication of shoots were started in MS+ 3 mg/l BAP media but the growth tends to be slow and the media started to contaminate so the subculturing was done a little earlier to the media containing 5 mg/l BAP. But the growth of explants in subcultured media failed. Second subculture was done for the explants growing in the media containing MS+ 3 mg /l Kinetin. subculturing was done in media containing 3 mg/l kinetin. The growth was positive and the length of the explant was up to 0.96 cm. Third subculturing was done for media containing 5 mg/l kinetin. Explants are sub cultured to media containing 5 mg/l Kinetin. The growth in 5 mg/l kinetin was overserved to be faster compared o other media and the length of shoot was overserved to be 1.96 cm.

Rhizogenesis

Attempts were made to induce roots in the developed shoot cultures in the same media where it was cultured as well as in the medium containing IBA and IAA. Rooting was not induced in any of the medium investigated. When transferred to the rooting medium with IBA and IAA, leaf drop was noticed in almost all the cultures even after repeated subculturing. Rooting was tried with media containing 2 mg/l IAA, 2 mg/l IBA, 3 mg /l IBA, 3 mg/l IAA and with 4 mg/l IBA and 4 mg /l IAA but nothing Howes positive response and no root growth is observed.

Table 2: Screening of basal media and basal media with kinetin and BAP

S. No	Media	Morphogenic response of shoot tip explants(%)	Morphogenic response of nodal segments(%)	Mean
1.	MS media	17.5	20.25	18.87
2.	MS media + BAP	12.50	28.30	20.4
3.	MS media + Kinetin	19.00	38.60	28.8
	Grand mean	16.33	29.05	

Table 3: Effect of BAP on multiple shoot induction of *Khaya senegalensis*

S. No	Treatments	Percent culture with shoot induction(%)	Average number of shoots/explants	Length of shoots
1.	MS Basal	11.25	1.25	0.69
2.	MS + 1 mg/l BAP	15.00	2.00	0.93
3.	MS + 2 mg/l BAP	15.00	1.7	0.43
4.	MS + 3 mg/l BAP	18.75	1.63	1.04
5.	MS + 4 mg/l BAP	16.25	1.43	1.23
6.	MS + 5 mg/l BAP	20.00	2.03	1. 4
7.	MS + 6 mg/l BAP	17.50	2.5	0.76
8.	MS + 7 mg/l BAP	27.50	2.75	2.04
9.	MS + 8 mg/l BAP	17.50	1.5	1.42
10.	MS + 9 mg/l BAP	23.75	1.8	1.22
11.	MS + 10 mg /l BAP	18.75	2.2	1.32
	Grand mean	18.30	1.954	1.179

Table 4: Effect of Kinetin of multiple shoot induction of *Khaya senegalensis*

S. No	Treatments	Percent culture with shoot induction(%)	Average number of shoots / explants	Length of shoots (cm)
1.	MS Basal	11.25	1.70	0.69
2.	MS + 1 mg/l Kin	23.75	2.20	1.90
3.	MS + 2 mg/l Kin	20.00	1.40	1.75
4.	MS + 3 mg/l Kin	22.50	1.60	2.20
5.	MS + 4 mg/l Kin	25.00	2.40	2.15
6.	MS + 5 mg/l Kin	32.50	4.00	2. 90
7.	MS + 6 mg/l Kin	22.50	1.90	2.31
8.	MS + 7 mg/l Kin	23.75	2. 10	2.65
9.	MS + 8 mg/l Kin	20.00	2.30	1.49
10.	MS + 9 mg/l Kin	22.50	2.60	2.20
11.	MS + 10 mg/l Kin	30.75	3.02	2.14
	Grand mean	22.05	2.12	1.938

Best sterilization technique: Among the sterilization technique used the best and successful sterilant was 5% sodium hypochlorite. The response of cultures using sodium hypochlorite is far better compared to the Mercuric chloride. Sterilzation technique using sodium hypochlorite responded well when brisk wash of ethanol for 30 second is done prior to the treatment with sodium hypochlorite. The contamination of bottles and test tubes were low with sodium hypochlorite compared to Mercuric chloride.

Conclusion

Among the media tested, MS medium supplemented with kinetin was found to be optimal and ideal compared to other media such as basal media and basal + BAP with 28.8% Morphogenic response (%). Among the two explants investigated for shoot induction, nodal explants showed the maximum morphogenic response of 29.05% compared to shoot tip explants. For sterilizing the explants, 5% Sodium hypochlorite solution for five minutes was found to be the best treatment and yielded maximum survival percentage compared to 0.1% Mercuric chloride. Compared to MS basal medium, treatment with MS+7.0 mg/l BAP was found to be best treatment. The best treatments for obtained shoot induction was MS medium supplemented with MS+7.0 mg/l BAP recorded highest percent shoot induction (27.50%), number of shoots per explants (2.75) and average shoot length (2.04).Among the various concentration of kinetin investigated MS + 5 mg/l Kinetin recorded highest percent shoot induction with (32.50%) and shoots per ex-plant (4.00) and highest average shoot length (2.90cm).But multiple shoot induction responded well in MS + 10 mg/l Kin. The shoots sub-cultured in MS medium supplemented with auxins such as IBA and IAA did not induce rhizogenesis.

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