Micropropagation of a Forest Tree *Sterculia alata*

Final Report of a Minor Project of UGC

Submitted by

Dr Nishi Kumari, Assistant Professor

Department of Botany

Mahila Mahavidyalaya

Banaras Hindu University

Varanasi- 221005

UGC Reference No.: F. No. 32-422/2006 (SR) **Project code: P-04/149**

2016

Annexure - IV

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
NEW DELHI – 110 002

Utilization certificate

Certified that the grant of Rs.45,000/- (Rupees Forty Five Thousand only) received from the University Grants Commission under the scheme of support for Minor Research Project entitled "Micropropagation of a forest tree *Sterculia alata*" vide UGC letter No. F. 32-422/2006 (SR) dated Feb. 24, 2007 has been fully utilized (Rs. 44,559/-) for the purpose for which it was sanctioned and in accordance with the terms and conditions laid down by the University Grants Commission.

SIGNATURE OF PRINCIPAL INVESTIGATOR (Seal)	**THE REGISTRAR (Seal)**	**STATUTORY AUDITOR (Seal)**

Annexure –III

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
NEW DELHI – 110 002.

Final Report of the work done on the Minor Research Project.

1. Project Report No. : Final

2. UGC Reference No.F.32-422/2006 (SR) dated Feb. 24,2007

3. Period of report: from 1/4/2007 to 31/3/2009

4. Title of research project: Micropropagation of a forest tree *Sterculia alata*

5. (a) Name of the Principal Investigator: Dr Nishi Kumari

   (b)Deptt. : Department of Botany, MMV

   (c) University/College where work has progressed: Banaras Hindu University

6. Effective date of starting of the project: 1/4/2007

7. Grant approved and expenditure incurred during the period of the report:

   a. Total amount approved: Rs. 60,000/- (amount released- Rs.45,000/-)
   b. Total expenditure: Rs. 44,559/-
   c. Report of the work done: Enclosed

i. Brief objective of the project: To develop regeneration protocol of *Sterculia alata* either through organogenesis or embryogenesis.

ii. Work done so far and results achieved and publications, if any, resulting from the work (Give details of the papers and names of the journals in which it has been published or accepted for publication): Communicated manuscript enclosed

iii. Has the progress been according to original plan of work and towards achieving the objective. If not, state reasons: Yes

iv. Please indicate the difficulties, if any, experienced in implementing the project: Although somatic embryos germinated into plantlets, but the percent germination was low.

v. If project has not been completed, please indicate the approximate time by which it is likely to be completed. A summary of the work done for the period (Annual basis) may please be sent to the Commission on a separate sheet: Enclosed

vi. If the project has been completed, please enclose a summary of the findings of the study. One bound copy of the final report of work done may also be sent to University Grants Commission. Enclosed

vii. Any other information which would help in evaluation of work done on the project. At the time of completion of the project, the first report should indicate the output, such as

(a) Manpower trained: One Ph. D student

(b) Ph. D. awarded: Three [One Ph. D student worked on same topic and awarded degree in 2013]

(c) Publication of results: Part of thesis, one manuscript communicated (enclosed)

(d) Other impact, if any: Helped in creating basic facility to the laboratory

SIGNATURE OF
THE PRINCIPAL INVESTIGATOR **REGISTRAR** **PRINCIPAL**

Annexure- V

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
NEW DELHI – 110 002

STATEMENT OF EXPENDITURE IN RESPECT OF MINOR RESEARCH PROJECT

1. Name of Principal Investigator: Dr Nishi Kumari

2. Deptt. of Principal Investigator: Department of Botany, Mahila Mahavidyalaya, Banaras Hindu University

3. UGC approval Letter No. and Date: F. No. 32-422/2006 (SR) dated Feb.24, 2007

4. Title of the Research Project: Micropropagation of a forest tree *Sterculia alata*

5. Effective date of starting the project: 1/4/2007

6.a. Period of Expenditure: From 1/4/2007 to 31/3/2009
b. Details of Expenditure:

S. No.	**Item**	**Amount Approved Rs.**	**Expenditure Incurred Rs.**
i	Books& Journals	Nil	Nil
ii	Equipment	30,000	29,570
iii	Contingency	5,000	4989
iv	Field Work/Travel	Nil	Nil
v	Hiring Services	Nil	Nil
vi	Chemicals & Glassware	Nil	Nil
vii	Overhead	25,000 (received- Rs 10,000/-)	10,000/-
viii	Any other items	Nil	Nil

(c) Staff: Not Applicable

S.No	**Items**	**From To**	**Amount Approved (Rs.)**	**Expenditure Incurred (Rs.)**
1.	Honorarium to PI (Retired Teachers) @ Rs. 18,000/-p.m.: Not Applicable			
2.	**Project fellow**: i) **NET/GATE qualified**-Rs. 16,000/- p.m. for initial 2 years and Rs. 18,000/- p.m. for	Not Applicable		

the third year.
ii) **Non-GATE/Non-NET**- Rs. 14,000/-
p.m. for initial 2 years and Rs. 16,000/-
p.m. for the third year.

2. If as a result of check or audit objection some irregularly is noticed at later date, action willbe taken to refund, adjust or regularize the objected amounts.

3. Payment @ revised rates shall be made with arrears on the availability of additional funds.

4. It is certified that the grant of Rs.45,000/- (Rupees Forty Five Thousand Only) received from the University Grants Commission under the scheme of support for Major Research Project entitled "**Micropropagation of a forest tree *Sterculia alata***" vide UGC letter No. F.32-422/2006 (SR) dated April 26, 2007 has been fully utilized (Rs. 44559/-) for the purpose for which it was sanctioned and in accordance with the terms and conditions laid down by the University Grants Commission.

SIGNATURE OF
PRINCIPAL INVESTIGATOR **REGISTRAR** **PRINCIPAL**
(Seal) **(Seal)**

Annexure – VIII

UNIVERSITY GRANTS COMMISSION
BAHADUR SHAH ZAFAR MARG
NEW DELHI – 110 002

PROFORMA FOR SUBMISSION OF INFORMATION AT THE TIME OF SENDING THE FINAL REPORT OF THE WORK DONE ON THE PROJECT

1. Title of the Project: Micropropagation of a forest tree *Sterculia alata*

2. NAME AND ADDRESS OF THE PRINCIPAL INVESTIGATOR

   Dr NISHI KUMARI, DEPARTMENT OF BOTANY, MMV, BHU

3. NAME AND ADDRESS OF THE INSTITUTION: Department of Botany, Mahila Mahavidyalaya, Banaras Hindu University

4. UGC APPROVAL LETTER NO. AND DATE: F. No. 32-422/2006 (SR) dated Feb. 24, 2007

5. DATE OF IMPLEMENTATION: 1/4/2007

6. TENURE OF THE PROJECT: 1/4/2007- 31/3/2009

7. TOTAL GRANT ALLOCATED: Rs. 60,000/-

8. TOTAL GRANT RECEIVED: Rs. 45,000/-

9. FINAL EXPENDITURE: Rs. 44559/-

10. TITLE OF THE PROJECT: Micropropagation of a forest tree *Sterculia alata*

11. OBJECTIVES OF THE PROJECT: To develop regeneration protocol of *Sterculia alata* either through organogenesis or embryogenesis.

12. WHETHER OBJECTIVES WERE ACHIEVED: Yes, a regeneration protocol of *Sterculia alata* was developed through somatic embryogenesis. Immature zygotic embryos were selected as explants and somatic embryos were formed from the calli of zygotic embryo. Ontogeny of somatic embryos was studied by microtomy method. Genetic fidelity of somatic embryos were also done by RAPD method.

13. ACHIEVEMENTS FROM THE PROJECT: *Sterculia alata* is a forest tree, which grows very fast and therefore, it can be used for afforestation programme. Somatic embryogenesis is an efficient micropropagation method for large scale

production of plants. So, present protocol will certainly help in forest restoration purpose, It is the first report of somatic embryogenesis in *Sterculia alata.*

14. SUMMARY OF THE FINDINGS:
    A protocol of somatic embryogenesis was developed by using immature zygotic embryos of *Sterculia alata* Roxb. Most responsive embryogenic calli were induced by 3 mm size of zygotic embryos, when the explants were cultured on Murashige and Skoog's medium supplemented with 2, 4-D (2.0 mg/l) and BAP (0.1 mg/l). The embryogenic calli were transferred to MS basal medium after three weeks treatment in induction medium. Maximum frequency of somatic embryogenesis was observed by adding 400 mg/l glutamine into the MS basal medium. Maximum maturation of somatic embryos was found on MS basal medium containing 0.01 mg/l Abscisic acid. Maximum 63.73 % of mature somatic embryos were able to germinate on the modified woody plant medium with 3 % sucrose but the conversion frequency was only 7.07 %. Ontogeny of somatic embryos was studied histologically. The Random amplified polymorphic DNA was used to analyze genetic fidelity of different stages of somatic embryos and also to identify the problem in conversion if any at genetic level. Somatic embryos regenerated from immature zygotic embryos showed no aberration in RAPD banding patterns with respect to donor explants, thus showing absence of somaclonal variation in present regeneration protocol.

15. CONTRIBUTION TO THE SOCIETY: Being a fast growing forest tree, its multiplication and large scale plantation is possible through present regeneration protocol. It can be used for afforestation purposes. Similarly, nutshell of the tree is being used for the production of activated carbon, which is highly useful for treatment of waste water. Therefore, large scale plantation can be useful for commercial production of activated carbon.

16. WHETHER ANY PH.D. ENROLLED/PRODUCED OUT OF THE PROJECT: Yes, the work is part of thesis of Ph.D student.

17. NO. OF PUBLICATIONS OUT OF THE PROJECT: One paper communicated (enclosed)

(PRINCIPAL INVESTIGATOR) **(REGISTRAR)** **(PRINCIPAL)**
(Seal) **(Seal)**

Detailed Report

Abstract

A protocol of somatic embryogenesis was developed by using immature zygotic embryos of *Sterculia alata* Roxb. Most responsive embryogenic calli were induced by 3 mm size of zygotic embryos, when the explants were cultured on Murashige and Skoog's medium supplemented with 2, 4-D (2.0 mg/l) and BAP (0.1 mg/l). The embryogenic calli were transferred to MS basal medium after three weeks treatment in induction medium. Maximum frequency of somatic embryogenesis was observed by adding 400 mg/l glutamine in MS basal medium. Maximum maturation of somatic embryos was found on MS basal medium containing 0.01 mg/l Abscisic acid. Maximum 63.73 % of mature somatic embryos were able to germinate on the modified woody plant medium with 3 % sucrose but the conversion frequency was only 7.07 %. Ontogeny of somatic embryos was studied histologically. The Random amplified polymorphic DNA was used to analyze genetic fidelity of different stages of somatic embryos and also to identify the problem in conversion if any at genetic level. Somatic embryos regenerated from immature zygotic embryos showed no aberration in RAPD banding patterns with respect to donor explants, thus showing absence of somaclonal variation in present regeneration protocol.

Abbreviations: BAP - 6-Benzylaminopurine; MS - Murashige and Skoog; 2, 4-D - 2, 4 Dichlorophenoxyacetic acid; ABA- Abscisic acid; RAPD- Random amplified polymorphic DNA.

Introduction

Sterculia alata Roxb. (family-Sterculiaceae) is commonly known as Buddha's coconut. It is found in India and various parts of South East Asia. It is very tall, erect, deciduous tree. The tree is used for afforestation and reclamation due to its easy adaptability and rapid growth rate even under degraded and wasteland conditions in the tropics. In India, the seeds are eaten and the plant is used medicinally by tribal communities. The seeds have narcotic properties and used as a substitute for opium. The nut shell of tree is used to prepare activated carbon with zinc chloride which has the ability to absorb phenols from waste water. Sterculynic acid has been isolated from the seed oil and identified as 8, 9-methyleneoctadec-8-en-17-ynoic acid. The tree is propagated by the winged seeds which are produced in large number but seeds show low percentage of germination. Conventional method for the propagation of this tree species is inefficient for large scale plantation. Thus, there is a need to develop an efficient regeneration system of *Sterculia alata*.

Somatic embryogenesis is an efficient tool for propagation. It is also used for biochemical and molecular studies of the regulation of embryo development in plants. Regeneration of plants through somatic embryogenesis is preferred for genetic transformation in woody perennials because somatic embryos originate from single cells and they are bipolar structures possessing both root and shoot meristems. The development of regeneration methods for large scale plantation of forest tree species has become very necessary to control fast decline of forest reserves. Regeneration of plants via somatic embryogenesis is considered to be an efficient approach for clonal plant propagation. The regeneration protocol in other species of *Sterculia* i.e. *Sterculia*

urens has been already developed by both organogenesis and somatic embryogenesis methods [Sunnichan et al., 1998]. There are unpublished reports on somatic embryogenesis of *Sterculia alata* [Pandey, 1998; Tripathi, 2012]. The present work reports induction of somatic embryogenesis from immature zygotic embryos of *Sterculia alata*.

Although somaclonal variation is not desirable in clonal propagation, but such variation has been reported among somatic embryo derived regenerants of some plant species. For industrial and commercial applications of regeneration protocol, there is a need to detect such variations especially heritable ones. As the trees are perennial and are having long juvenile phase, such variations may not be detected at early stages. Many workers have assessed these variations in various plant species through morphological, biochemical and molecular analysis. Therefore, Random Amplified Polymorphic DNA (RAPD) analysis was done to monitor the genetic fidelity of somatic embryos, as use of this marker is technically simple, quick to perform and requires very little plant materials. It is the first report of somatic embryogenesis in *Sterculia alata* Roxb.

Materials and Methods

Explant material, surface sterilization, media and culture condition

Immature fruits were collected from the tree of *Sterculia alata* growing in the campus of Banaras Hindu University, Varanasi during first and second week of September (Fig.1A). Seeds (Fig.1C) were isolated from the fruits (Fig.1B) and washed in running tap water for 10 min and then treated with 1 % (v/v) cetrimide (a detergent and antiseptic; ICI, India) with 2–3 drops of Tween 20 (surfactant; Hi Media, India) for about 14-15 min. Surface sterilization was carried out with 0.05 % mercuric chloride (disinfectant; Merck, India) for 4–5 min after a brief rinse in 70 % ethanol under aseptic condition. Seeds were then rinsed thrice with autoclaved double distilled water. Zygotic embryos (Fig.1D) were dissected aseptically from the seeds and cultured on MS medium. Different sizes (1-5 mm) of zygotic embryo explant were taken for culture initiation. Media supplemented with varying concentrations (0.1, 0.5, 1.0, 2.0, 3.0 or 5.0 mgl^{-1}) of different growth regulators (2, 4-D, NAA, IAA, BAP, Kn) and combinations of BAP & Kn (0.001, 0.01, 0.1, 0.5 mgl^{-1}) with 2, 4-D (2.0 mgl^{-1}) were tried for the induction of callus. The pH of the medium was adjusted to 5.8 ± 0.02 and the medium was autoclaved for 15 min at 121ºC temperature and 1.1 kg cm^{-2}pressure. Cultures were maintained at 25± 2 ºC with 16/8 h (light/dark) photoperiod at a photon flux of 50–70 mmol m^{-2} s^{-1} from white fluorescent tubes.

Induction, development, maturation and germination of somatic embryos

Dissected zygotic embryos were cultured on MS medium with various concentrations of auxins and cytokinins. After callus initiation, cultures were transferred for callus proliferation either to medium of same composition or MS basal medium. To standardize the period of treatment with growth regulators, the explants were transferred to MS basal media (maintenance medium) after different time durations from induction media. To enhance the number of globular structures (pro- embryogenic masses) and somatic embryos, different concentrations of sucrose (1, 2, 3, 4, 5 %), L-glutamine (100, 200, 300, 400, 500 & 600 mg/l) and L- proline (50, 100, 200, 300, 400 mg/l) were tried in embryo induction media. To optimize maturation, globular embryos were transferred to MS medium supplemented withvarying concentrations of sucrose (0

– 7 %) and ABA (0.001-1.0 mg/l). For germination, different strengths of MS (M1, M2, M3 & M4), different concentrations of sucrose (1, 2, 3, 4,5 & 7 %), various concentrations of ABA (0.001, 0.01, 0.1 & 1.0 mg/l) and different media (MS, WPM & modified WPM) were tried.

Histological studies

Somatic embryos of different stages were fixed for 24 h in 40% formaline –acetic acid - 95 %ethanol (1/1/18, v/v/v), dehydrated in a graded ethanol series and embedded in paraffin. Serial sections of ten to fifteen micron thickness were cut with the help of rotary microtome. Sections were double stained with safranin and fast green and then dehydrated through alcohol and xylene series. Finally, slides were mounted with DPX mountant. The observations of slides were done under phase contrast microscope (Nikon, Japan).

Experimental design and data analysis

Twenty five replicates were used for each treatment and the experiment was repeated thrice. The frequency of embryogenesis was calculated as the percentage of cultures showing at least one somatic embryo and intensity of embryogenesis was calculated as the mean number of somatic embryos produced per responsive explant in a particular treatment. The number of somatic embryos of different stages formed from each responding explant was counted under a stereo-microscope (Nikon, SMZ-2T, Japan). Data were analyzed through SPSS (version 16) software and the mean separations were carried out using Duncan's multiple range tests ($P<0.05$).

DNA extraction and PCR amplification

Genomic DNA was extracted from the different stages (globular, cotyledonary and germinated) of somatic embryos and mother explants according to method by Dellaporta et al [1983] with minor modifications. The concentration of DNA was determined by a UV–Visible spectrophotometer (Perkin Elmer 2380) and quality of genomic DNA was checked following electrophoresis on 0.8 % agarose gel. Twenty five random deca-nucleotide primers were used to analyze the genetic variations among the samples. Polymerase chain reaction (PCR) was performed in a volume of 25 µl reaction mixture containing 1 µl template DNA (80 ng), 2.5µl 10x PCR buffer, 1.5 µl MgCl2 (1.5 mM), 1 142 µl of dNTPs (25 mM), 1 µl random primer (10pM), 0.5 µl Taq polymerase (three units) and 17.5 µl sterile distilled water. DNA amplification was carried out in a DNA thermal cycler (Biorad, USA). The PCR program consisted of an initial denaturation for 4 min at 94 ºC, then 38 cycles of 1 min denaturation at 94 ºC, 1 min annealing at 33 ºC and 1 min extension at 72 ºC with a final extension at 72 ºC for 5 min. The samples were stored at 4 ºC until analysis was carried out. Amplification with each primer was repeated twice to confirm reproducibility of the results. The amplified samples were analyzed by electrophoresis in 1.2 % agarose gels using Tris–acetic acid–ethylene di amine tetra-acetic acid buffer and stained with ethidium bromide. The number of bands was recorded using a gel documentation system (AlphaImager TM 3400).

Results

Somatic embryogenesis induction

Immature zygotic embryos of 3 mm size (Graph 1) were found most responsive for induction of callus. On the medium, the explants showed swelling and increase in size after 7-10 days of inoculation (Fig 2: B & C). MS medium supplemented with different concentrations of the auxins or cytokinins alone showed callusing, but medium with 2, 4-D (2.0 mg l^{-1}) was most responsive for callus initiation (71.60 + 2.13%). No callusing was observed on media supplemented with (0.1& 0.5) mg l^{-1}IAA and 0.1 mg l^{-1} Kn. Response of callus initiation was maximum on 2, 4-D (2.0 mg l^{-1}) + BAP (0.1 mg l^{-1}) containing medium (Fig 2), whereas less response of callusing was observed on the media containing different combinations of 2,4-D (2.0 mg l^{-1}) and Kn. Three week pulse treatment was observed as the most favourable for induction of embryogenic callus (Fig 3A). The callus became compact and non-embryogenic after maintaining on the same initiation medium. Therefore, callus was transferred to MS basal medium after three weeks. Addition of L-glutamine and L-proline in maintenance medium enhanced the formation of globular structures. As compared to control, L-glutamine (400 mg/l) in the medium showed enhancement in maximum frequency of embryogenesis with maximum number of globular structures per culture whereas L-proline (100 mg/l) showed slight improvement in the formation of globular structures (Table 1). The best medium for somatic embryo formation was observed on MS medium supplemented with3 % sucrose and 400 mg/l L- glutamine.

Development and maturation of somatic embryos

Somatic embryo formation was reported after 7 week of culture initiation. Most of the cultures showed formation of asynchronous growth of somatic embryos (Fig. 4: A-D). Full strength agar solidified MS medium supplemented with 3 % sucrose and 400 mg/l glutamine was favorable for development and maturation of somatic embryos (Table 2). During development and maturation, anomalies like fused embryo, pluricotyledony and recurrent embryogenesis were seen in somatic embryos (Fig. 4A, 8E & 7: A-E). To overcome these anomalies and for maximum maturation, different percentages of sucrose and ABA concentrations were tried. Among different sucrose concentrations, 3 % was best whereas on 5 % and 7 % sucrose supplemented medium most of the somatic embryos got vitrified. Somatic embryos treated with 3 % sucrose + ABA (0.01 mgl^{-1}) showed optimum percent maturation (Table 2).

Germination and conversion of somatic embryos

Cotyledonary somatic embryos showed increase in size and elongation of hypocotyls and root formation on MS basal medium (Fig 6C). It takes 4-6 weeks for the formation and elongation of shoot meristem and 5-8 weeks for the emergence of first two leaves. As germination on MS basal medium was very low, therefore somatic embryos were transferred to different strengths of MS medium, MS medium supplemented with different concentrations of sucrose & ABA and different media types (Table 3). The best response in terms of germination and conversion was observed in modified WPM medium with 3 % sucrose. Since none of the plantlet grew beyond first five or six leaf therefore plant development from somatic embryos was incomplete (Fig. 6E). For further study, there is a need to optimize protocol especially for germination and normal plantlet formation.

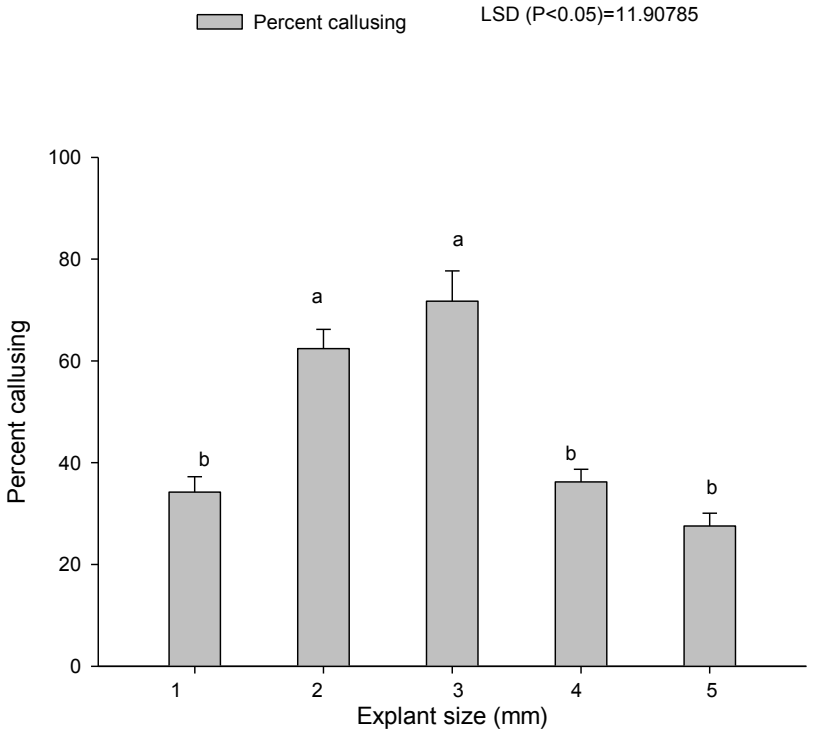
Histological analysis of the somatic embryos

Embryogenic callus showed formation of globular structures (pro-embryogenic masses) (Fig. 5A & B). The cells of globular structures were densely nucleated cytoplasmic cells with no intercellular spaces (Fig.5C). Embryo formation occurred at the surface of such structures. These embryos showed various stages of development i.e. globular, heart and cotyledonary stages (Fig.5 D, E & F). Fusion of embryo and procambial strand, poor development of shoot meristem and recurrent embryogenesis can be easily seen in histological sections of various stages of culture (Fig 8: A-F).

Monitoring the genetic fidelity by RAPD

In order to identify the problem of conversion at genetic level (if any), different stages of somatic embryos (globular, cotyledonary, and germinated embryos) were screened for RAPD analysis. For this RAPD analysis, 25 random primers were used (Table 4). The results were scored as patterns of bands obtained from the different stages of somatic embryos and compared with the mother explants (Fig 9). All the 25 random primers tested, each primer produced distinct amplification profiles displayed the same banding pattern of all the samples. The total number of amplification products related by polymerase chain reaction was 181 bands (7.24 bands per primer). From the representative profile of the somatic embryo samples and the control, it was obvious that the different stages of somatic embryos showed identical RAPD profiles (i.e. no polymorphism was observed). The results confirmed the genetic fidelity of different stages of somatic embryos and the problem of conversion was not at genetic level.

Graph 1: **Effect of explants size on induction of callus**



Medium: 0.8% agar-solidified full-strength MS medium + 3% sucrose + 2, 4-D (2.0 mgl^{-1}). Data represent the Mean ± SE of three repeated experiments, each with twenty four replicates. Mean values followed by different letter are significantly different from each other and sharing the same letters do not differ significantly at $P < 0.05$ according to Duncan's multiple range test.

Table 1: Effect of different factors (sucrose, L-Glutamine and L-proline) on frequency of somatic embryogenesis and formation of globular structures

Sucrose (%w/v)	L-Proline (mg/l)	L-Glutamine (mg/l)	Frequency of embryogenesis (%)	Number of globular structures
0	0	0	0.0 ± 0.00d	0.00 ± 0.00d
1	0	0	35.57 ± 1.75c	7.23 ±1.15c
2	0	0	52.31 ± 1.61b	19.42 ±1.92b
3	0	0	80.47 ± 0.36a	30.74 ± 1.01a
4	0	0	55.05 ± 4.70b	17.65 ±1.36b
5	0	0	32.42 ± 3.26c	8.29 ± 0.67c
3	0	0	80.47 ± 0.36ab	30.74 ± 1.01a
3	50	0	82.42 ± 0.42a	32.10 ± 1.01a
3	100	0	83.65 ± 1.07a	33.62 ± 0.92a
3	200	0	74.97 ± 2.51b	22.65 ± 1.55b
3	300	0	60.88 ± 3.69c	13.21 ± 1.48c
3	400	0	40.87 ± 0.69d	7.06 ± 0.61d
3	0	0	80.47 ± 0.36b	30.74±1.01c
3	0	100	81.38 ± 0.21bc	32.30±1.24c
3	0	200	83.05 ± 0.42bc	36.73±0.84b
3	0	300	85.31 ± 0.50b	38.79±0.58b
3	0	400	91.11 ± 0.68a	45.23±1.15a
3	0	500	64.15 ± 2.94c	23.00±1.55d
3	0	600	42.46 ± 1.50d	12.05±1.80e

Medium: 0.8% agar-solidified full-strength MS medium + Different concentration of various factors. Values represent the Mean ± SE of three repeated experiments, each with twenty four replicates. Mean values followed by different alphabet under different treatments within a column are significantly different from each other at $P \leq 0.05$ (Duncan's multiple range test)

Table 2: Effect of different concentrations of sucrose and ABA on maturation of somatic embryos

Sucrose (%, w/v)	ABA (mg/l)	Mature embryos (Mean ± SE)
0	0	0.00 ± 0.00f
1	0	5.56 ±0.57e
2	0	12.87 ±1.52d
3	0	48.00 ± 1.32b
4	0	32.14 ±1.87b
5	0	22.31 ± 1.62c
7	0	13.42 ± 1.20d
3	0	48.00 ± 1.32b
3	0.001	62.98 ± 1.32a
3	0.01	65.53 ± 2.91a
3	0.1	44.54 ± 2.34b
3	1.0	22.79 ± 1.48c

Medium: 0.8% agar-solidified full-strength MS medium + Different concentration of sucrose and ABA. Experiments for each factors was conducted separately. Values represent the Mean ± SE of three repeated experiments, each with twenty four replicates. Mean values followed by different alphabet under different treatments within a column are significantly different from each other at $P \leq 0.05$ (Duncan's multiple range test)

Table 3: Effect of different type of medium and strength of medium on germination and conversion of somatic embryos

Type of medium	Strength of medium	Germination of somatic embryos (Mean ± SE)	Conversion of somatic embryos (Mean ± SE)
MS		23.88 ± 0.99c	5.48 ± 0.40a
WPM		54.89 ± 3.33b	5.91 ± 0.68a
Modified WPM		63.73 ± 0.83a	7.07 ± 0.29a
	M1	24.09 ± 0.86a	6.41 ± 0.58a
	M2	16.39 ± 1.20b	0.00 ± 0.00c
	M3	11.73 ± 1.05c	0.00 ± 0.00c
	M4	18.86 ± 0.75b	4.64 ± 0.24b

M1, 0.8% agar-solidified full-strength MS medium; M2, 0.8% agar-solidified half strength MS medium; M3, 0.8% agar-solidified Quarter strength MS medium; M4, 0.8% agar-solidified Modified (Half strength macro and iron EDTA and full strength micronutrients and organics) MS medium, Modified WPM (WPM macrosalts and MS microsalts and organics). Mean values sharing the same letter do not differ significantly ($P<0.05$) according to Duncan's multiple range test.

Table-4 The nucleotide sequences of primers used for RAPD analysis

Primer	5'-3'	No of bands	Primer	5'-3'	No of bands
OPK-1	CATTCGAGCC	08	OPV-11	AGACGATGGG	08
OPK-3	CCAGCTTAGG	06	OPV-17	GACGTGGTGA	07
OPK-5	TCTGTCGAGG	09	OPV-20	AGCGTGGTGA	09
OPL-1	GGCATGACCT	07	OPW-1	CTCAGTGTCC	08
OPL-2	TGGGCGTCAA	11	OPW-2	ACCCCGCCAA	06
OPL-4	GACTGCACAC	06	OPW-3	GTCCGGAGTG	05
OPL-5	ACGCAGGCAC	07	OPW-4	CAGAAGCGGA	07
OPL-20	TGGTGGACCA	08	OPY-1	GTGGCATCTC	07
OPN-1	CTCACGTTGG	09	OPY-2	CATCGCCGCA	08
OPN-2	ACCAGGGGCA	09	OPZ-1	TCTGTGCCAC	05
OPN-3	GGTACTCCCC	07	OPZ-3	CAGCACCGCA	09
OPN-4	GACCGACCCA	06	OPZ-4	AGGCTGTGCT	03
OPO-6	CCACGGGAAG	06			

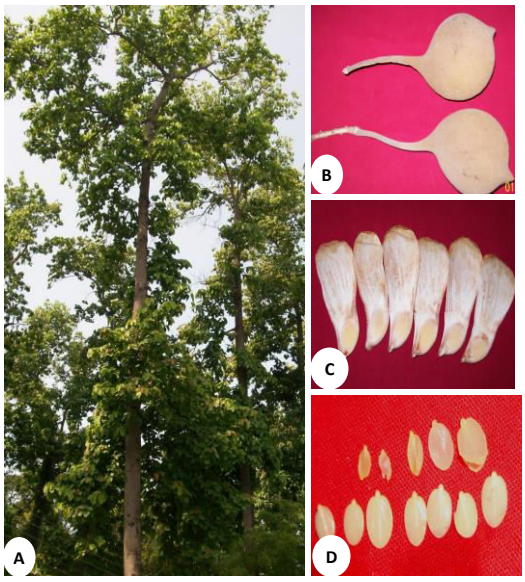


Fig.1 : A. Tree selected as donor plant, B. Fruits, C. Winged seeds, D. Zygotic Embryos

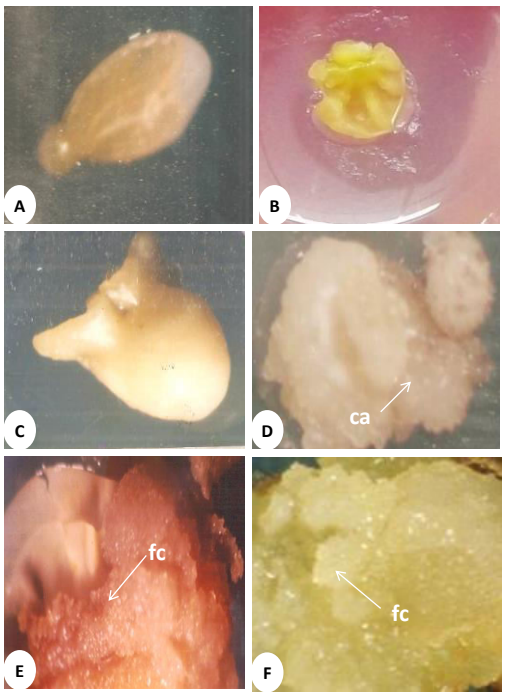


Fig.2: (A-F) Callus initiation from zygotic explants
ca- callus; fc- friable callus

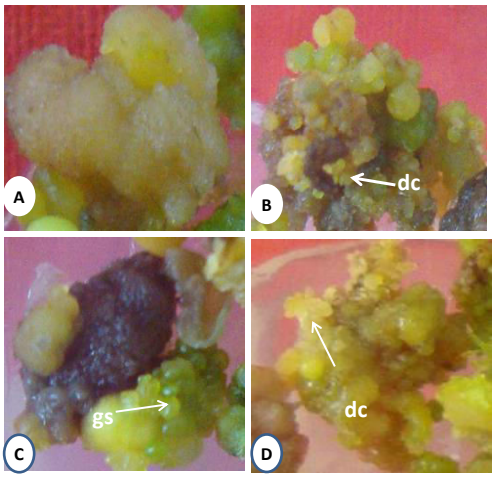


Fig. 3: A. Embryogenic callus, B-D. Somatic embryo formation
gs- globular structure, dc- dicotyledonary embryo

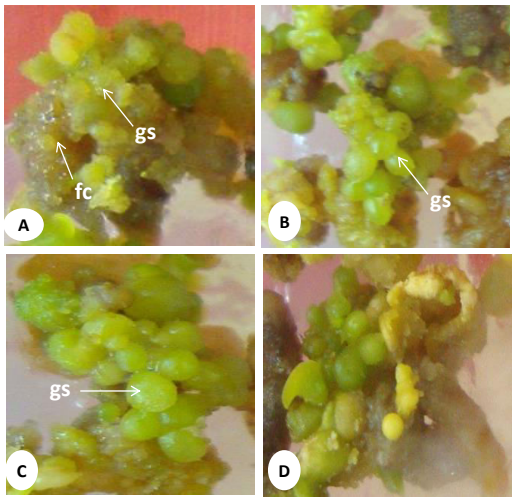


Fig. 4: (A-D) Asynchronous development of embryos
gs- globular structure, fc- fused cotyledonary embryo

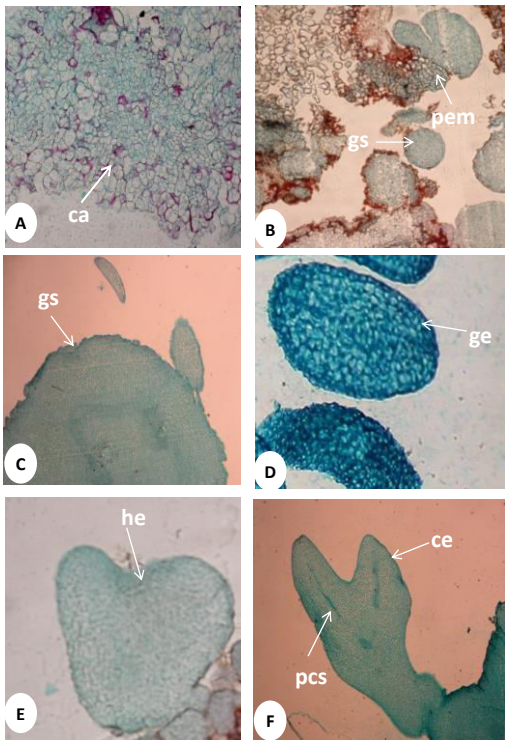


Fig. 5: A. Callus (ca) , B. Proembryogenic masses (pem), C. globular structure (gs) D-F Different stages of embryos [ge- globular embryo, he- heart stage, ce- cotyledonary embryo, pcs- procambial strands]

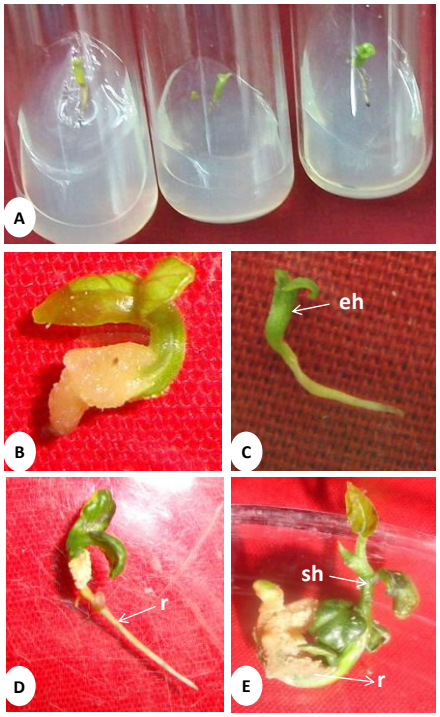


Fig. 6: (A-E) Various stages of germination

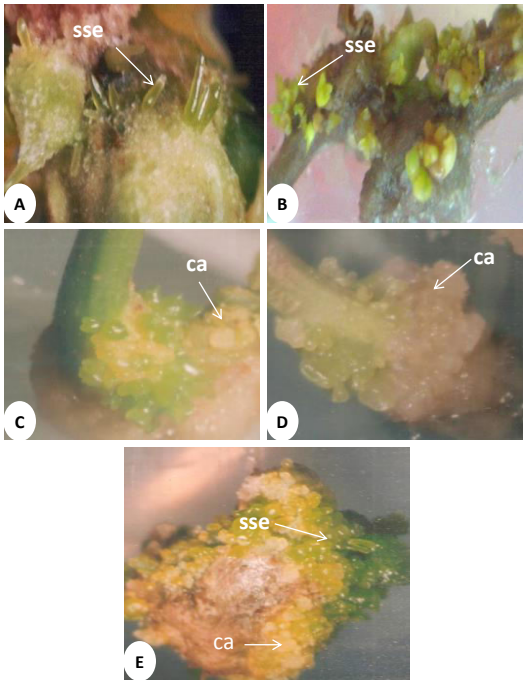


Fig. 7: (A-E) Secondary somatic embryogenesis
sse- secondary somatic embryo, ca- callus

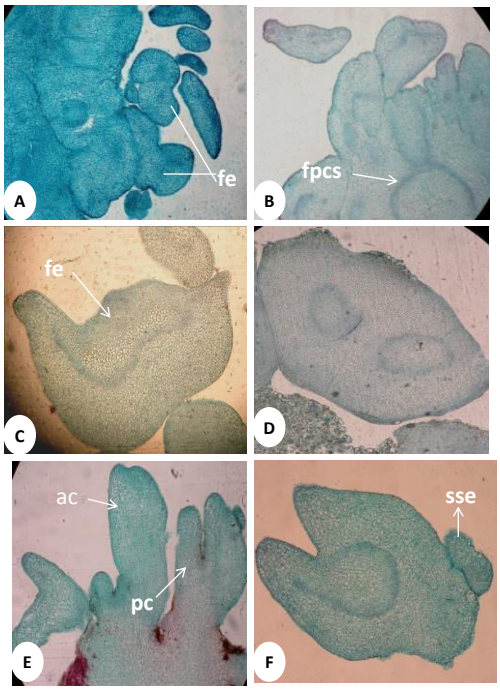


Fig. 8: (A-F) Abnormality in somatic embryos [fe- fused embryo, fpcs- fused procambial strand, ac- abnormal cotyledon, sse- secondary somatic embryo]

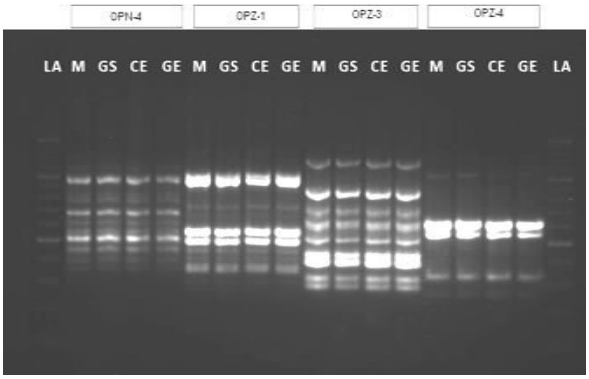


Fig. 9

Annual Report

[Annexure III, 7(v)]

First Year Achievement (2007-2008)

(i) Procurement of equipments- Air Conditioner & Refrigerator.
(ii) Culture Initiation by culturing zygotic explants on media containing various concentrations and combinations of growth regulators.
(iii) Callus initiation, callus proliferation & initiation of somatic embryos.
(iv) Maturation and germination of somatic embryos.
(v) One Ph.D student was trained to do above work.

Second Year Achievement (2008- 2009)

(i) To study various factors and optimization of factors.
(ii) Compilation of data.
(iii) Fixation of various materials for microtomy.

Achievements after completion of project (2009 onwards)

(i) To make permanent slides of callus, different stages of somatic embryos to understand ontogeny of somatic embryos and to study embryos histologically.
(ii) RAPD analysis to check genetic fidelity of somatic embryos.
(iii) The work became a part of thesis of Ph.D student (Tripathi, 2012).
(iv) Manuscript was communicated for publication (One research paper is already communicated and under revision).