

***IN VITRO* APPROACHES TO THE ESTABLISHMENT AND
MULTIPLICATION OF TEAK (*TECTONA GRANDIS* L.)**



MUHAMMAD AKRAM

**DEPARTMENT OF BOTANY
UNIVERSITY OF THE PUNJAB
LAHORE, PAKISTAN**

***IN VITRO* APPROACHES TO THE ESTABLISHMENT AND
MULTIPLICATION OF TEAK (*TECTONA GRANDIS* L.)**

**A THESIS SUBMITTED TO THE UNIVERSITY OF THE PUNJAB IN PARTIAL
FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY**

By

MUHAMMAD AKRAM

**DEPARTMENT OF BOTANY
UNIVERSITY OF THE PUNJAB
LAHORE, PAKISTAN**

JULY, 2010

**TO MY
LOVING PARENTS &
MY SINCERE TEACHERS**

TABLE OF CONTENTS

Title	Page No.
CERTIFICATE.....	i
ACKNOWLEDGEMENTS	ii
ABBREVIATIONS.....	iii
SUMMARY.....	v
LIST OF TABLES.....	viii
LIST OF FIGURES.....	xi
CHAPTER 1 (INTRODUCTION).....	1
CHAPTER 2 (LITERATURE REVIEW).....	7
2.1 Seed dormancy.....	8
2.2 Vegetative propagation.....	12
2.3 Tissue culture studies in teak.....	15
2.3.1 Micropropagation.....	15
2.3.2 Callus induction, somatic embryogenesis and plant regeneration.....	19
2.4 Novel micropropagation methods.....	22
2.3.1 Shoot forcing and forcing epicormic buds.....	22
CHAPTER 3 (MATERIALS AND METHODS).....	28
3.1 Procurement of plant material.....	28
3.2 Preparation of stock solutions and culture media.....	28
3.2.1 Preparation of MS and WPM stock solutions.....	28
3.2.2 Stock solutions of plant growth regulators (PGRs).....	28
3.2.3 Preparation of Quick-Dip auxin solutions.....	29
3.3 Preparation of MS or WPM basal media.....	29
3.4 Sterilization.....	29
3.4.1 Sterilization of glassware.....	29
3.4.2 Sterilization of working area.....	30
3.4.3 Sterilization of surgical tools.....	30
3.4.4 Sterilization of tissue culture media.....	30
3.4.5 Sterilization of glasshouse media.....	30
3.4.6 Filter sterilization of PGRs.....	30
3.5 Culture conditions.....	31

3.6 Histological studies.....	31
PLAN OF WORK	32
3.7 Estimation of viability, dormancy and germination of teak seeds.....	32
3.7.1 Fruit size grading.....	32
3.7.2 Seed status by X-rays radiography.....	32
3.7.3 Seed status by cutting test.....	33
3.7.4 Seed dormancy.....	33
<i>Fresh water scarification</i>	33
<i>Mechanical scarification</i>	33
<i>Stratification</i>	33
3.7.5 Seed germination tests.....	34
<i>Seed germination in soil</i>	34
<i>Fleshy seed germination in trays under glasshouse conditions</i>	34
<i>In vitro seed germination</i>	34
3.8 <i>In vitro</i> establishment of fleshy teak seeds.....	35
3.8.1 Effect of cytokinins on <i>in vitro</i> seed germination and.....	35
morphogenesis	
3.9 Vegetative propagation.....	35
3.9.1 Propagation by mature shoot cuttings.....	36
3.10 Propagation from forced softwood shoots.....	36
3.10.1 Effect of glasshouse media and environment on forcing softwood.....	36
shoots from epicormic buds of large stem segments	
3.10.2 Softwood shoot forcing in solution from dormant shoot tips.....	37
3.10.3 <i>In vitro</i> establishment of epicormic softwood shoots.....	38
3.11 Somatic embryogenesis and plant regeneration from shoot tips derived from forced epicormic shoots.....	41
3.11.1 Explant preparation and culture conditions.....	38
3.11.2 Callus induction.....	38
3.11.3 Callus subculture, proliferation and callus biomass.....	39
3.11.4 Somatic embryogenesis.....	40
3.11.5 <i>In vitro</i> rooting and acclimatization.....	41
3.12 Shoot organogenesis from <i>in vitro</i> germinating seedlings.....	41
3.12.1 Effect of different culture conditions and media for callus	

induction.....	41
3.12.2 Effect of phytohormones for callus induction.....	42
3.12.3 Shoot regeneration and shoot elongation.....	42
3.12.4 <i>In vitro</i> rooting and acclimatization.....	42
3.13 Statistical analyses.....	43

RESULTS

CHAPTER 4.1 (VIABILITY, DORMANCY AND GERMINATION OF TEAK SEEDS)	44
PART-A: SEED VIABILITY AND DORMANCY	44
4.1.1 Grading of fruits on the basis of size and weight.....	44
4.1.2 Seed status by X-Ray radiography.....	48
4.1.3 Seed status by cutting test.....	50
4.1.4 Seed dormancy and germination.....	52
4.1.4.1 Fresh water scarification.....	52
4.1.4.2 Mechanical scarification.....	52
4.1.4.3 Stratification.....	53
4.1.4.4 <i>In vitro</i> seed germination on agar or sterilized sand medium.....	53
PART-B <i>IN VITRO</i> SEED GERMINATION ON DIFFERENT CYTOKININS	57
4.1.5 Effect of cytokinins on <i>in vitro</i> seed germination.....	57
4.1.6 Effect of cytokinins on seedling growth.....	60
4.1.7 Effect of cytokinins on seedling morphology and shoot bud induction...	62
4.1.8 Effect of different cytokinins on seedling hyperhydricity.....	66
CHAPTER 4.2 (VEGETATIVE PROPAGATION BY MATURE SHOOT CUTTINGS)	68
4.2.1 Rooting of cuttings.....	68
4.2.2 Callusing and sprouting.....	70
4.2.3 Steckling ability of rooted cuttings.....	72
CHAPTER 4.3 (SOFTWOOD SHOOT FORCING/FORCING EPICORMIC BUDS OF TEAK AND STUDIES ON THEIR ROOTING POTENTIAL	75
4.3.1 FORCING EPICORMIC BUDS.....	75
4.3.1.1 Effect of light or shade conditions on forcing softwood shoots.....	75
4.3.1.2 Effect of growth environments on forcing softwood shoots.....	78

4.3.1.3 Effect of growth media on forcing softwood shoots.....	80
4.3.1.4 Effect of growth seasons.....	82
4.3.1.5 Vigour of softwood shoots.....	82
4.3.2 FORCING DORMANT SHOOT TIPS OF TEAK IN SOLUTION.....	84
4.3.2.1 Effect of environments.....	84
4.3.2.2 Effect of growth seasons.....	86
4.3.3 ROOTING OF SOFTWOOD SHOOTS.....	88
CHAPTER 4.4 (CALLUS INDUCTION, SOMATIC EMBRYOGENESIS AND PLANT REGENERATION FROM SOFTWOOD SHOOTS OF TEAK).....	89
4.4.1 Morphogenic callus induction.....	89
4.4.2 Callus subcultures.....	92
4.4.3 Callus proliferation.....	95
4.4.4 Callus fresh and dry weights.....	96
4.4.5 Somatic embryogenesis.....	97
4.4.6 Shoot regeneration.....	100
4.4.7 Shoot elongation.....	102
4.4.8 <i>In vitro</i> rooting and acclimatization.....	104
CHAPTER 4.5 (SHOOT REGENERATION VIA ORGANOGENESIS FROM CALLUS CULTURES OF <i>IN VITRO</i> GERMINATING SEEDLING OF TEAK).....	107
4.5.1 Effect of culture conditions on callus induction.....	107
4.5.2 Effect of basal medium for callus induction.....	108
4.5.3 Effect of phytohormones on callus induction.....	109
4.5.4 Morphological features of callus cultures.....	111
4.5.4.1 Callus morphology from leaf explant.....	111
4.5.4.2 Callus morphology from internodal explant.....	114
4.5.4.3 Callus morphology from cotyledon explant.....	116
4.5.5 Shoot regeneration from nodal explants.....	118
4.5.6 Shoot elongation.....	122
4.5.7 <i>In vitro</i> rooting and acclimatization.....	123
CHAPTER 5	126
(DISCUSSION).....	

CHAPTER 6 (LITERATURE CITED)	149
ANNEXURES	171
Annexure-1	171
Formulation of MS Medium (Murashige and Skoog, 1962) for the Preparation of Stock Solutions	
Annexure-2	172
Formulation of WPM Medium (Lloyd and McCown, 1981) for the Preparation of Stock Solutions	
Annexure-3.....	173
Preparation of Stock Solutions for MS (Murashige and Skoog, 1962) Medium	
Annexure-4	174
Preparation of Stock Solutions for WPM (Lloyd and McCown, 1981)) Medium	
Annexure-5.....	176
Preparation of stock solutions of Plant Growth Regulators (PGRs)	
Annexure-6.....	178
Preparation of an IBA or NAA alone or in combination Quick-Dip Method solution rooting experiments	
Annexure-7.....	179
Preparation of one liter MS medium	
Annexure-8.....	179
Preparation of one liter WPM medium	

CERTIFICATE

This is to certify that the research work entitled "*In vitro* approaches to the establishment and multiplication of teak (*Tectona grandis* L.)" described in this thesis by **Mr. Muhammad Akram** is an original work of the author and has been carried out under my direct supervision. I have personally gone through all the data, results materials reported in the manuscript and certify their correctness and authenticity. I further certify that the material in this thesis has not been used in part or full in a manuscript already submitted or in the process of submission of partial or complete fulfillment of the award of any other degree from an institution. I also certify that the thesis has been prepared under my supervision according to the prescribed format and I endorse its evaluation for the award of Ph.D degree through the official procedures of the University of the Punjab, Lahore.

Supervisor:



(Dr. Faheem Aftab)
Associate Professor,
Department of Botany,
University of the Punjab,
Lahore-54590.

Date: July 28, 2010

ACKNOWLEDGEMENTS

I deem it great honor and privilege to record deep sense of gratitude to my worthy supervisor **Dr. Faheem Aftab**, Associate Professor, Department of Botany, University of the Punjab, Lahore, for his sincere efforts, guidance, encouragement and his faith and confidence in me, for lending me a 'free hand' to work in the lab, providing everything necessary during the course of work. Also, his scholarly directions, keen interest and most importantly his caring and affectionate behavior was the real source of inspiration for me for the completeness of this project.

I extent my zealous thanks to **Prof. Dr. Khan Rass Masood**, Chairman, Department of Botany, University of the Punjab, Lahore, for providing me much needed facilities to complete my Ph.D research work. I am also thankful to the chairman for giving me great opportunity of teaching at the department, financial support and his keen interest for the establishment of tissue culture-raised teak orchard in PU Botanical Garden.

I acknowledge my sincere thanks to **Prof. Dr. Shahida Hasnain**, Former Dean Faculty of life sciences, for her encouraging behavior during the course of work.

I am thankful to **Dr. Javed Iqbal (Professor Emeritus)**, Department of Botany and Director, School of Biological Sciences, University of the Punjab, Lahore, for giving me confidence to work on this project.

I am also thankful to **Dr. Humera Afrasiab**, Assistant Professor, Department of Botany, for her valuable suggestions, and thanks to all other faculty members.

My sincere thanks go to **Ex. Vice Chancellor, University of the Punjab, Lahore, Gen. Arshad Mahmood**, who appreciated our research work and awarded us cash prize. I acknowledge **Higher Education Commission (HEC), Pakistan** for providing facilities under the project No. 1155 granted to **Dr. Faheem Aftab**.

To my all lab fellows, more importantly **Mr. Zahoor Ahmad, Ms. Adeela Haroon, Dr. Neelma Munir and Ms. Arifa Khalid** for their helping hands.

I am also thankful to my parents, brothers and sisters for their tremendous patience and selfless support during my study.

Muhammad Akram

ABBREVIATIONS/UNIT ABBREVIATIONS

μM	Micro Molar
μm	Micrometer
$\mu\text{mol m}^{-1}\text{s}^{-1}$	Micromole per meter per second (Light intensity)
2, 4-D	2, 4-Dichlorophenoxyacetic acid
2iP	Isopentenyl adenine
8-HQC	8-hydroxyquiniline citrate
ABA	Abscissic acid
ABT	1-aminobenzotriazole
Ads	Adenine sulphate
BA	N^6 -Benzyleadenine
Cm	Centimeter
cm^2	Square centimeter
cm^3	Cube Centimeter
CMF-PT	Changa Manga Forest Plus Trees
CMF-TS	Changa Manga Forest Teak Stand
CPPU	N_1 -(2-chloro-4-pyridyl)- N_3 -phenylurea
CrO_3	Chromic acid
df	Degree of Freedom
DKW	Driver and Kuniyuki (1984) medium
DMRT	Duncan's Multiple Range Test
DW	Dry weight
EDTA	Ethylene diamine tetraacetate
FW	Fresh weight
g	Gram
g^{-1}	Gram per liter
GA_3	Gibberellic acid
h	Hour
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
IAA	Indole-3-acetic acid
IAAsp	Indol-3-acetyl-aspartic acid

IBA	Indole-3-butyric acid
IPA	N ⁶ - (2-isopentyl) adenine
Kin	Kinetin
KNO ₃	Potassium Nitrate
L.	Linneus
LSD	Least Significant Difference
M	Molar
mg	milligram
min	Minute
ml	Milliliter
mM	Milli molar
mm	Millimeter
MS	Murashige and Skoog (1962) basal medium
MW	Molecular weight
NAA	Naphthaleneacetic acid
NaOCl	Sodium hypochlorite
NaOH	Sodium Hydroxide
Ø	Diameter
PGRs	Plant Growth Regulators
pH	Hydrogen ion concentration
ppm	parts per million
PUBG	Punjab University Botanical Garden
PVP	Polyvinyl polypyrrolidone
RH	Relative humidity
SE	Standard Error
SWS	Softwood shoots
TDZ	Thidiazuron (<i>N</i> -phenyl- <i>N'</i> -1,2,3-thidiazol-5-ylurea)
UV	Ultra violet light
V/V	Volume per volume
W/V	Weight per volume
WPM	Woody plant medium
\bar{x}	Mean
$\bar{\bar{x}}$	Grand mean
Zt	trans-Zeatin

SUMMARY

SUMMARY

Propagation and multiplication of teak, *Tectona grandis* L., was investigated by using the conventional methods of seed germination and rooting of cuttings, and *in vitro* techniques of callus induction and plant regeneration. Teak fruits were collected from different provenances and graded on the basis of their size. Most of the fruits were of 6-8 mmØ. A total mean number of fruits of 2560 ± 89.5 , 2870 ± 71.1 and 3356 ± 105.5 per kilogram were collected from CMF-TS, CMF-PT and PUBG, respectively. The large-sized fruits of mean diameter of $\geq 17\text{mmØ}$ were heavier ($\bar{x}=0.58 \pm 0.02$ g) than the smaller ones (12-16mm, 9-11mm or 6-8mmØ). Most of the fruits were one-seeded as analyzed by X-rays radiography collected from either fruit size grade. After fresh water scarification of seeds, the mean seed germination under nursery conditions was 44.3 percent. The rate of *in vitro* seed germination was 100 % on MS medium supplemented with $0.22 \mu\text{M N}^6$ -benzyladenine (BA). After 40 days of culture, the *in vitro* germinated seedlings had 4.4 ± 1.2 cm long shoots and 4.7 ± 1.3 cm long roots. On MS medium with $0.8 \mu\text{M}$ thidiazuron an average of 27.4 ± 4.8 shoot buds were formed on intact seedlings, which further developed into shoots. Mature shoot cuttings were also used for developing a simple and reproducible method for clonal propagation of teak. For this purpose, mature shoot cuttings were treated with IBA or NAA or in combination. After 60 days of planting, the best rooting (80 %) was obtained with a combination of $14.76 + 14.76$ mM (IBA + NAA) as compared with 4.92 IBA or 9.84 mM NAA. Softwood shoots were forced from latent epicormic buds on various media under various environmental conditions. It was found that continuous light ($35\mu\text{mm}^{-1}\text{s}^{-1}$) at 25 ± 2 °C enhanced softwood shoot production ($\bar{x}=2.7 \pm 1.3$) as compared with glasshouse ($\bar{x}=2.1 \pm 1.0$) or natural environment ($\bar{x}=1.8 \pm 0.9$). Potting medium had a significant role in

softwood shoot production; the highest ($\bar{x}=3.4 \pm 0.4$) shoots were formed on coco peat as compared with sand or sawdust during autumn season after 38 days of initial potting. The forcing solution of 8-hydroxyquinoline citrate, 2 % sucrose, 10 μM TDZ in combination with 2 μM each of IBA and gibberellic acid was effective in shoot forcing from dormant shoot tips with 56.6 % success. Softwood shoots were used for further studies on rooting and axillary shoot proliferation. Softwood cuttings treated with IBA + NAA (3000 + 3000 μM) had the highest rooting (89.3 %) with an average of 5.5 ± 0.05 roots. Addition of glutamine and PVP were equally effective with 60 % establishment of shoot apices on MS medium containing 10 μM BA and 5 μM NAA and produced on average 43 ± 3.6 shoots. In the present study, as compared with other auxins (2, 4-D, Dicamba, Picloram or NAA), thidiazuron (0.1 or 1 μM) was found to be highly effective for callus induction, the highest (100 %) callus induction was obtained after 35 days from shoot-tip explants taken from forced-softwood shoots. Calluses were creamy, nodular and embryogenic with high proliferation. After 63 days of the culture, 100 % of the calli underwent somatic embryogenesis with an average 36.4 ± 6.6 globular somatic embryos on MS medium supplemented with 8 μM TDZ, 2.2 μM BA and 5mM ascorbic acid. The rate of shoot regeneration was 100 % with an average of 16.4 ± 2.6 after culture up to 120 days on the same medium. Shoots were elongated and rooted (70 %) on half-strength MS medium supplemented with 8 μM IBA and 8 μM NAA, and were acclimatized successfully under field conditions. In another experiment, highest rate of callus induction (100 %) was achieved from cotyledon as compared with that derived from node, internodes or leaf explants of *in vitro* germinated 50 day old seedlings. For callus induction MS medium was better than Woody Plant Medium (WPM). Thidiazuron (1 or 4 μM) gave highest callus induction response (93.3 ± 8.2 %) as compared with woody plant medium (WPM) for callus induction. Thidiazuron (1 or 4 μM) induced

highest callus induction response of 100 %. Thidiazuron was better than the other phytohormones, 2, 4-D, Dicamba, NAA and Picloram. A high frequency of shoot regeneration (100 %) was obtained on medium supplemented with a combination of 8 μ M Thidiazuron and 0.5 μ M IBA; also the highest number of shoots (\bar{x} =42.3 \pm 4.2) was obtained on the same medium after 120 days of culture. Shoots were elongated by transferring them on half-strength MS basal medium supplemented with IBA and NAA. Best rooting (\bar{x} =75.4 %) was obtained on medium containing 8 μ M IBA and 8 μ M NAA after 35 days of culture; a mean number of 1.6 \pm 0.7 and mean root length of 3.2 \pm 0.1 cm. The rooted shoots were transferred to plastic pots or trays filled with mixer of sand : soil : peat moss of 2 : 1 : 1 and kept in a growth room at 25 \pm 2 $^{\circ}$ C for a month and then shifted to a glasshouse at 28 \pm 5 $^{\circ}$ C for an another 28 days. The plants in baby glass jars were covered with polythene sheet to maintain high relative humidity, and after 28 days these were transferred to soil in large plastic pots (25 x 30 mm). After 4 months of growth, these were planted in field plot in botanical garden. The results showed that *in vitro* plant regeneration can be used to complement the conventional propagation of teak from seed and rooted cuttings.