Characterization of candidate plus trees (CPTs) of *Pongamia pinnata* (L.), a versatile legume from North Guwahati, Assam

A THESIS

submitted by

VIGYA KESARI

for the award of the degree

of

DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOTECHNOLOGY

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

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Dedicated to my parents

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DEPARTMENT OF BIOTECHNOLOGY

STATEMENT

I do hereby declare that the matter embodied in this thesis is the result of investigations carried out by me in the Department of Biotechnology, Indian Institute of Technology Guwahati, India, under the supervision of Dr. Latha Rangan.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

Date: 04 May, 2010 **Vigya Kesari**

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

DEPARTMENT OF BIOTECHNOLOGY

CERTIFICATE

It is certified that the work described in this thesis entitled "**Characterization of candidate plus trees (CPTs) of *Pongamia pinnata* (L.), a versatile legume from North Guwahati, Assam**" by Vigya Kesari for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision in the Department of Biotechnology, Indian Institute of Technology Guwahati, India, and this work has not been submitted elsewhere for a degree.

Date: 04 May, 2010

Dr. Latha Rangan
Associate Professor
(Thesis Supervisor)
Department of Biotechnology
IIT Guwahati
Guwahati - 781 039, India

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Date: 04 May, 2010 **Vigya Kesari**

Abstract

Plant source for fuel that replaces fossil fuels is a topical subject and has gained prominence as ‘‘Biofuel crops’’. *Pongamia pinnata*, commercially important tree species used to produce biofuels, is known for its multipurpose benefits and its role in agro-forestry. *Pongamia* development as a multi-potent legume crop in IIT Guwahati in collaboration with Sila Forest Range, North Guwahati, Assam was attempted in the present study. The overall objectives of the thesis were divided into 6 distinct chapters that includes systematic candidate plus tree (CPT) identification, growing nursery, mass multiplication of identified CPTs using vegetative propagation and tissue culture approach, diversity evaluation among elite genotypes using SDS-PAGE, RAPD, ISSR and AFLP, collecting seeds from identified CPTs, extracting oil, examining medicinal and biodiesel aspects, morpho-biomolecular characterization of seeds by exploitation of advanced biotechnological tools and exploring symbiosis between *Rhizobium* and *Pongamia* by identification of novel *Rhizobium pongamiae* from root nodules CPT.

In first chapter, 10 CPTs belonging to populations 6 and 10 were identified based on morphological markers (vegetative and reproductive) using combined analysis over locations by CROPSTAT software. Identified CPTs were then multiplied using seed propagation technique in nursery bed. The best genotype identified was NGPP 46 with respect to pod-seed traits and total oil content. Hexane extraction yielded maximum oil content from seeds (33 %) compared to petroleum ether (30 %). When varying the seed to solvent ratio, no significant difference was noticed on the total oil yield for an individual tree, although the recovery of solvent and time taken for oil extraction reduced to significant

level at higher ratio. Identified CPTs can be further included in programmes aimed at genetic improvement of the species.

Second chapter has been divided into two sub-objectives. First one includes examining the amenability of vegetative propagation and effect of maturation in CPT, NGPP 46 through rooting of stem cuttings treated with varying concentrations and combinations of auxins. All auxin treatments promoted sprouting and at lower concentrations triggered/enhanced rooting of cuttings. The effectiveness was in the order of IBA>NAA>IAA when applied singly. IBA at 4.92 mM was found to be most effective as the rooting percentage and number of roots were significantly higher ($p<0.01$) than in control. However higher concentrations of auxins above 7 mM in general inhibited the rooting of cuttings. The interaction among auxins was found to be effective in root induction and differentiation and the most stimulating effects were observed in three-component mixture (IAA 1.42 mM + IBA 4.92 mM + NAA 1.34 mM). For further study mature stem cuttings of ten CPTs were subjected to the best responding auxins application to compare their adventitious rooting ability. Significant differences were observed for sprouting (26 - 86.67%) and rooting (56.67 - 93.33%) behaviour. Rooting after nine weeks was more than 96% from NGPP 26, 27 and 46, in IAA 1.42 mM + IBA 4.92 mM + NAA 1.34 mM and was approximately 4 times that of control. Auxin treatment increased number of roots (2.32) and root length (22.23 cm) but has negligible effect in the number of shoots per rooted cuttings. Cuttings harvested during January showed maximum rooting while the sprouting was better during October. Second sub-objective was *in vitro* propagation of *Pongamia pinnata* which includes two major aspects *i.e.* regeneration efficiency and genetic clonality. Woody Plant (WP) Medium and Murashige and Skoog's (MS) medium supplemented with different

concentrations and combinations of plant growth regulators were screened for high frequency regeneration using nodal segment culture and axenically grown seedlings of elite genotype of *P. pinnata*. Percentage response from mature nodal segments of *P. pinnata* were highly dependent on the season, with greater than 68% of culture developing adventitious shoots during spring. Woody Plant Medium supplemented with benzyladenine (22.2 µM) and kinetin (2.3 µM) gave the greatest response to initiation and multiplication. The multiplication rate of 11 shoots/explant with an average shoot length of 3.0 cm was observed. Multiplied shoots started to produce roots in the multiplication medium itself containing BA and NAA but subsequent establishment was poor. The rooting response was enhanced in ½-strength MS media with indole-3-butyric acid (2.5 µM). Rooted plants were hardened successfully in glass house with 70% survivability. RAPD and ISSR markers were employed to determine the genetic fidelity of *in vitro*-raised plantlets.

Third chapter assessed the genetic variability and relatedness among 10 CPTs of *P. pinnata* using bio-molecular markers. SDS-PAGE analysis revealed presence of 15-20 unique polypeptide fragments of molecular size ranged from 150 kDa to 14 kDa in immature and mature stages of seed development. SDS PAGE of total soluble seed storage proteins showed no variability among 10 CPTs at a particular stage of seed development. For molecular genetic investigations a modified protocol for isolation of genomic DNA using SDS extraction step, followed by precipitation and purification to remove polysaccharides, proteins and polyphenols which are abundant in storage tissues like seeds was standardized. Spectrophotometric and electrophoretic analysis indicated that the isolated DNA was highly pure and of high molecular weight amenable for PCR amplification and restriction endonucleases. Further, the application and informativeness of PCR based

molecular markers (RAPD, ISSR and AFLP) to assess the genetic variability and relatedness among 10 CPTs of *P. pinnata* were investigated. Polymorphism rate of 10.48, 10.08 and 100 % were achieved using 18 RAPD, 12 ISSR and 4 AFLP primer combinations respectively. . Polymorphic information content (PIC) varied in the range of 0.33-0.49, 0.18-0.49 and 0.26-0.34 and average marker index (MI) value obtained were 7.48, 6.69 and 30.75 for polymorphic RAPD, ISSR and AFLP markers respectively. Based on Nei's gene diversity and Shannon's information index, inter population diversity (h_{sp}) was highest as compared to intra population diversity (h_{pop}) and the gene flow (N_m) ranged from a moderate value of 0.607 to a high value of 6.287 for three DNA markers. Clustering of individuals was not similar when RAPD and ISSR derived dendrogram analysis were compared with AFLP. Mantel test cophenetic correlation coefficient was higher for AFLP (r=0.98) than that of ISSR (r=0.73) and RAPD (r=0.84). Molecular markers discriminated the individuals efficiently and generated a high similarity in dendrogram topologies derived using UPGMA, although some differences were observed. The 3-dimensional scaling by principal coordinate analysis (PCA) supports the result of clustering.

Fourth chapter has been divided into three sub-objectives. First sub-objective includes analysis of oil and antimicrobial activity from seed oil of CPT, NGPP 46. The highest oil yield (33%) from seeds was recovered in n-Hexane. Physico-chemical properties of crude oil of CPT established suitability for its use as a potential biofuel crop. The total monounsaturated fatty acid (oleic acid 46%) present in seed oil was more in comparison to polyunsaturated fatty acid (33%) as analyzed by GC-MS. Seed oil also showed inhibition against the tested fungal and bacterial cultures. However, the efficacy of antimicrobial activity of the seed oil at four concentration levels (50%, 80%, 90% and 100%) against

various pathogenic indicators was found to be concentration-dependent. The obtained results confirmed the use of seed oil from well characterized CPT of *Pongamia* as diesel fuel and in pharmaceuticals. Second sub-objective established characterization of seed development in CPT, NGPP 46 with respect to phenotypic traits, protein profile and storage reserve accumulation and utilization (SEM and TEM) during maturation and germination respectively. Seeds were collected starting from 90 days after flowering (DAF) and in an interval of 40-45 days thereafter till maturity (350 DAF). The polypeptide banding pattern during seven stages of maturation showed differences in expression pattern of three main polypeptide bands (MW 50 kDa, 18 kDa and 14 kDa). Globulins are the main seed storage proteins that includes legumins and vicilins. The developmental expression of seed protein exhibited inverse relationship for seven distinct stages of seed maturation with germination. In the third and final sub-objective of this chapter the temporal and spatial expression of desaturase and oleosin genes has been examined in different vegetative (leaf and root) and developing seed tissues by semi-quantitative RT-PCR. The temporal pattern of gene expression varied dramatically between the two classes of gene. Desaturase gene expression was relatively expressed in all stages of seed tissues whereas oleosin gene expression was detectable from stage IV (225 DAF). Of the three desaturase genes FAD2 and FAD3 showed similar, relatively uniform patterns of spatial expression in all the tissues. It is suggested that, in developing *P. pinnata* seeds, the accumulation of storage oil and oleosin is not concomitant but that the eventual deposition of oleosin onto the surfaces of storage oil bodies is essential for their stability during seed desiccation.

In chapter five, gram-negative, rod-shaped, non-spore forming fast growing aerobic bacterium (VKLR01^{T}) was isolated from the root nodules of CPT using YEM medium at 28

^{o}C and pH 7.0. On the basis of 16S rRNA gene sequence similarity, strain VKLR01^{T} was shown to belong to the class *Proteobacteria*, most closely related to *Rhizobium* sp. and *Agrobacterium tumefaciens* (98 % similarity to respective type strains). The strain grew well in the presence of 2 % NaCl, positive for oxidase, catalase and nitrate reduction text and produced acid during fermentation of sucrose, mannitol and lactose. The strain shares a unique CFA profile of predominately branched fatty acids (65.92 % $C_{18:1\ \omega7c}$, 5.67 % $C_{16:1\ iso1}$, 2.80 % $C_{13:\ 1}$, 2.41 % $C_{16:1\ \omega7c}$ /$C_{16:1\ \omega\ 6c}$, 0.52 % $C_{17:1\ \omega\ 8c}$) that supported further the affiliation of VKLR01^{T} to the genus *Rhizobium*. The DNA G+C content of the strain VKLR01^{T} was 59.1 mol% and the major quinone was Q-10. DNA-DNA hybridization with *Agrobacterium radiobacter* DSM 30147^{T} (relatedness of 51.9% with strain VKLR01^{T}) and physiological and biochemical tests allowed genotypic and phenotypic differentiation of strain VKLR01^{T} from described *Rhizobium* species with validly published names. Strain VKLR01^{T} therefore represents a novel species, for which the name *Rhizobium pongamiae* sp. nov. is proposed (type strain VKLR01^{T}= DSM ID No 09-1048)

Finally an attempt was made to renew and revive the interest on applied aspects of *Pongamia* in local communities through an awareness program. The objectives underlined in this study bring knowledge and information to the scientific community for further tree improvement and also stimulated forest officials of North Guwahati to undertake initiatives to enhance its cultivation in the marginal lands. Thus the present research provides the solid foundation to understand the economic, ecological value and association of the *Pongamia*. The findings can be helpful in efforts to improve *P. pinnata* for the desirable traits to make this species as an economically viable biodiesel crop.

Contents

Abbreviations

ACP	Acyl Carrier Protein
AFLP	Amplified Fragment Length Polymorphism
AOAC	Association of Official Agricultural Chemists
ASTM	American Society for Testing and Materials
ATP	Adenosine Triphosphate
BA	Benzylamino purine
BCKV	Bidhan Chandra Krishi Viswavidyalaya
CCS	Chaudhary Charan Singh Agricultural University
CD	Collar Diameter
cDNA	Complementary DNA
Cl	Chloride
cm	Centi meters
CPTs	Candidate Plus Trees
CRIDA	Central Research Institute for Dryland Agriculture
CSAUA&T	Chandra Shekhar Azad University of Agriculture & Technology
cst	centistroke
CTAB	Cetyl Trimethyl Ammonium Bromide
DAF	Days After Flowering
DBT	Department of Biotechnology
DMRT	Department of Medical and Research Technology
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribo Nucleic Acid
dNTPs	Deoxy Nucleotide Triphosphates
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DW	Dry Weight
EDTA	Ethylene Diamine Tetra-acetic Acid
EN	European Nation
EPS	Exopolysaccharide
EtBr	Ethidium Bromide
EtOH	Ethyl Alcohol
FAD	Fatty Acid Desaturase
g	Grams
GC	Gas Chromatography
GC-MS	Gas Chromatography Mass spectrophotometry
GITC	Guanidinium Isothiocyanate
GOI	Government of India
h	Hours

ha	Hectare
HAU	Haryana Agricultural University
HPLC	High performance liquid chromatography
IAA	Indole 3-acetic Acid
IBA	Indole 3-butryic acid
ICRISAT	International Crops Research Institute for Semi-arid Tropics
IGAU	Indira Gandhi Krishi Vishwavidyalaya
IIT	Indian Institute of Technology
ISSR	Inter Simple Sequence Repeat (ISSR) Markers
ISTA	International Seed Testing Association
JNKVV	Jawaharlal Nehru Krishi Vishwa Vidyalaya
Ka	Kanamycin
Kb	Kilo base
KCl	Potassium Chloride
kDa	Kilo Dalton
KH_2PO_4	Potassium Dihydrogen Phosphate
$KMnO_4$	Potassium Permanganate
m	Meter
M	Molar
MEGA	Molecular Evolutionary Genetics Analysis
$MgCl_2$	Magnesium Chloride
MIC	Minimum Inhibitory Concentration
min	Minutes
MJ kg^{-1}	Mega Joule per Kilogram
mL	Microliters
mM	Millimolar
Mn	Manganese
MPKV	Mahatma Phule Krishi Vidyapeeth
mRNA	Messenger RNA
MS	Murashige & Skoog Medium
MTCC	Microbial Type Culture Collection
NAA	α-Napthalene Acetic Acid
NaCl	Sodium Chloride
NAS	National Academy of Sciences
NBRI	National Botanical Research Institute
NCBI	National Centre for Biotechnology Information
NG	North Guwahati
NGO	Non Governmental Organization
NGPP	North Guwahati *Pongamia pinnata*
NOVOD	National Oil Seeds and Vegetable Oils Development
NRCAF	National Research Centre for Agroforestry

NTSYS	Numerical Taxonomy System of Multivariate Statistical Programs
OPA	Operon Primers
OsO_4	Osmium tetroxide
P	Phosphorous
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDKV	Dr.Panjabrao Deshmukh Krishi Vidyapeeth
PGRs	Plant Growth Regulators
Ph	Potentiometric Hydrogen Ion Concentration
PI	Principal Investigators
PIC	Polymorphic Information Content
PMSF	Phenyl Methane Sulfonyl Fluoride
ppm	Parts per million
QI	Quality Index
QTLs	Quantitative Trait Loci
R&D	Research & Development
RAPD	Random Amplification of Polymorphic DNA
RAU	Rajendra Agricultural University
RNA	Ribose Nucleic Acid
rRNA	Ribosomal RNA
RT-PCR	Real Time Polymerase Chain Reaction
S	Sulphur
SACPD	Stearoyl-acyl carrier protein desaturase
SAD	Stearoyl-ACP desaturase
SAS	Statistical Analysis System
SCAR	Sequence Characterized Amplified Region
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SE	Standard Error
SEM	Scanning Electron Microscopy
SPSS	Self-Propelled Semi-Submersible
SSR	Simple Sequence Repeats
TAGs	Triacylglycerols
TBE	Tris Borate EDTA
TBOs	Tree born Oil seeds
TEM	Transmission Electron Microscopy
TFRI	Tropical Forest Research Institute
TNAU	Tamil Nadu Agricultural University
Tris HCl	Tris Hydrochloride
TS	Transverse Section

TYE	Trypton yeast extract
UAS	University of Agricultural Sciences
UV	Ultra Violate
VI	Volume Index
w/v	Weight/Volume
WP	Woody plant
wt	Weight
YEM	Yeast Extract Monitol
µg	Micrograms
µM	Micro Meter
^{0}C	Degree Centigrade
2,4-D	2, 4-Dinitro-1-trifluoromethoxy-benzene

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Introduction

Introduction

Members of the Leguminosae, comprising about 19,000 species form the third largest plant family on earth and play an important role in maintaining our ecosystem. The importance of this family in the world economy needs no emphasis. Leguminous plants are very diverse in morphology, habitat, and ecology, ranging from Arctic annuals to tropical trees. They probably evolved approximately 60 million years ago, early in the tertiary period and their evolutionary success can be largely attributed to their ability to provide broad economic importance worldwide such as food, fuel, fodder and agricultural importance due to its symbiotic association with bacteria known as rhizobia.

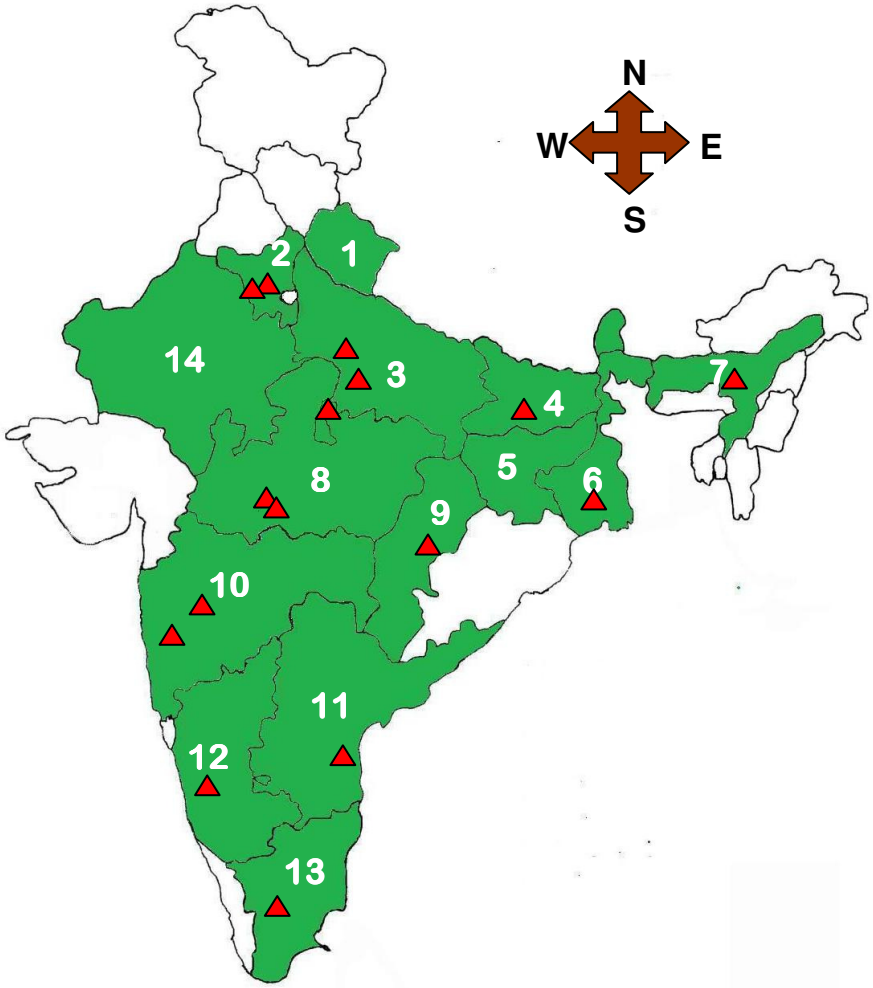
Improper land use and population pressure over several years has resulted in extensive degradation of agricultural land that has resulted in large tracts of wasteland in India (approximately 55 million ha). On the other hand, population explosion, greater urbanization and land reforms have resulted in the depletion of coal and natural resources. Increasing price of petroleum products and increasing concerns about oil production are likely to have serious implications for the automobile industry in the future. The answer to the above requirement is to search for an alternative to the fast depleting reserves of fossil fuel from renewable natural resources (Martini & Shell, 1998; Srivastava & Prasad, 2000). This has resulted in strengthening the research and development in the areas of biofuel. The current manufacturing cost of ethanol and biodiesel in India is about Rupees 21 /L, roughly the same as petrol and diesel (Nigam & Agarwal, 2004). This puts biofuels in a favourable position for meeting India's energy needs, especially as the cost of petroleum is expected to continue its upward trend. India is the fourth largest ethanol producer after Brazil, the United States and China, its average annual ethanol output amounting to 1,900 million litres with a distillation capacity of 2,900 million L/ year (Mittelbach & Remschmidt, 2004). For a 5 % ethanol blend in petrol nationally, the ethanol required would be 810 million litres in 2011-2012 (Garafalo, 2004). Current capacity can potentially satisfy this demand. However, the demand for diesel is five times higher than the demand for petrol in India. But while the ethanol industry is mature, the biodiesel industry is still in its infancy (Mandal, 2004). India's current biodiesel technology of choice is the transesterification of vegetable oil. The government has formulated

an ambitious National Biodiesel Mission to meet 20 % of the country's diesel requirements by 2011-2012. Since the demand for edible vegetable oil exceeds supply, the Government of India (GoI) has given a top priority for promotion and use of non-edible oils for production of biodiesel (Planning Commission, Government of India, 2003). Biodiesel has become more attractive because of its concerns from air pollution, global warming, other environmental and sustainability issues (MacLeana & Laveb, 2003; Ramadhas et al. 2004). Further emphasize is on use of forest and non-forest wastelands for plantation of non-edible oil seed bearing plants. Some of the plant species that produce oil seeds and can grow on marginal lands are getting special attention as biodiesel. *Pongamia* is one such species that falls into this category (Daniel, 2001). *Pongamia pinnata* (L.) Pierre (Synonyms *Derris indica* Lam., Bennet, *Pongamia glabra* Vent., & *Cytisus pinnaus* L.) tree belonging to family Fabaceae, sub family, Papilonaceae, popularly known as 'Karanj' or 'Karanja' in Hindi is known for its multipurpose benefits and as a potential source of biodiesel (Meera et al. 2003; Sharma & Singh, 2008).

Pongamia pinnata – Habit and habitat

Distribution and ecology

The natural distribution of *Pongamia* is along coasts and river banks in India and Burma. They are native to humid and subtropical environments including Asian subcontinent and lowlands in the Philippines, Malaysia, Australia, the Seychelles, the United States and Indonesia. Natural reproduction is by seed and common by root suckers (Duke, 1983). *Pongamia* tree is cultivated throughout India except temperate regions (Fig. 1). Large number of trees has been planted on roadside, railway tracks, canal banks and open farm lands during last two decades. *Pongamia* can grow on most soil types ranging from stony to sandy to clayey and grows to elevations of 1200 m (Daniel, 2001). The plant thrives well in areas having an annual rainfall from 500- 2500 mm, with the maximum temperature ranging from 27 to 38 °C and the minimum from 1 to 16 °C. Mature trees can also withstand water logging, are resistant to high winds, drought, and salinity but are susceptible to freezing temperatures (Gilman & Watson, 1994). The tree does not grow well on dry sands.



Promising states	
1. Uttaranchal	8. Madhya Pradesh
2. Haryana	9. Chattisgarh
3. Uttar Pradesh	10. Maharastra
4. Bihar	11. Andhra Pradesh
5. Jharkhand	12. Karnataka
6. West Bengal	13. Tamilnadu
7. Assam	14. Rajasthan

Research organizations (▲)	
▪CCS, HAU,	▪JNKVV, Jabalpur
▪RRS, HAU,	▪IGAU, Raipur
▪NBRI, Lucknow	▪MPKV, Rahuri
▪NRCAF, Jhansi	▪PDKV, Akola
▪CSUAT, Kanpur	▪IIT Guwahati
▪RAU, Samastipur	▪UAS, Dharwad
▪BCKV, Nadia	▪CRIDA, Hyderabad
▪TFRI, Jabalpur	▪TNAU, Mettupalayam

Figure I. *Pongamia* plantations in India.

Green shade indicates the states where *Pongamia* grows naturally and plantations are being carried out. Also indicated are the prominent research organizations engaged in plus tree identification and cultivation of *Pongamia* germplasm (*Source: NOVOD report*).

http://www.novodboard.com/Publications.htm; *http://biodiesel.nedfi.com/pages/introduction/objectives.php*

Pongamia is common along waterways or seashores, with its roots in fresh or salt water and grows well in full sun or partial shade. However maximum growth rates are observed on well drained soils with assured moisture.

Botany

Pongamia is a medium sized hardy tree that generally attains a height of about 8-10 m and a trunk diameter of more than 50 cm (Fig. 2A). The trunk is generally short with thick branches spreading into a dense hemispherical crown of dark green leaves. The bark is thin gray to grayish- brown, and yellow on the inside (Daniel, 2001). The taproot is thick and long extending 10 m into the ground to extract water from far beneath the ground surface for its need without competing with other crops. Therefore it flourishes in dry areas where agriculture is unproductive and in poor or saline soils. The alternate, compound pinnate leaves consist of 5 or 7 leaflets which are arranged in 2 or 3 pairs, and a single terminal leaflet. Leaflets are 5-10 cm long, 4-6 cm wide, and pointed at the tip. Buds and flowers are borne on raceme inflorescence (Fig. 2B & C). Flowers are pink, light purple, or white (Fig. 2D). Pods are elliptical, 3-6 cm long and 2-3 cm wide, thick walled, and usually contain a single seed rarely two, elliptical or reniform 1.7 -2.0 cm long and 1.2 -1.8 cm broad, wrinkled, with reddish brown leathery testa (Fig. 2E-I). Seeds are 10-20 cm long and light brown in color (Fig. 2J). The tree starts yielding at the age of 4-7 years (ICRISAT, 2007) and seed yield varies from 10-250 kg/tree. Regarding yield, there are varying estimates: 5-8 tonnes/ha and the oil productions are 1.5- 2.4 tonnes/ha, depending upon the soil and climatic conditions. The seeds are reported to contain on an average about 28 - 34 % oil with high percentage of polyunsaturated fatty acids (Sarma et al. 2005).

Applications

Pongamia pinnata a versatile oleaginous tree has many attributes and multiple applications. *Pongamia* has attracted the world's attention as a sustainable substitute for petroleum products and its ability to grow and reclaim marginal lands (Scott et al. 2008). The tree is also valued for its other beneficial effects in medicine (Brijesh et al. 2006; Kumar et al. 2007a).

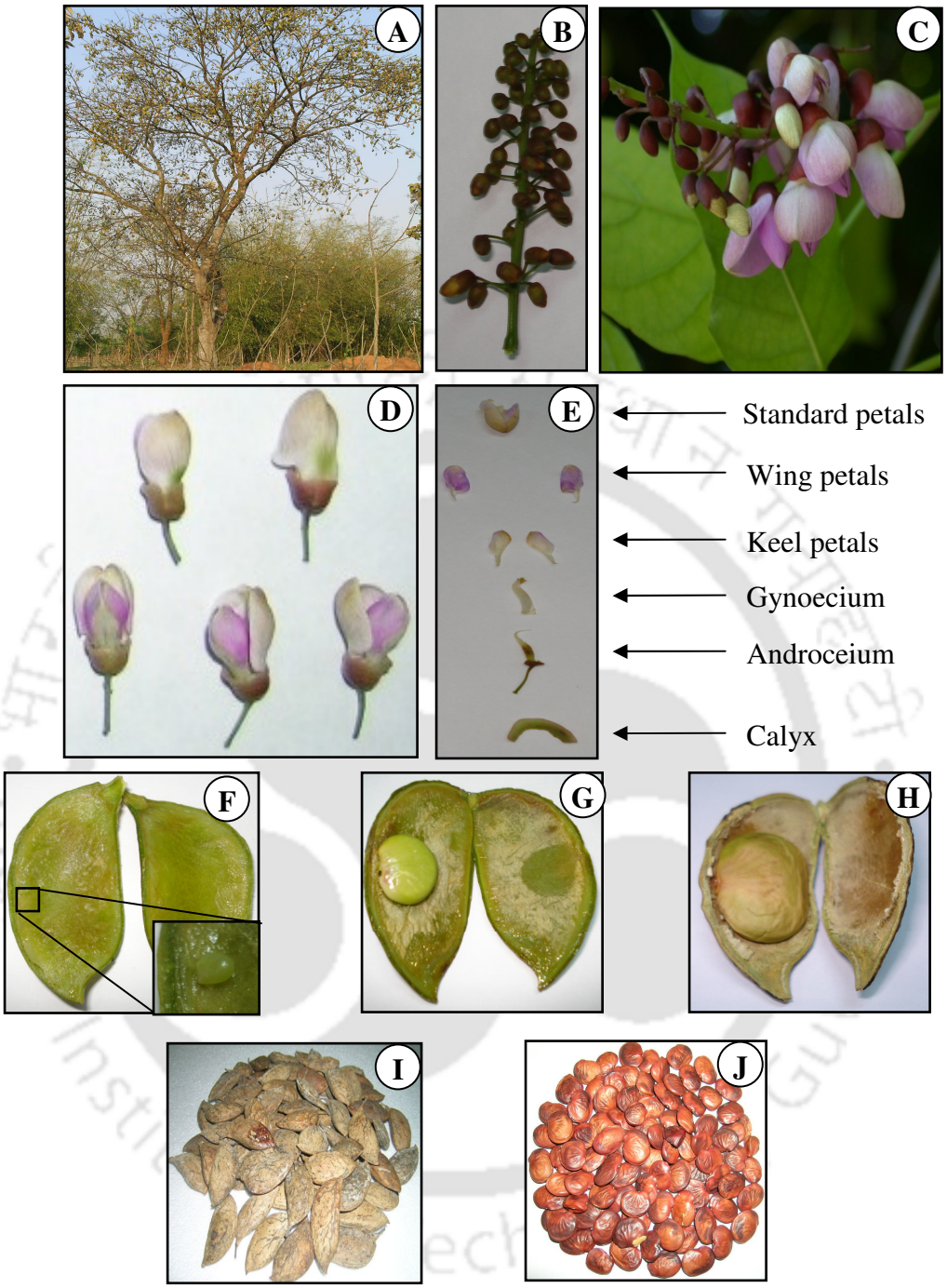


Figure II. *P. pinnata* habitat and habit.

A. tree occurring in North Guwahati, Assam, India; **B.** characteristic raceme inflorescence (buds); **C.** characteristic raceme inflorescence (flowers); **D.** individual flowers, flowering in *Pongamia* at Assam occur in May; **E.** standard petal, two identical wings and two identical keel petals; **F-H.** pod with growing seed; **I.** ripened pods, each pod weighs about 3-5 g; **J.** matured seeds; seed maturation takes roughly 9 months, each seed weighs about 1.5- 2.5 g.

It has been used as folk medicinal plant, particularly in Ayurvedha and Siddha systems of Indian medicine (Meera et al. 2003). All parts of the plant have been used as a crude drug for the treatment of tumours, piles, skin diseases, itches, abscess, painful rheumatic joints, wounds, ulcers, diarrhea etc. (Shoba & Thomas, 2001; Meera et al. 2003). More recently, the effectiveness of *Pongamia* as a source of biomedicines has been reported, specifically as antimicrobial and therapeutic agents targeting host pathways and processes (Brijesh et al. 2006). Besides, it is well known for its application as animal fodder, timber and fish poison (Cribb & Cribb, 1981; Bottoms, 2000). It has also been recognized to possess applications in agriculture and environmental management, with insecticidal and nematicidal activity (Chopade et al. 2008). The tree itself is nitrogen fixing and so can grow on waste land or unproductive land and is adaptable to wide agro-climatic conditions (Scott et al. 2008; Uddin et al. 2009). The seeds or nuts of the tree are its most useful product. The non-edible oil extracted from seeds used as fuel, lubricant and in soap making. The cake is non-edible; it can be used as organic fertilizer and is rich in nitrogen and micronutrients. It can also play a role in rural economy by generating huge manpower employment during various stages of its cultivation as well as during downstream processing (Shrinivasa, 2001).

Present research in *Pongamia*

Previous scientific information available about leguminous tree *P. pinnata* mainly describes its use in agroforestry and medicine. Research on this crop has greatly expanded during the last 19th century and has continued this trend at an accelerated pace ever since its role as a feedstock for biodiesel production was defined and confirmed (Shrinvasa, 2001; Vivek & Gupta, 2004; Sharma & Singh, 2008; Mukta et al. 2009). It can play a role in rural economy by generating huge manpower employment both during various stages of its cultivation as well as during downstream processing (Shrinivasa, 2001).

In spite of these numerous favorable attributes, even half the complete potential of *P. pinnata* is yet to be realized. Selection for economically important traits such as high seed yield, high oil content and desirable fatty acid composition is the prerequisite to make this species as an economically viable biodiesel crop. Knowledge of genetic variation in seed morphology and oil content will be of great potential in tree improvement programs.

Unfortunately not much work has been done systematically on germplasm conservation and utilization. Therefore, the present study was undertaken for the identification of CPTs, standardizing propagation techniques and assessment of the genetic stability of the micro-propagated plants through RAPD markers for production of elite varieties of clones throughout the year for afforestation programs. Genetic diversity studies among CPTs needs to be done since that could be the only starting point for breeding purpose and genetic improvement of the species having high seed yield to tease out for its oil content and quality, the most important aspect for biodiesel. The very fact that *Pongamia* has adapted itself to a wide range of edaphic and ecological conditions suggests that there exists considerable amount of genetic variability to be exploited for potential realization. Conventional taxonomic techniques in conjunction with molecular biology tools may go a long way in providing accurate and powerful way of analyzing genetic relationship in the genus *Pongamia*. Though a few studies on morphological based characterization of *Pongamia* species have been attempted (Kaushik et al. 2007), not much has been done on molecular characterization. Genetic fingerprinting studies will also provide the scope to pursue genomics research in *P. pinnata* that are almost neglected in this versatile oleaginous legume.

Although chemical composition and bioassay of the seed oil of this plant has been previously reported separately (Meera et al. 2003; Scott et al. 2008), but there has been no systematic study or concerted efforts to characterize the oil content from the elite genotype and its efficacy against range of microorganisms acting as pathogenic indicators. In current study best reporting stable genotype CPT of *P. pinnata* was progressed further with the following objectives: (i) extraction and quantification of the total oil yield from the seeds using different solvents and their azeotropic mixtures (ii) to analyze and compare the physico-chemical properties of the seed oil of n-Hexane and ethyl acetate fraction (iii) to determine the fatty acid profile of the fraction yielding maximum oil and, (iv) to investigate the antimicrobial activity of the seed oil against referential bacterial and fungal strains. *Pongamia* seeds contains on an average of 30-40 % protein and 32-39 % oil (NOVOD, 2009), but both genetic and environmental factors can strongly affect the seed composition. *Pongamia* proteins are a complex polymorphic mixture of polypeptides, and like many

species of the Leguminosae, it may contain four important groups of proteins: the enzymes involved in metabolism, structural proteins including both ribosomal and chromosomal, membrane proteins and the storage proteins. Almost no information is currently available about the different aspects of seed development in *Pongamia*. The seed maturing process represents unique events during its development and is the result of a series of biochemical and physiological changes. In addition, localization of lipid bodies at different developmental stages within the oilseeds using microscopic technique will provide a tool for the identification of appropriate targets for biotechnological engineering of seed oils and lipid biosynthesis in near future in *Pongamia*. This challenging task will require tools in the fields of classical and modern biotechnology that can be used to elucidate the traits of significance. Semi-quantitative analysis by RT-PCR and electrophoretic pattern of polypeptides of different floral organ and seeds of different developmental stages would give information regarding the gene predominance in organ that will bring valuable references for further research on seeds of *Pongamia*.

The present research also investigated the rhizobium that is symbiotically associated with *P. pinnata*. Rhizobia are a diverse group of bacteria that live in symbiosis with legumes in root nodules (Ahmad et al. 1984). Due to the ability of rhizobia to fix atmospheric nitrogen these trees are particularly considered in afforestation and soil erosion control programs. The objective of this research therefore was also to determine the identity of the rhizobia species nodulating the roots of native *P. pinnata* and its further characterization based on its phenotypic, biochemical and molecular studies.

Unless the value of plant genetic resources is known among local communities there may not be much of interest in management. Therefore value addition to a plant thereby strengthening the livelihoods of the local communities would be an effective mode for propagation and management of forest resources. In the current study an attempt was made to renew and revive the interest in local communities, holder of traditional knowledge through means of awareness program on various applied aspects of *Pongamia* and its significance.

Specific objectives

Based on an extensive literature survey on availability and versatile applicability of *Pongamia pinnata* in India, the present study was focused on the identification of candidate plus trees (CPTs) from North Guwahati, Assam and its further multipurpose characterization to make it a potential biofuel crop termed "Indian Tree of Biofuel". In order to establish its potential applications in industries as a sustainable energy source, for cleaning environment, medicinal plant and reclamation of waste lands following were the detailed investigations performed in the present research:

1. Systematic germplasm collection and evaluation for the identification of CPTs of *Pongamia pinnata* from study site North Guwahati, Assam.
2. Standardization of micropropagation protocols in combination with conventional techniques to raise its large scale cultivation in the wasteland.
3. Standardization of modified DNA isolation protocol and characterisation of CPTs of *P. pinnata* for its genetic variability.
4. Total seed oil extraction and physicochemical analysis with regard to its biodiesel potentiality.
5. Antimicrobial assay of crude seed extracts for its bio-efficacy.
6. Study the variation in protein profiling pattern at different stages of seeds development, germination and in different organs such as leaves, flowers, roots and pods of tagged CPT.
7. Identification, localization and distribution pattern of storage reserves (i.e. protein carbohydrate and lipids) by using electron microscopic techniques (SEM and TEM) in seeds at different stages of sexual seed development and germination to focus on the synthesis of the reserves from the first signals of cell deposition up to seed maturity in identified elite genotype.
8. Transcript analysis of fatty acid genes involved in triacylglycerol biosynthesis during developmental stages of sexual seed development by semi-quantitative transcript profiling.
9. Characterization of root nodulating *Rhizobium* species for its symbiotic association with *Pongamia*.
10. Awareness and extension activities for dissemination of information.

Significance

1. Studies in systematic characterization of *Pongamia pinnata*, a multipurpose oleaginous legume tree species has significant economic, ecological and environmental benefits.
2. Immense potential lies for improvement of productivity by selection of appropriate elite genotypes based on visible markers (morphological and reproductive) that could be the starting point for breeding purpose having high seed yield to tease out for its oil content and quality, the most important aspect for biodiesel.
3. Micropropagation will speed up mass multiplication and generation of superior true-to-type planting material, thus can complement the ongoing activities of conventional propagation to raise its cultivation in the wasteland. *In vitro* studies will also help in further studies with regard to genetic engineering and thus genetic improvement of the biodiesel crops for the desired traits.
4. Exploration of genetic variation in plant population, seed morphology and oil content of 10 identified CPTs of *P. pinnata* will enable to understand, and hence, better management and subsequent utilization for tree improvement programmes.
5. SDS-PAGE of different floral organ and seeds of different developmental stages would give information regarding protein predominance in organs and pattern of seeds maturation that will give an impetus to further research on seeds of *Pongamia*.
6. Semi-quantitative analysis by RT-PCR will enable to know the genes involved in fatty acid metabolism. It will also shed light on regulatory pattern as studies were performed during different developmental stages of seed maturation in the present work.
7. Localization of seed storage reserves at different developmental stages in oilseeds using microscopic technique will provide a tool for the identification of appropriate targets for biotechnological engineering of seed oils and lipid biosynthesis in near future.
8. An additional benefit of *P. pinnata* than other biodiesel plants such as *Jatropha curcas*, *Azadirachta indica*, *Mesua ferrea* etc. is its involvement in the reclamation of marginal lands due to its symbiotic association with *Rhizobium* species. Thus *P. pinnata* will provide not only alternative renewable energy source but could be a significant plant for developing countries to improve the fertility and productivity of virgin soils.


भारतीय प्रौद्योगिकी संस्थान गुवाहाटी
Indian Institute of Technology Guwahati

Chapter 1

Chapter 1

Identification of candidate plus trees (CPTs) based on morphometric traits and seed oil in *Pongamia pinnata*

1.1 Introduction

To increase the biodiesel production it's important to have an elite genotype of *P. pinnata* bearing high oil-yielding seeds. The candidate plus tree (CPT) is an individual tree of *P. pinnata* possessing superior morphological characters (vegetative and reproductive characters) than other individuals of the same species.

The ideal tree in a tree improvement/breeding programme is the high seed yielding plants that are resistant to diseases and pests. Selection of the best phenotypes leads to improved seed production of desirable traits. *Pongamia* are well adapted to harsh weather conditions though there is a need to domesticate them for cultivation under different production systems on degraded lands and community wastelands. Oil content and seed yield in *Pongamia* vary considerably and can be attributed to a number of factors such as genotype, climatic conditions and soil fertility. Thus, its growth and productivity depends on the availability of geographical and edaphic factors. Variation in seed morphology and oil content of *P. pinnata* is of great potential in tree improvement programs. Assessment of these resources for seed yield and oil content has not been done yet. Since the species has always been treated as avenue tree, efforts towards domestication through identification of elite genotypes have been very limited. Selection for economically important traits such as high seed yield, high oil content and desirable fatty acid composition is the prerequisite to make this species as an economically viable biodiesel crop.

So far, no concerted efforts have been made in promotion of *Pongamia* which is well acclimatized to various agro-climatic conditions of the country. In other words, no work has been done on elite genotype identification, germplasm conservation and utilization in

Pongamia. The first step in the plus tree selection is to state the selection criteria. Once the selection criteria have been clarified, the next procedure is to choose a proper system of selection. The selection is usually based on a set of criteria rather than one single criterion. In current chapter superior trees were selected for various morphological (vegetative and reproductive) markers using a combined analysis across populations for two consecutive seasons. The selected trees were multiplied using seed propagation technique. In the second stage of analysis, CPTs were assessed for seed and pod traits, the two most important aspects with regard to oil. Study was also undertaken to extract the oil with different solvents from the seed and to consider the factors influencing the rate of extraction.

1.2 Review of Literature

In this chapter literature on plus tree selection programme in *P. pinnata* or other economically important woody tree species has been reviewed to gather the information available that can form the guidelines to initiate tree improvement programme in *Pongamia*. Conventional tree breeding consists of the following two steps: phenotypic selection of individual plus tree followed by establishment of selected plus trees in the seed orchards for production of improved seeds which can be used for high-quality plantation (FAO, 1985; Zobel & Talbert, 1984; Edwards, 1999). Identifications of woody tree species based on vegetative characters is a common practice to distinguish between tree families, genera or species as reproductive traits are expressed late in its life cycle (Hargreaves, 2006).

Characterization and selection of CPT's is essential for the improvement of *P. pinnata* in addition to experiments on controlled crossing among selected genotypes (Mukta & Sreevalli, 2010). Selection of best planting stock in *Pongamia* was attempted from seven biogeoclimatic zones in Tamil Nadu, India (Kumaran & Surendran, 1999). Seed was collected from 28 single-parent families and germinated. Six-month-old seedlings were evaluated for stock quality and significant variation was recorded for morphological parameters viz. height, root length, root collar diameter, number of leaves, number of nodules as well as stem, leaf and root dry weight. Three new ratios, sturdiness quotient (SQ), volume index (VI) and quality index (QI), were calculated based on seedling height (H), root collar diameter (CD) and dry weights (DW) as further tests of seedling quality, where

SQ = H/CD, VI = CDXH, and QI =DW/(H/CD +DW/DW). There was significant variation among the 28 single-parent families for these three ratios. A lower SQ and higher VI and QI revealed the best quality of the seedling to be planted out. The single-parent family 3 (Anaikatty, Coimbatore) recorded the lowest value (3.50) for SQ and a minimum QI of 1.03 and also the second highest value for VI (361.81), making it the best planting stock among the 28 families tested. Manjare et al. (2003) surveyed 10 districts of Maharashtra, India and studied 216 trees based on seed productivity and pest resistance. Among these, 13 plus trees were identified for high oil content (>40 %). Highest seed oil content of 49.8 % was reported. Kaushik et al. (2007) identified 40 CPTs of *P. pinnata* based on the morphometric and qualitative traits from different locations of Haryana state as a starting material for production of quality seedlings for mass afforestation in different forestry and agroforestry programmes. The study was focused on pod-seed traits and high oil content from the selected 40 CPTs which revealed promise in their further exploitation for plantation and selection for improvement. Authors also observed variability in oil content from 32.6 to 44 % among the selected CPTs. In another such similar studies for 75 germplasm accessions of *P. pinnata* collected from Andhra Pradesh, oil content was more than 40 % in 15 % of the accessions with highest value of 46 % similarly seeds collected from six locations within three agroclimatic zones of neighbouring state, Tamil Nadu the oil content varied from 26 to 38.2 % (Kumar et al. 2003).

Under the National Network on Integrated Development of *P. pinnata* in India, the survey of potential areas and identification of CPTs was undertaken by 10 participating centres in the States of Andhra Pradesh, Tamil Nadu, Chhattisgarh, Madhya Pradesh, Maharashtra, Uttar Pradesh, Rajasthan and Karnataka. After selection, seeds/cuttings were collected from these sources. The seeds were characterized for their morphological features like seed length, diameter, test weight and oil content (%). The numbers of CPTs identified by various research organizations in respect of Karanja are mentioned in Table 1.1.

Selection of plus trees based on morphometric traits have been reported earlier in tree and other biofuel crops. Mishra (2009) identified candidate plus phenotypes of *Jatropha curcas* L., a potential biodiesel plant using method of paired comparisons. Sharma et al. (2001) reported 40 CPTs of *Pinus roxburghii* from different parts of Garhwal Himalaya in

India. The improvement in germination and seedling growth through seed size manipulation (seed weight and oil content) has been reported in other oil yielding trees aiming at agro forestry programmes (Ponnammal et al. 1993). In A*zadirachta indica* A. Juss. (Neem) seed morphology (seed length and 20 seed weight) and oil content were reported from five provenances of northern and western India to identify ecotypes with high oil yield that could be selected for mass multiplication and subsequent incorporation into tree improvement programs (Kaur et al. 1998).

Table 1.1. The numbers of CPTs of *P. pinnata* identified by various research organizations.

Sl. No.	Name of Research Institutes	No. of CPTs /seed source identified	Oil content range (%)
1	MPKV, Rahuri,	261	33.32-42.60
2	NRCAF, Jhansi	52	30.28-41.41
3	UAS, Dharwad	10	28.98-42.79
4	TFRI, Jabalpur	68	24.42-49.32
5	BCKV, Nadia (W.B.)	15	---
6.	RAU, Samastipur, Bihar	25	---
7.	JNKVV, Jabalpur	62	28.11-41.54
8.	TNAU, Mettupalayam	65	26.28-33.77
9.	CSAUA&T, Kanpur	45	29.70-39.34
10	CCS, HAU, Hisar	41	26.35-37.17
11.	RRS, HAU, Bawal	39	32.57-44.07
12.	NBRI, Lucknow	16	---
13.	CRIDA, Hyderabad	139	24.83-45.08
14.	PDKV, Akola	53	28.29-46.42
15.	IGAU, Raipur	18	34.13-43.01
	Total	938	---

Source: 4th NOVOD REPORT (2009)

1.3 Methods

1.3.1 Selection of CPTs

Ten locations with 5 random trees from each locations were scored for various morphological and reproductive characters viz; girth; height; number of leaves/g weight; seed germination (%); number of buds/inflorescence; number of flowers/inflorescence and number of seeds/inflorescence for two consecutive seasons in the month of April-June (2006-2007) from North Guwahati. Geographical features and location of the populations studied is marked in Table 1.2 & Fig. 1.1. Trees grown in one location is considered to be a population. To predict the performance of the candidate trees with respect to the traits studied, a combined analysis across populations (locations) was conducted using CROPSTAT for Windows Version 6.1. (ftp://ftp.cgiar.org/icis/cropstat/).

Table 1.2. Geomorphological features of the study site, North Guwahati, Assam.

Characteristics	North Guwahati
State	State of Assam, North Eastern region of India
Latitude and longitude	26 ° 14' 6" N & 91 ° 41' 28" E
Area	Sila Forest Range, North Guwahati
pH	5.0-7.5
Number of rainy days	45-60 days
Annual rainfall	152 to 324 cm, with fairly heavy rainfall during May-Oct.
Temperature	Fluctuates between 10 ˚C to 38 ˚C with maximum summer temperature between 30 ˚C-38 ˚C min. winter temperatures between 17 ˚C to 10 ˚C.

Two stage analyses were performed on populations of *Pongamia*. In the first stage, best populations were chosen based on the morphological and reproductive traits. The following linear model across populations was used for each season.

$$y_{ij} = \mu + p_i + e_{ij}$$

y_{ij} is the response from the j^{th} replicate of i^{th} population (location), μ is the over all mean, p_i is the effect of the i^{th} population and e_{ij} is the error. A critical minimum value, which constitutes the average of all populations, was given for each trait. Individuals performing

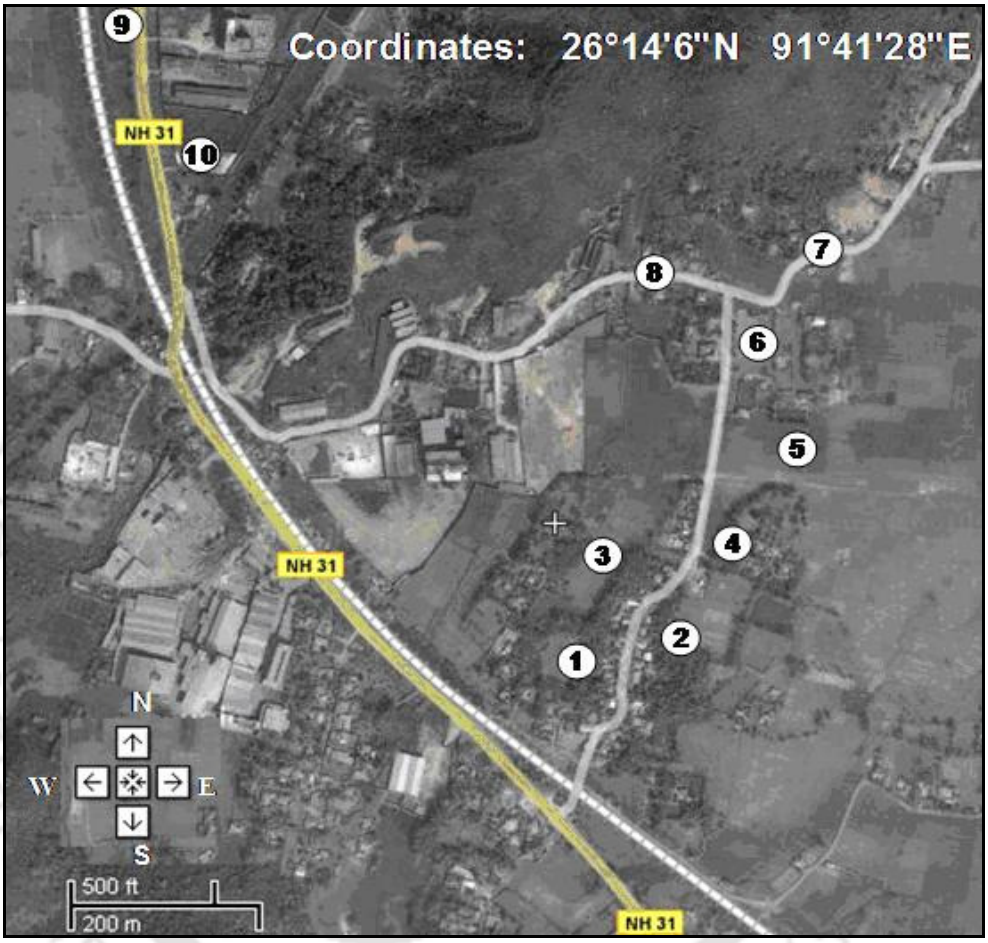


Figure 1.1. Geographic map locations of *Pongamia* populations used in the current study. Each population is indicated by the circle. Letter inside marker indicates population group.

above average for 75 % of traits were tagged as the CPTs. Table 1.3 gives the selection criteria for CPT identification. The populations were the performance was above than the mean (highlighted in bold face) using the model were tagged as best population. In the second stage, the trees from the chosen populations were evaluated. A total of 75 pods (three replications) of each CPT (the trees from each chosen population), were taken and average was computed for the pod and seed characters. Analysis of variance was carried out using the following model.

$$y_{jk} = \mu + t_j + r_k(t_j) + e_{jk} \qquad j = 1\ldots10,\ k = 1\ldots3$$

j indexes the trees and k the replicates within trees. y_{jk} is the response from the k^{th} replicate of the j^{th} tree, μ is the over all mean, t_j is the effect of the j^{th} tree, $r_k(t_j)$ is the effect of the k^{th} replicate within the j^{th} tree and e_{jk} is the error.

1.3.2 Clonal seed propagation

Mature seeds from the CPTs identified based on characters indicated in Table 1.3 & 1.4 were used for clonal seed propagation. Seeds were rinsed in EtOH (25 %) solution for 10 minutes and washed with distilled water 2-3 times before being placed in polythene bags containing sand and clay at the ratio of 1:4 in mist chamber at 28±2 ^{o}C with a relative humidity of 70-80 % respectively. Germination percentages were recorded according to the ISTA (2003) guidelines. After 2 months successfully established saplings were kept under shady areas in nursery (Sila Forest Range, NG) for hardening and subsequently field transferred.

1.3.3 Extraction of oil

Mature ripened seeds of identified CPT (based on growth and number of seeds) were collected during the month of April-June from the study site. Care was taken to collect the dry seeds directly from marked trees. The seeds were cleaned and dried in an oven at 50 ^{o}C for 48 h. Dried seeds were weighed and grounded manually. The powdered seeds (sufficient

for three extractions to be taken from each sample, each extraction being 100 g of powdered material) were packed in polythene bags and stored at 4 ^{o}C until further analysis. The total oil from the seeds was extracted following the soxhlet extraction method as per the standard AOAC (American Oil Chemical Society) procedure (1995) using n-Hexane as a solvent. Hot water extraction was also used for extraction of the oil where 100 g powdered seed was immersed in n-Hexane, agitated gently in a conical flask for 24 h. The residue was allowed to settle and supernatant was decanted, distilled at 65 ^{o}C to remove the solvent completely. The final volume of the oil was measured (% v/w) and also the final weight of the solid material left was noted. The experiments were repeated thrice and the mean values calculated for individual CPTs**.**

1.4 Results and discussion

1.4.1 Selection of CPTs based on vegetative and reproductive traits

Table 1.3 describes the critical minimum value (mean performance of the populations as per the linear model mentioned in M & M for various vegetative and reproductive traits from different populations of *Pongamia* tagged from the study site. The trees, which were performing above this average value for 75 % of the characteristics in both seasons, were the CPTs. Thus here the candidate plus tree is an individual tree of *P. pinnata* possessing superior morphological and reproductive characters than other individual of the same species. The data comprises morphological and reproductive traits measured on 50 trees and each of the 50 trees is a different genotype. There are 10 locations and 5 trees were scored from each location and the best locations (populations) with respect to the measured trait were chosen at the first stage. Similar studies were performed by Rao et al. (2001) for genetic enhancements from natural populations of mangrove trees growing in Pitchavaram, India. Thus ten CPTs were identified based on the morphological markers; NGPP 26, NGPP 27, NGPP 28, NGPP 29, NGPP 30, NGPP 46, NGPP 47, NGPP 48, NGPP 49 and NGPP 50 the first five belonging to population 6 and the next five belonging to population 10 respectively (Fig. 1.2). Full data could not be generated for three trees (NGPP 41, NGPP 42 and NGPP 43) of population 9 as they were cut due to its approachment on National Highways. Percentage conversion of seeds from flowers were also recorded that varied from 12 % (NGPP 21) to 59 % (NGPP 27).

Figure 1.2. Candidate plus trees of *P. pinnata* from North Guwahati

A. NGPP 26; **B.** NGPP 28; **C.** NGPP 30; **D.** NGPP 47; **E.** NGPP 46; **F.** NGPP 49.

Table 1.3. Identification of CPTs based on morphological and reproductive characters in *P. pinnata*

Population[a]	Morphological Character[b]								Reproductive Character[b]					
	Girth (m)		Height (m)		No. of leaves/ g weight		Seed germination (%)		No. of buds/ inflorescence		No. of flowers/ inflorescence		No. of seeds/ inflorescence	
	S1	*S2*	*S1*	*S 2*	*S 1*	*S 2*	*S 1*	*S 2*	*S 1*	*S 2*	*S 1*	*S 2*	*S 1*	*S 2*
P1	0.5	0.5	5.6	6.0	1.5	1.6	75.4	71.0	48.8	47.5	33.8	34.2	7.7	8.0
P2	0.7	0.7	7.5	7.9	2.0	2.1	76.4	72.0	48.0	46.7	32.5	32.9	8.0	8.4
P3	0.6	0.7	6.1	6.5	1.8	1.9	70.0	74.8	47.9	46.6	31.8	32.2	7.7	8.1
P4	0.6	0.7	6.9	7.3	1.6	1.7	60.1	65.1	51.6	50.2	32.6	33.0	7.2	7.6
P5	0.6	0.7	6.5	6.9	1.8	1.9	70.5	76.3	52.8	51.4	30.7	31.1	5.3	5.6
P6	**1.2**	**1.3**	**9.1**	**9.7**	1.7	1.8	**71.8**	**74.6**	**54.6**	**53.0**	**35.5**	**36.0**	**7.9**	**8.3**
P7	0.6	0.7	6.4	6.8	1.6	1.7	72.6	69.8	52.8	51.4	30.8	31.1	6.6	7.0
P8	0.6	0.7	4.4	4.7	1.6	1.7	69.8	67.0	49.7	48.3	28.7	29.0	6.2	6.5
P9	1.2	1.0	5.8	5.5	1.5	1.6	72.9	70.1	52.4	50.9	35.8	36.2	6.0	6.3
P10	**0.9**	**0.9**	**5.3**	**5.7**	1.7	**1.9**	**72.2**	**74.4**	**55.6**	**54.0**	**34.3**	**34.7**	**7.2**	**7.6**
Mean[b]	0.8	0.8	6.4	6.7	1.7	1.8	71.2	71.5	51.4	50.0	32.7	33.0	7.0	7.3
SE	0.14	0.14	0.66	0.72	0.15	0.16	5.73	6.01	5.09	4.95	4.20	4.25	1.05	1.11
d.f.	40	37	40	37	40	37	37	37	37	37	37	37	37	37
LSD (5 %)	0.40	0.41	1.89	0.72	0.43	0.16	16.40	6.01	14.58	4.95	12.04	4.25	3.01	1.11

[a] Each population consists of 5 genotypes. A population comprises of trees from a location.

[b] Mean of the predicted values for each trait across populations.

S 1 and *S 2* represents two seasons.

1.4.2 Pod and seed traits in CPTs

The study of pod and seed characters with oil content of the natural populations is often considered to be useful step in the study of the genetic variability (Kaushik et al. 2007). Mean performance of CPTs variation for pod and seed traits is shown in Table 1.4. Analysis of variance indicated significant differences ($p<0.05$) among the CPT for all pod and seed characters. Maximum pod length (5.41 cm) was observed in NGPP 26 and NGPP 27 and minimum (4.88 cm) in NGPP 49. Pod thickness also varied significantly ($p<0.05$) among all the CPTs. Maximum seed length (2.35 cm) was observed in NGPP 46 and minimum (2.00

cm) in NGPP 47. Hundred pod and seed weights were also determined for this CPTs. Hundred-pod weight varied from 329.36 g in NGPP 26 to 440.78 g in NGPP 46. The seed-pod ratio varied from 0.5 to 0.58. CPT, NGPP 46 was found to be the best on the basis of pod-seed characters, which reveal its promise in further exploitation for plantation and selection for improvement. Considerable differences and significant correlation has been reported in pod-seed morphological characters among various seed sources of *Pongamia* CPTs (Kumaran, 1991; Kaushik et al. 2007). The identification of an association between pod and seed traits could provide a significant contribution to the genetic improvement of this species. Further improvement in germination and seedling growth through seed size manipulation (seed weight and oil content) has been reported in oil yielding trees aiming at agro forestry programmes (Ponnammal et al. 1993; Kaushik et al. 2003).

Table 1.4. Mean performance for pod and seed traits in identified CPT of *P. pinnata*

Genotype	Pod Characters[b]			Seed Characters[b]		
	Breadth (cm)	Length (cm)	Thickness (cm)	Breadth (cm)	Length (cm)	Thickness (cm)
NGPP 26	2.47	5.41	1.29	1.73	2.02	0.86
NGPP 27	2.48	5.41	1.28	1.70	2.23	0.99
NGPP 28	2.52	5.26	1.30	1.76	2.30	0.95
NGPP 29	2.50	5.26	1.29	1.76	2.29	0.96
NGPP 30	2.55	5.32	1.30	1.64	2.06	0.95
NGPP 46	**2.83**	**5.40**	**1.27**	**2.05**	**2.35**	**0.98**
NGPP 47	2.45	5.39	1.29	1.72	2.00	0.86
NGPP 48	2.48	5.38	1.28	1.70	2.26	0.98
NGPP 49	2.46	4.88	1.22	1.64	2.16	0.90
NGPP 50	2.57	5.38	1.31	1.66	2.04	0.95
SE[e]	0.013	0.047	0.008	0.017	0.029	0.009
5 % LSD 18d.f.	0.039	0.14	0.022	0.05	0.09	0.03

[e] SE is the standard error

[b]Three replications from each CPT and 25 pods in each replicate were computed for the pod and seed characters.

1.4.3 Clonal seed multiplication of CPTs

Identified CPTs were used further for clonal seed propagation and oil analysis. Seeds germinated within 10 days in all CPTs. Pre soaking in water overnight was found to enhance the rate of germination. Percentage of germination varied from 46 % (NGPP 17) - 95 % (NGPP 46). It was interesting to note that the young germinated seedling showed a typical pattern of phyllogenesis; that is production of compound leaf formation preceded by simple leaf production. The typical pattern of phyllogenesis in the neonatal and regenerating *P. glabra* was reported by Subbuswamy et al. (1978). Germinated saplings reached a height 45 cm within two months of growth and were transferred to forest area for successful establishment (Fig. 1.3). The area chosen for transfer of young saplings of CPTs of *Pongamia* were the marginal wastelands in North Guwahati. Experiments are underway to determine the genetic gain through the first cycle of selection from the seed orchards developed from the seeds of CPTs of this species.

1.4.4 Oil extraction and yield

Genotypes with high oil content need to be selected so that a high seed yield really translates into high oil production. Thus the right choice of oil extraction technique is very important process for the biodiesel production. Seed oil extracted from CPTs varied in yields using different extraction procedure. As shown in Table 1.5 Soxhlet extraction yielded best results with maximum yield being closer to 33 % using n-Hexane. The values expressed as % v/wt in Table 1.5 are the mean values of experiment repeated thrice per CPT. Advantage of solvent extraction technique is that the solvent can be recovered during the process and is easy for implementation. Solvent extraction technique has been employed in many potential biodiesel crops (Akintayo, 2004; Kaushik et al. 2007; Shrivastava, 2006). n-Hexane extraction from the NGPP 46 yielded the best results and was subjected to further studies.

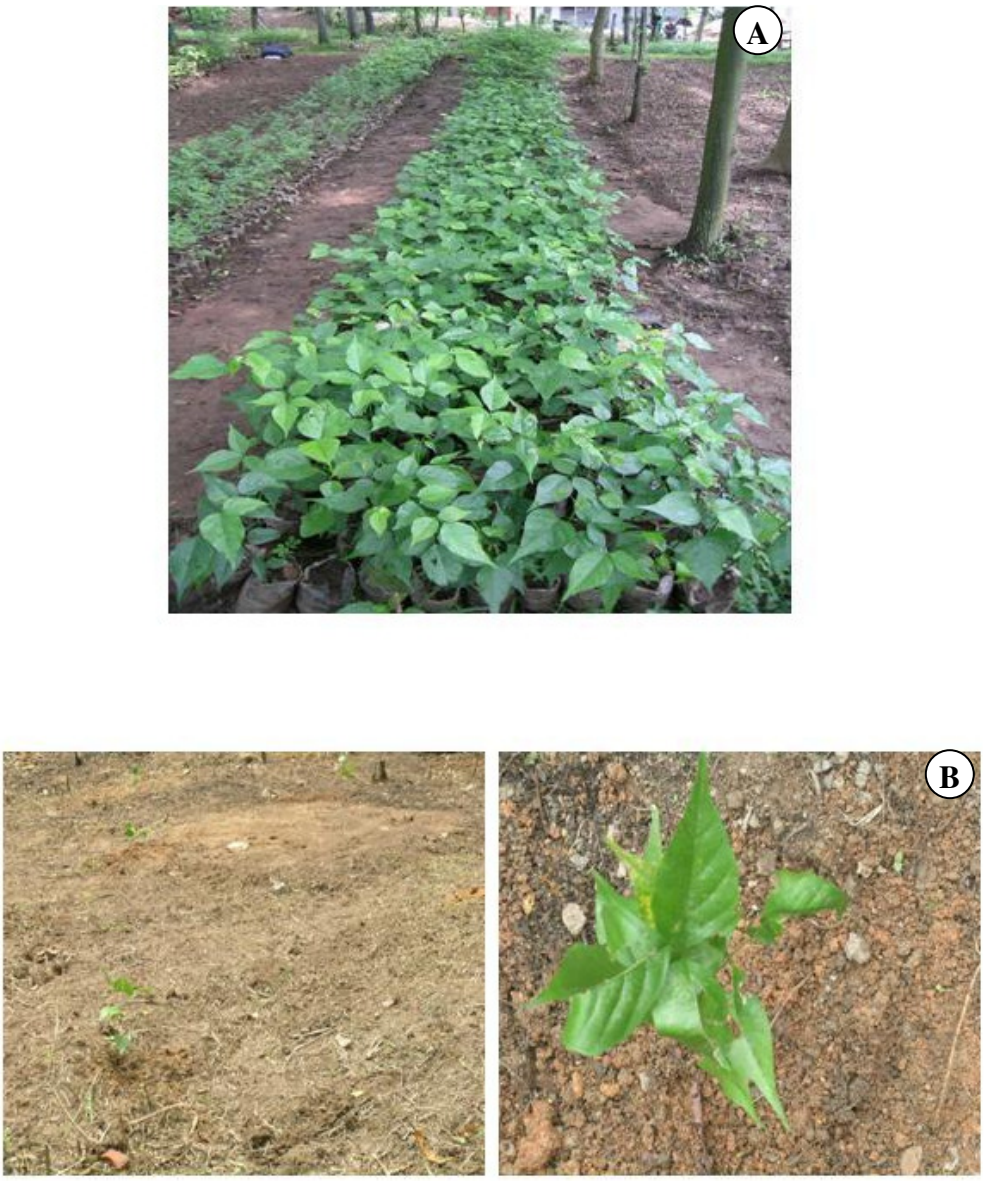


Figure 1.3 Hardening and field transfer of *Pongamia* seedlings.

A. Seed bed nursery; **B.** Field transfer at Sila Forest Range (spacing of 5m x 5m).

Table 1.5. Extraction of oil from seeds of *P. pinnata*[a]

CPT	Soxhlet Extraction (% vol/wt)[b]	Hot H_2O Extraction (% vol/wt)[b]
NGPP 26	28.5 ± 0.21	22.5 ± 0.35
NGPP 27	29.0 ± 0.29	23.0 ± 0.77
NGPP 28	29.0 ± 0.35	22.5 ± 0.65
NGPP 29	32.5 ± 0.21	23.5 ± 0.66
NGPP 30	31.5 ± 0.29	23.0 ± 0.45
NGPP 46	**33.0 ± 0.21**	**23.5 ± 0.74**
NGPP 47	29.0 ± 0.37	22.5 ± 0.50
NGPP 48	28.5 ± 0.16	22.0 ± 0.61
NGPP 49	32.0 ± 0.43	23.0 ± 0.45
NGPP 50	31.0 ± 0.42	23.0 ± 0.57

[a] *n-Hexane for extraction*

[b] *Three replications and each replicate is 100 g of powdered seeds from the tagged CPTs.*

Each value = mean ± SD

1.4.4.1 Factors affecting rate of extraction

Various factors are known to influence the rate of extraction. Seed to n-hexane ratio has been found to be critical parameter when using the Soxhlet apparatus for solvent extraction technique. Oil extraction has been found to be temperature dependent (Bera et al. 2006). However, keeping temperature constant and altering seed to n-hexane ratio resulted in differences in the recovery of solvent and time taken for oil extraction, although the oil yield remained the same for an individual tree. The yield of oil was somewhat higher than the yield obtained by simple solvent extraction technique like hot water extraction and ultrasonication (Shrivastava, 2006). Additional solvent does not improve the oil yield but reduces the duration of the process. The variation of experimental time on the Soxhlet apparatus with seed to solvent ratio is shown in Table 1.6. Results shows that, as more solvent is used, the time for oil extraction decreases and gives a golden yellow colour of the oil. Quality and particle size of the seed are another determining parameters affecting the oil yield by solvent extraction technique. If seeds are not dried adequately than it will not be crushed properly. All moisture should be removed before crushing. Similarly particle size

should be small enough of 0.2 – 0.6 mm to allow greater interfacial area between the solid and liquid to allow higher rate of transfer of material. The result showed that properly dried seeds and smaller sized particle gave better amount of oil yield.

1.4.4.2 Influence of solvent

The choice of solvent used in extraction also affects the oil yield and recovery of solvent. The liquid chosen should be a good selective solvent and its viscosity should be sufficiently low for it to circulate freely. The total oil from the seeds was extracted following Soxhlet extraction using petroleum ether and n-Hexane (at their boiling point) by varying seed to solvent ratio. The seed oil yield varied between the two solvent systems used. Maximum oil yield of 33 % was obtained when the seed was extracted with n-Hexane (50:300) (Table 1.6) whereas 300 ml of petroleum ether yielded 30 % of oil from the same amount of dried seeds. Thus, n-Hexane extraction gave higher oil yield and recovery of solvent compared with extraction with petroleum ether, however the latter method takes less time for oil extraction using Soxhlet apparatus and is more economical. Seed oil extracted from *S. brachiata* also varied in yields with the two solvent systems, n-Hexane giving the best result (Eganathan et al. 2006).

Table 1.6. Comparison between n-Hexane and petroleum ether on the oil yield[a]

Seed: Solvent	**n-Hexane[b]**			**Petroleum ether[b]**		
	Time (min)	Oil yield (% vol/wt)	Solvent Recovery (% of sol.)	Time (min)	Oil yield (% vol/wt)	Solvent Recovery (% of sol.)
1:4	75	33.0 ± 0.16	79.0 ± 0.16	65	30.1 ± 0.40	76.0 ± 0.56
1:5	71	33.1 ± 0.24	81.5 ± 0.14	58	30.0 ± 0.32	77.0 ± 0.69
1:6	**60**	**33.2 ± 0.35**	**82.5 ± 0.43**	**50**	**30.1 ± 0.35**	**77.5 ± 0.08**
1:7	56	33.1 ± 0.08	84.0 ± 0.40	44	30.1 ± 0.43	80.0 ± 0.35
1:8	51	33.0 ± 0.29	85.0 ± 0.43	40	30.0 ± 0.42	82.0 ± 0.53

[a]*NGPP 46*

[b]*The experiments repeated thrice and the mean values calculated.*

Each value = mean ± SD

1.5 Conclusion

In view of limited supply of natural fossil fuel, *Pongamia* is undoubtedly one of the key source-species for combating the crisis and a potential source of viable biodiesel. As a long-term goal, work has been initiated at Centre for Energy, IIT Guwahati where the emphasis is to develop and to characterize genetic material of value in the development of environmentally friendly biodiesel sources. The study reported here has applied significance. This is the first study on any systematic characterization to identify CPT, multiplication and seed oil analysis from tagged trees of *P. pinnata*. Ten CPTs belonging to populations 6 and 10 were identified based on morphological markers (vegetative and reproductive) at first stage analysis. Identified CPTs were multiplied using seed propagation technique. In the second stage screening, NGPP 46 was identified to be the best genotype based on pod - seed traits and total oil content. Hexane extraction yielded maximum oil content from seeds (33%) compared to petroleum ether (30%). It may be useful to have information with regard to *P. pinnata* seed and yield trait in a systematic manner from populations of a particular region for any future analysis.

From the results of current study on CPT identification and oil yield varying different parameters from the seeds of this species could be helpful in encouraging tree breeding and commercialization of the same in areas suitable for its growth. It also has important implications for germplasm utilisation. Also, it may establish a case study of genetic enhancement of biodiesel species. However it is important to note that the selection of plus tree should be site specific since plant characters, both quantitative and qualitative will vary from place to place.

Before an applied breeding programme is undertaken, basic information generated based on the morphometric traits to identify suitable seed sources with high oil content in *Pongamia sp* is essential. Such studies will not only pave the way for designing a molecular breeding programme for oil traits in *Pongamia,* but will also help to enhance our capacity to undertake precision breeding in biodiesel crops.


भारतीय प्रौद्योगिकी संस्थान गुवाहाटी
Indian Institute of Technology Guwahati

Chapter 2

Chapter 2

Mass multiplication by conventional and biotechnological tools in CPTs

2.1 Introduction

Improper land use and population pressure over several years has resulted in extensive degradation of agricultural land that has resulted in large tracts of wasteland (approximately 55 million ha) in India (Saxena, 2002). On the other hand, population explosion, greater urbanization and land reforms have resulted in the depletion of coal and natural resources. The problem is further compounded by the fact that the demand for energy and oil is on rise and there is a greater need to explore alternative sources of oil. This has resulted in strengthening the R&D in the areas of biodiesel. Nearly 20 % of land in India can be considered largely as wasteland where little agriculture is practiced. Some of this land could be used for growing non edible tree borne oil seeds (TBOs). To increase the biodiesel production it's important to have unlimited feed stocks of *Pongamia* bearing, high oil-yielding seeds. In addition, large scale plantation of clonal stocks of elite genotype needs to be done to encourage aforestation programme. Inspite of high valued agroforestry, environmental, medicinal and industrial characteristics research on cultivation and propagation in CPTs of *Pongamia* is limited.

Tree breeding involves selection of superior germplasm followed by large-scale propagation of true-to-type plus trees in the orchard for the production of improved seeds, which can be used for high quality plantations. Conventional propagation through vegetative means forms an integral part of tree improvement programme that provides the best planting stock with highest genetic quality that is not always the case with sexually propagated progenies. The advantage of vegetative propagation technique is that the desirable traits are perfectly preserved and provide a given number of progeny of selected individuals in about half the time of seed orchards. Thus the growing of cuttings through vegetative means offers the cost effective mass production of pathogen free uniform plants of elite genotype. The plants can moreover be raised

throughout the year and the plantable stock for some species can be obtained in shorter time than those raised through seeds. Biotechnological means i.e. *in vitro* propagation in CPTs of *P. pinnata* will not only speed up mass multiplication and generation of superior planting material of true-to-type plants but can also aid in complementing the ongoing activities of conventional propagation to raise its large scale cultivation.

Therefore, the present study on genetic enhancement of potential biodiesel plant *P. pinnata* is based on the above principle in which characteristics plus trees namely NGPP 26, NGPP 27, NGPP 28, NGPP 29, NGPP 30, NGPP 46, NGPP 47, NGPP 48, NGPP 49, and NGPP 50 identified from North Guwahati, Assam as mentioned in chapter 1 (Kesari et al. 2008) were progressed further for mass multiplication through both conventional (vegetative propagation) and biotechnological (*in vitro* propagation) means. The whole experiment of the present chapter was divided into three sub objectives, first was vegetative propagation from the best identified genotype of *Pongamia* to examine the rooting ability of mature stem cuttings and regulation by different phytohormones. Rooting was evaluated on stem cuttings treated with varying concentrations and combinations of natural and synthetic auxins viz; indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), and naphthalene acetic acid (NAA) respectively. Study was also performed to see the synergistic effects on two and three-component mixtures of auxins and effect of maturation in rooting. The second sub objective was to investigate the effect of genotypic variability and auxins application influencing the rooting behavior from mature stem cuttings of 10 tagged CPTs growing in their natural habitat. Final and third sub objective was to establishing extensive plantations comprising high-yielding elite lines through *in vitro* technique. The establishment of genetic stability of *in vitro* regenerated plants will be an essential requisite for large-scale clonal forestry. Despite the advantages of the *in vitro* propagation, genetic instability has been observed in micropropagated species; hence it is necessary to establish a system that produces genetically stable and identical plants, especially in the case of commercial plant species such as CPTs of *P. pinnata*. *In vitro* propagation of CPTs of *P. pinnata* was undertaken with the following sub sub-objectives: (1) to mass multiply using different explant sources and, (2) to establish a disease-free material for further studies. In this sub-objective of this chapter the effect of Woody Plant Media (WP) (Lloyd & McCown, 1980) traditionally used

in the multiplication of tree species, and MS media (Murashige & Skoog, 1962) were investigated for multiplication of CPTs of *P. pinnata*. A comparison of initiation and multiplication of different explants in WP and MS with different hormone combinations was also attempted in this chapter under consideration. In addition, RAPD and SDS-PAGE analysis of the micropropagated plants for genetic stability assessment were also performed.

The overall aim of the chapter was to develop a suitable technique exploiting both conventional and biotechnological methods for the large scale production of superior clonal stock for testing and plantation establishment of CPTs of *P. pinnata*. The results clarify and allow the optimal use of the knowledge and methods developed in promoting domestication of elite genotypes of *Pongamia* in the wastelands or unproductive lands.

2.2 Review of Literature

Tree breeding can be both by sexual and asexual means. Sexual method of propagation through seeds has the limitation that the seedlings raised, do not fully resemble the mother plant and often essential superior qualities or plus-traits of a mother plant fails to get transmitted to the young ones. Furthermore, there are chances that seeds exhibit dormancy or they have poor viability and long generation time before attaining maturity (Somashekar & Sharma, 2002). On contrary, in asexual mode of propagation which uses the vegetative parts such as stem cuttings, the progeny always resembles the mother plant in all respects and thus helps in maintaining the special characters and plus qualities of the mother plant (Henrique et al. 2006).

The successful introduction and subsequent expansion of plantings of any new crop species is reliant on the ability to develop simple and reliable methods for the propagation of large number of plants. The different methods adopted for mass multiplication are vegetative propagation through cuttings, air layering and grafting and *in vitro* propagation through direct organogenesis or indirect organogenesis via undifferentiated mass of tissues. Vegetative propagation an integral part of tree improvement programmes, ensures quick genetic gains through mass multiplication of selected genotypes and establishment of clonal seed orchards (Surendran et al. 2003). This technique has additional benefits in cross pollinated species like

P. pinnata in producing true to type plants with shorter juvenile period leading to early productivity (Raju & Rao, 2006). *Pongamia* can be successfully propagated through seeds and cuttings (Handa et al. 2005; Singh et al. 2005). The major drawback of this tree species is that they exhibit a very long flowering cycle of 4-7 years depending on the environmental conditions (ICRISAT, 2007). There are few reports on vegetative propagation of *P. pinnata*.

Branch cuttings form the most widely used propagules for vegetative propagation through the application of synthetic auxins to induce adventitious rooting. Application of auxins has been reported to induce rooting seasonally or throughout the year in many species (Davies, 1984; Nanda, 1984; Nanda & Kochhar, 1984). In most tree species exogenous application of natural and synthetic auxins facilitates adventitious root production from branch cuttings (Haissig et al. 1992; Kevers et al. 1997; Singh et al. 2004; Henrique et al. 2006). IBA has been recommended as the best among different auxins (IBA, IAA and NAA) for root induction in cuttings of *P. pinnata*. However, the concentration advised varies among different studies (Negi & Tiwari, 1984; Palanisamy & Kumar, 1997; Palanisamy et al. 1998; Mishra et al. 2001; Karoshi & Hegde, 2002). This may be due to the differences in experimental conditions as well as genotypic variability. The factors affecting adventitious rhizogenesis also include organic salts *viz*. $KMnO_4$, KCl and KH_2PO_4. Inorganic salts of P, S, Cl and Mn significantly influence IAA ionization and adventitious rhizogenesis with P and S salts having a more pronounced effect (Ansari et al. 2004). Sprouting of cuttings and rooting response ranged between 70 and 100 % at 500–800 ppm of IBA and a cluster of many (50–67) thin and long roots emerged at the basal region of the cuttings (length 25–30 cm) (Negi & Tiwari, 1984; Palanisamy & Kumar, 1997). However, Karoshi & Hegde (2002) reported a very high concentration of IBA (2500 ppm) for induction of rooting in 80 % cuttings indicating that there may be genotypic variability for this response. Adventitious rooting in shoot cuttings and seasonal variations on adventitious rooting in branch cutting of *P. pinnata* were carried out by Palanisamy et al. (1998). Authors concluded that spring season becomes the best season for root induction in the presence of exogenous auxins. Ansari et al. (1998) reported that season of collection had a significant influence on rooting. Their study showed that maximum rhizogenesis coincided with the emergence of new sprouts in spring in semi-hardwood cuttings obtained from 10 to 15-year-old trees of *P. pinnata*. Poor root induction occurred

during summer and early winter. Age of the mother tree is another factor affecting root induction with juvenile plant material totally lacking or possessing very poor rhizogenic ability (Negi & Tiwari, 1984).

Grafting is an effective technique to overcome the problem of long juvenile period as well as assurance of good productivity owing to elite scions. In grafting, one year old seedlings of *Pongamia* can be used as root stock. The scions can be collected from an elite genotype with the same dimensions as that of the root stock. A wedge shaped cuttings can be made and inserted in the scion made on root stock and kept under polyhouse for 2 months. The grafts can be then be taken and placed in hardening chamber. Wedge grafting has been found to be most successful using a 3-month old seedling raised in polybags as the stocks and semi-hardwood scions of 12-15 cm length (Karoshi & Hedge, 2002). Direct planting of seeds in the field and *in situ* grafting with a scion from high yielding trees after 9 months has also been recommended (Wani & Sreedevi, 2007). Multiplication of elite *P. pinnata* by grafting has been found to be easier than propagation by cuttings.

Air layering is another effective method of vegetative propagation in *P. pinnata*. Also called as "Chinese Layering" where roots are produced in small branches by applying root producing hormones and rooting media. The method can be employed to tertiary branches of *Pongamia* without much damage to the mother plant.

Pongamia is easily propagated through seeds (Singh et al. 2005) either by direct sowing in the nursery bed/ polybags during July-August or *in situ* sowing of seeds in the plantation field, but the seed germination rate is very slow, and progeny are of highly heterozygous. Potting mixtures consisting of sand, soil and manure in the ratio of 1:1:1 have been found to yield good results for large scale production of quality planting material of *P. pinnata* (Arjunan et al. 1994; Handa et al. 2005). Sowing of fresh seeds is recommended as germination rate decreases on storage. The germination of *Pongamia* is hypogeal i.e. in normal seedling; the cotyledons remain beneath the germination medium while the plumule pushes upwards and emerges above. Seedlings attain a height of 25-30 cm in their first growing season and transplanting to the field has to be completed at the beginning of the next rainy season when seedlings are 60 cm in height (Daniel & Hegde, 2007). Bold and cleaned

seeds are sown in advanced well prepared seedbeds. The seeds are dibbled in the nursery beds at the spacing of 7.5 cm x 15 cm during onset of hot weather. Mulching of beds is also found helpful for moisture conservation. The knowledge of germination and pre-treatment is essential for reproducibility of uniform results. The most commonly used pre-treatment are soaking of seeds in hot water, growth regulators or salt solutions (Ramamoorthy et al. 1989; Kumar et al. 2007b). Soaking of seeds in IBA (0.15 mM) or GA_3 (0.06 mM) for 24 h enhances germination (NOVOD, 2008). Seedlings develop large root systems. *P. pinnata* produces root suckers profusely. This characteristic makes it unsuitable for agro-forestery. It has the potential to become a weed if not managed properly. This species can be regenerated by coppice management (Misra & Singh 1989).

Scientific literature and case-studies have suggested various plantation models for large-scale *Pongamia* plantations (Misra & Singh, 1987; Naidu & Swamy, 1993). These range from blocks of monoculture plantations in community lands and less productive wastelands, with intercropping in agro-forestry models on productive lands to boundary, row plantations along the agricultural fields, and strip plantations along rail, road, and canals. Whatever be the model adopted, a large array of spacing between plants is recommended apart from considering locale specific factors to maximize the yield output (Bhojvaid, 2008). Many State Governments, NGOs, and multinationals in India have shown great interest in *Pongamia* plantations in the last decade. However there is no systematic study to record the extent of *Pongamia* plantations. The planting density (number of plants per hectare) in different models, soil type, and intensity of post-planting operations such as irrigation will determine planting costs. Resultantly, the reported/ published plantation cost is approximately Rs 25,000/ha on a yearly basis (NOVOD, 2008) (http://www.tn.gov.in/policynotes/agriculture). Wide array of subsidies on plants, plantations, and other related activities has been announced by NOVOD and many state governments (Fig. 2.1). The figure showed that total money spent in the plantation of *P. pinnata* in 1 ha land was Rs 117321 approx. Once the harvesting of fruits started in the 5^{th} year the money spent was Rs. 2400 approx., and in the successive years i.e. 6^{th}, 7^{th}, 8^{th}, 9^{th}, 10^{th}, 11^{th}, it increases to 60, 50, 40, 30, 20 and 10 % of the previous year respectively and from 12^{th} to 14^{th} year it becomes constant.

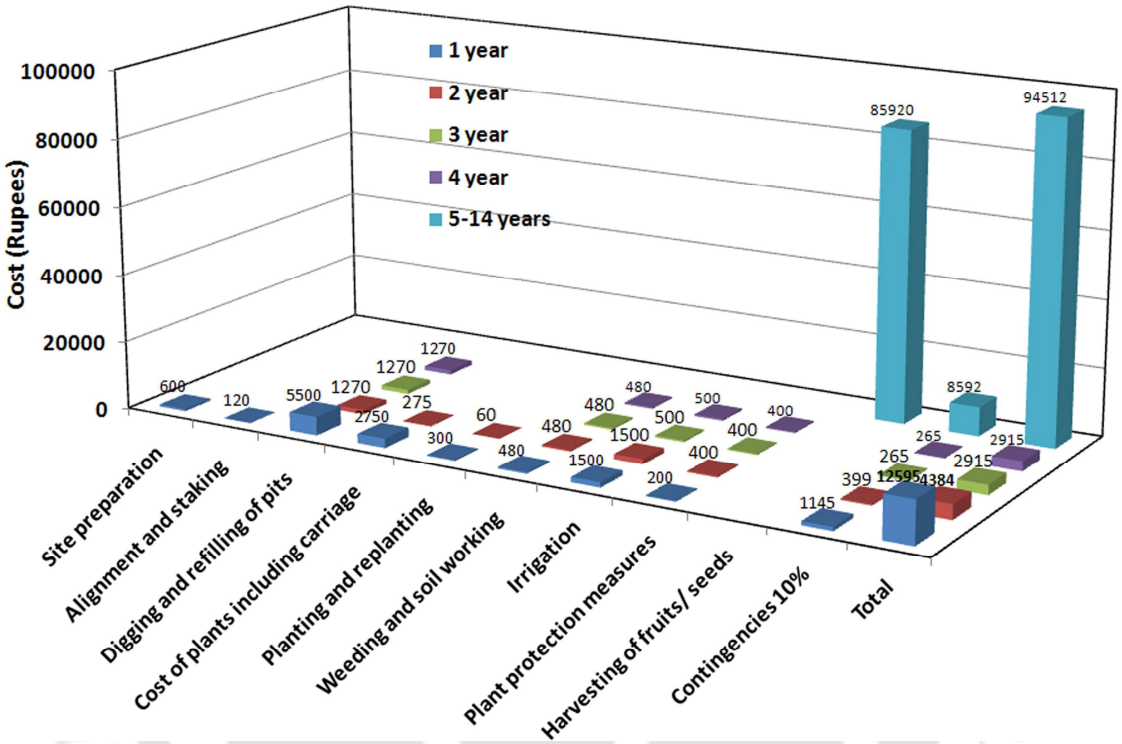


Figure 2.1. Cost of plantation of *P. pinnata* in one hectare land.

(*Source: Modified from NOVOD 2008 Report*)

Micropropagation of tree species offers a rapid means of producing clonal planting stock for afforestation, woody biomass production, and conservation of elite and rare germplasm (Anis et al. 2005). This could be useful in selection, genetic improvement programs and conservation efforts of genetic diversity within a species (Mesén et al. 2001). The amenability for tissue culture in *P. pinnata* has not been extensively studied. *In vitro* regeneration system plays a very important and crucial role for the improvement of *P. pinnata* as micropropagation protocols ensure the production of promising/elite planting stock on a large scale. Efforts to standardize protocols for micropropagation and *in vitro* regeneration via organogenesis and embryogenesis have increased in recent years as greater attention has been focused on the tree. Though the earliest report on tissue culture studies in *P. pinnata* dates to 1997, it is limited to callus induction from juvenile explants (Ranjan, 1997). The tree is slow to respond and mature explants require elaborate sterilization procedures. However, success has been reported for whole plant regeneration and hardening through de novo caulogenesis (Sujatha et al. 2008) and through micropropagation (Sujatha & Hazra, 2007).

The earliest report on tissue culture describes callus induction from hypocotyls, stem, leaf and cotyledon explants on Gamborg's B5 medium (Gamborg et al. 1968) supplemented with various growth regulators. Root differentiation occurred with the incorporation of different hormones and coconut water. De-embryonated cotyledons were used as explants for studies on regeneration (Sujatha et al. 2008). Shoot proliferation from cotyledonary nodes of seedling explants has been reported on medium incorporated with benzyl aminopurine (BAP) by Sujatha & Hazra (2006). A complete protocol was reported for the multiplication of *P. pinnata* using cotyledonary nodes derived from axenic seedlings by Sugla et al. (2007). Pre-conditioning of germinating seeds on MS medium with similar hormone concentration and addition of adenine sulphate enhanced the regeneration frequency (Srinivas & Rao, 2006; Srinivas et al. 2007). Micropropagation protocol from mature tree derived axillary meristems has been developed by Sujatha & Hazra (2007). They reported sprouting of axillary buds in 64 % explants on MS basal medium devoid of plant growth regulators.

Bimolecular analytical techniques like polypeptide and DNA polymorphisms profiling facilitates direct and reliable measurements to detect culture-induced variation at the DNA and protein level (Welsh & McClelland, 1990; Cloutier & Landry, 1994; Das et al. 2009; Sangwan

et al. 2003). RAPD analysis as a genetic markers to determine the extent of genetic variation or fiedility of regenerated plants has been used in plant and crops such as *Allium* (Al Zahim et al. 1999), *Lolium* (Wang et al. 1993), *Triticum* (Brown et al. 1993), *Beta* (Munthali et al. 1996), rice (Yang et al. 1999), sugarcane (Jain et al. 2005) where RAPD was used to detect somaclonal variation or purity. Of several molecular markers used for assessment, RAPD are the simplest, cheapest and appears to be useful for the analysis of genetic fidelity of *in vitro* propagated plants and have been well established in many tree species (Williams et al. 1990, Martins et al. 2004; Valladares et al. 2006). Polypeptide profiling (SDS-PAGE) is a useful biochemical marker system with functional/expressed gene diversity and has been more popularly applied to seed analysis for diagnostics and estimating outcrossing rates (Ferreira et al. 2000).

2.3 Methods

2.3.1 *Clonal multiplication in CPT, NGPP 46 from stem cuttings*

2.3.1.1 Selection of planting material

CPTs of *Pongamia* occurring across ten locations in North Guwahati, were evaluated based on morphological markers (vegetative and reproductive) to identify potential genotypes that can be included in programmes aimed at genetic improvement of the species (Table 2.1). Individuals performing above average for 75 % of traits were tagged as the CPTs (Kesari et al. 2008).

Table 2.1. Plus tree identification characters in *P. pinnata*[a].

Vegetative characters	Reproductive characters
• Girth of the main stem at breast level (m) • Plant height (m) • Number of leaves /g (wt) • Canopy size (m)	• Number of buds/inflorescence • Number of flowers/inflorescence • Number of seeds/inflorescence • Pod traits viz; Length (cm), Breadth (cm) and 100 Pod wt(g) • Seed traits viz; Length (cm), Breadth (cm) and 100 seed wt (g) Pod to seed ratio

2.3.1.2 Preparation of stem cuttings

Mature stem cuttings of characterized CPT of *Pongamia*, NGPP 46 were collected in the month of October from the study site near North Guwahati (Sila forest, Kamrup District, Assam, India). The leaves and shoot apices were excised and uniform leafless semi-hard wood cuttings (15-25 cm long and 0.5-1 cm diameter) comprising 3-4 nodes were prepared. The cuttings were dipped in 0.1 % aqueous bavistin (fungicide) for 10 minutes, subsequently distilled water washed and treated with root promoting auxins at the basal end (2 to 3 cm basal portion). The cuttings received distilled water (control) or treatments of IBA, IAA and NAA individually at different concentration ranging from 1.23 mM to 7.38 mM respectively for a maximum of 1 hr duration to determine the rooting capacity. Different combinations of above rooting hormones were also tried to see any possible synergistic effects in promoting the rooting ability from the stem cuttings. Cuttings from 2 month old seedlings of the same mother plant, raised in nursery bed were also used as source material for studying the effect of adventitious rooting and effect of juvenility treated with the best responding concentration. The top (apical) cut ends of the treated cuttings were sealed with paraffin wax to reduce the water loss by transpiration. Subsequently the cuttings were planted in poly-bags containing sand and clay at the ratio of 1:4 and kept in the mist chamber at 28±2 ºC and 70-80 % relative humidity. Light intensity was reduced to 22 % of ambient sunlight and day-length of 12±1 h throughout. Intermittent mist was supplied for 30 sec at every 15 minutes interval.

2.3.1.3 Data collection and statistical analyses

Eleven weeks after the mature stem cuttings of CPT were stuck, data were recorded for shoot and root traits viz; (i) number of cuttings that showed sprouting, (ii) number of shoots per sprouted cutting, (iii) maximum number of primary shoots, (iv) shoot length, (v) maximum length of primary shoot, (vi) number of cuttings that showed rooting, (vii) number of primary roots per rooted cutting, (viii) maximum number of primary roots, (ix) root length, and (x) maximum length of primary root.

The data were analyzed as a one-way classified data with sub sampling for the traits, number of shoots (ii), shoot length (iv) number of primary roots (vii) and root length (ix). The treatments were the different concentrations of the auxins. There were 26 treatments and three replications for each treatment. The sub samples correspond to the 15 cuttings within each replicate. The replications within the treatments do not have the same number of sub samples. ANOVA was performed on the weighted means to account for the variation in the relative contribution of the individual values to the treatment mean. The data was analyzed as a one-way classified data without sub sampling for the remaining traits. In both cases the following ANOVA model was used

$$y_{ij} = \mu + t_i + e_{ij}$$

where, y_{ij} is the j^{th} replication of the i^{th} treatment, μ is the overall mean, t_i is the effect of the i^{th} treatment and e_{ij} is the error. The SAS GLM (SAS Institute, 1999) procedure was used for the analysis.

2.3.2 Clonal multiplication in CPTs through mature stem cuttings

2.3.2.1 Preparation of stem cuttings

Mature stem cuttings from characterized 10 CPTs of *Pongamia* collected during the month of January from the study site were processed as per the method mentioned in section 2.3.1.1. The cuttings received distilled water (control) wash or treatments of the best responding concentrations viz., IBA (4.92), NAA (1.34) + IBA (4.92) and IAA (1.42) + IBA (4.92) + NAA (1.34) for a maximum of 1 hr duration to determine the rooting capacity.

2.3.2.2 Data collection and statistical analyses

Data was recorded for shoot and root traits as mentioned in section 2.3.1.2 after 9 weeks and the results were expressed as their mean ± SE. Each assay was performed three times containing 10 explants.

2.3.3 In vitro propagation in CPT

2.3.3.1 Direct organogenesis and genetic stability assessment

2.3.3.1.1 Plant material and culture conditions

Seeds and nodal segments of mature plant of *P. pinnata* collected from Sila Forest Range, North Guwahati, Assam (latitude 26°14'6" N and longitude 91°41'28" E), were used as starting material. The explants were washed under running tap water and subsequently treated with 2 % Tween 20 (w/v) for 5 min and rinsed with water until all traces of detergent were removed. Both ends of the explants exposed to sterilants were trimmed and were incubated in culture tubes (Borosil, India Ltd.) containing culture media. WP (Lloyd & McCown, 1980) and MS (Murashige & Skoog, 1962) media with 0.8 % agar (Himedia, UK) and 3 % sucrose were used as basal media for germination, initiation, and multiplication (Table 2.2). The pH of the media was adjusted to 5.8 and autoclaved at 121 ^{o}C for 15 min. Cultures were incubated at 25 ± 2 °C under 16 h/daylight. A light intensity of 40 µmol m^{-2} s^{-1} was provided by cool white fluorescent lights.

2.3.3.1.2 Effect of pretreatment and media type on seed germination/ shoot regeneration from seeds

Germination of seeds was observed after 2 weeks of incubation in culture medium. Germination rate was better on WP basal medium (70-91 %) as compared to MS basal medium (60-79 %). Presoaking facilitated leaching of phenolics from the seeds, prevented browning and caused swelling of seeds, thereby hastening the germination process by three weeks, as compared to the seeds which were not presoaked prior to inoculation (used as control). The best germination (91 %) was observed when seeds were soaked for 4-6 h prior to inoculation on WP basal medium without any hormones. Increasing the presoaking time to 12 or 24 h, did not increase the germination percentage further. Moreover, it resulted in contamination of cultures. Therefore, for seedling establishment seeds presoaked for 4-6 h were used. On an average, all seeds germinated within 2 weeks. However, growth was slow and it took about 4-6 weeks to develop into seedlings of 5-8 cm size.

Table 2.2. Nutrient composition of the media used in current study.

Nutrient composition	MS (1962) (mg/L)	WP (1980) (mg/L)
Major nutrients		
NH_4NO_3	1650	400
$(NH_4)_2SO_4$	0	0
KNO_3	1900	0
$Ca(NO_3)_2.4H_2O$	0	556
$MgSO_4.7H_2O$	370	370
$CaCl_2.2H_2O$	440	96
KH_2PO_4	170	170
K_2SO_4	0	990
Iron Stock		
$FeSO_4.7H_2O$	27.8	27.8
$Na_2EDTA.2H_2O$	37.3	37.3
Minor nutrients		
$MnSO_4.4H_2O$	22.3	22.3
$ZnSO_4.7H_2O$	8.60	8.60
H_3BO_3	6.3	6.2
KI	0.83	0
Na_2MoO_4	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.025
$CoCl_2.6H_2O$	0.025	0
Vitamins		
Inositol	100	100
Glycine	10.0	2.0
Thiamine	1.0	1.0
Nicotinic acid	1.0	0.5
Pyridoxin HCl	1.0	0.5

2.3.3.1.3 Initiation and multiplication

The explants used were adult field-grown nodal segments, and nodal and epicotyl segments decapitated from 4-6 weeks old germinated seedlings (size >5.0 cm). Different concentrations of BA and Kn were tried singly and in combination with NAA for shoot initiation, and multiplication. Of the several media combinations tried, those inducing the greatest response were recorded. Subculturing was carried out after 3 weeks, and the data were recorded after 6 weeks.

2.3.3.1.4 Rooting of micropropagules

The rooting response of micropropagules was tested on ½-strength MS media containing 0.8 % agar and different concentrations of NAA, IBA and IAA (0 – 5 µM). Single shoots were excised from multiple shoots, produced in optimal micropropagation medium (see the results section), transferred to 30 mL of each rooting medium, and incubated as above. Root production was scored at approximately two-week intervals, until the plants were ready for transfer to poly bags. The steps involved in tissue culture are summarized as flow diagram Fig. 2.2.

2.3.3.1.5 Transfer of plants to the greenhouse

Rooted micropropagules were removed from culture tubes and washed thoroughly with tap water to remove the adhering medium to minimize pathogen attack and subsequently were planted in poly-bags containing sand and clay at the ratio of 1:4, and kept in a mist chamber. Plants were hardened under a 16 h photoperiod at 28±2 ºC with a relative humidity of 75 % in the greenhouse. Intermittent mist was supplied for 30 sec at 15 min interval. The % survival was determined two months after transferring to the green house.

2.3.3.1.6 Genomic DNA extraction and RAPD analysis

For RAPD experiments, 7 regenerated plants were randomly selected from 60-day-old plantlets and mother plant were analyzed. Total genomic DNA was extracted from fresh tender leaves using SDS protocol with slight modifications (McCouch, 1992). One gram of leaf tissue was ground in liquid nitrogen and suspended in 10 mL of extraction buffer (100 mM Tris, 0.5 M NaCl, 50 mM EDTA) containing 1 % β-marcaptoethanol. The suspension was incubated at 65 ^{o}C in water bath for 30 min, extracted with 5 M potassium acetate and centrifuged at 2,795 x *g* at 4 ^{o}C for 30 min. The aqueous phase was precipitated with isopropanol and again centrifuged at 2,795 x *g* at 4 ^{o}C for 20 min. The pellet was dissolved in TE buffer (10 mM Tris HCl, 1 mM EDTA pH 8.0) and treated with RNAse. DNA was purified by ethanol precipitation. The quality and quantity of the extracted DNA was confirmed both spectrophotometrically and by running the extracted DNA on 1.0 % agarose gels containing 0.5 µg/mL of EtBr.

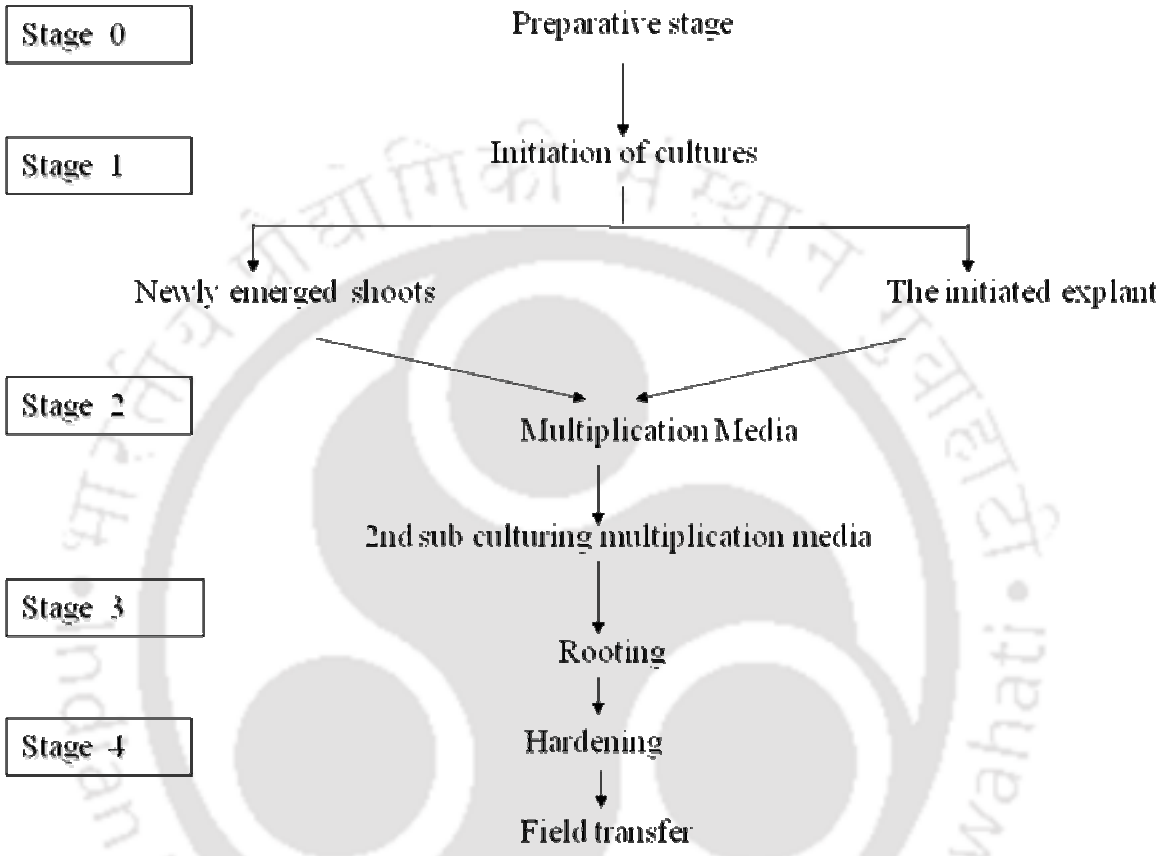


Figure 2.2. Flow scheme for the micropropagation studies.

PCR amplification of the genomic DNA was carried out using 20 arbitrary decamer oligonucleotide primers (Operon Tech, USA). Each reaction mixture of 20 µl contained 50 ng/µl of template DNA, 1 x assay buffer (100 mM Tris sulfonic acid, pH 8.8, 15 mM $MgCl_2$, 500 mM KCl and 0.1 % gelatin), 0.2 mM each dNTPs (Banglore Genei, India), 5 pM of each primer and 0.05 U of *Taq* polymerase (Banglore Genei, India). The reaction was performed in 0.2 ml microfuge tubes (Dialabs). PCR amplification was carried out in a Mini Thermal Cycler (Applied Biosystems, USA) programmed for 35 cycles. The first amplification cycle consisted of initial denaturation step of 5 min at 94 ^{o}C. This was followed by 35 cycles of 45 sec at 94 ^{o}C, annealing for 1 min at 32 ^{o}C, and extension at 72 ^{o}C for 1 min 30 sec. An additional cycle of 5 min at 72 ^{o}C was used for primer extension. The amplification products were electrophoresed in 1.5 % agarose gels in 0.5 x TBE (10 x stock contained 0.8 M Tris, 0.8 M Boric acid, 0.5 M EDTA). The gels were visualized and photographed under UV light by a gel documentation system (Bio Rad, USA). The size of amplification products was estimated using 1 Kb DNA ladder (Banglore Genei, India). PCR amplification was repeated twice and only primers producing reproducible bands were considered for analysis.

2.3.3.1.7 SDS-PAGE electrophoresis of total soluble proteins

Protein extraction was carried out for the same regenerated plants from which DNA was extracted. Young leaves were homogenized in 10 mM Tris-EDTA buffer (pH 8.0) containing 10 % SDS, 5 mM β-mercaptoethanol and 0.1 mg mL^{-1} phenylmethanesulphonylfluoride (PMSF). After centrifugation at 10,000 *g* for 10 min (4 ^{o}C), the supernatant was boiled for 10 min. Protein concentrations were measured using BSA as a standard by Bradford method (1976). Proteins were separated using discontinuous SDS-PAGE (12 % running gel, pH 8.8, and 5 % stacking gel, pH 6.8) at 4 ^{o}C according to Laemmli (1970). After electrophoresis, gels were stained overnight with 0.25 % Coomassie brilliant blue R250, destained, fixed, and photographed. Molecular weight of polypeptide was determined by the mobility of the standard proteins.

2.3.3.1.8 Data analysis

Three replicates (15 explants/ replicate) were inoculated per treatment to test the effects of medium consistency on multiplication rates. After three weeks of culture period, records were made on percentage of explant initiating shoot buds, mean number of multiple shoot buds and mean length of the longest shoot per explant. For rooting, percentage rooting, mean root number and mean root length per explant were measured after two weeks. All experiments were carried out independently and repeated thrice. The data were analyzed using one-way ANOVA (SPSS 16.0 version, 2007) and significant differences between treatment means were assessed using Duncan's multiple range test (DMRT) at a 5 % probability level ($P<0.05$).

Amplified DNA and polypeptide fragments were scored as present or absent both in the regenerated and in mother plants. Electrophoretic bands of low visual intensity that could not be readily distinguished as present or absent were considered as ambiguous and were not scored.

2.3.3.2 Indirect organogenesis

2.3.3.2.1 Plant materials for callusing

Leaf discs from young leaf and roots were used as explant materials. Pre-treatment of the explant and inoculation procedures were essentially the same as for the nodal explants except for the surface sterilization step, which was reduced to just 1 minute. Basal WP medium supplemented with auxins 2,4-D (0-5 mg) were tried for callus induction. In another set of experiments 2,4-D tried in combination with cytokinins BA (0-1 mg) and Kn (0-1 mg). A minimum of 10 replicates were maintained in each experiment and repeated thrice. Proliferating calli were subcultured on the same media combination repeatedly for 2-3 times before trying callus differentiation/organogenesis on different combinations of cytokinins and basal salts.

2.4 Results and discussion

The production of whole plants by vegetative and *in vitro* technique is an efficient, reliable and rapid strategy. This provides a faster method of large scale-propagation**.**

2.4.1 Effect of a range of auxin type on sprouting and rooting in CPT, NGPP 46

Auxins are well known to play a significant role in stimulating adventitious rooting from stem cuttings of tree species (Poupard et al. 1994; Tchoundjeu et al. 2004). It has been repeatedly confirmed that auxin is required for adventitious roots on stems and that the divisions of the first root initials are dependent on exogenous and endogenous levels of auxins (Ludwig-Müller, 2000; Kochhar et al. 2005). In addition to enhancing the rate of adventitious roots development, auxin application has been found to increase the number of roots initiated per rooted cutting in a variety of species (Aminah et al. 1995; Palanisamy et al. 1998).

In present study, cuttings from NGPP 46 developed sprouts within 10 days and adventitious roots within 3 weeks after planting, although there were significant differences in rooting and sprouting percentage between auxin-treated cuttings and control. All three natural and synthetic auxins resulted in a significant induction and growth of adventitious roots in cuttings. By week 11, there were significant differences ($p<0.05$) between the three auxin treatments (IBA>NAA>IAA) as the rate of rooting was strongly determined by the type of auxin. Among the treatments, 4.92 mM IBA induced maximum rooting (66.67 %), followed by 1.34 mM NAA (40.0 %) and 1.42 mM IAA (26.67 %) respectively when applied singly (Table 2.3). IBA at 4.92 mM was also found to enhance the number of roots by 3 fold, root length by 2 fold as compared to control (Fig. 2.3). IBA is the best auxin for general use because it is nontoxic to plants over a wide concentration range than NAA or IAA (Hartmann et al. 2002) and also effective in promoting rooting of a large number of plant species (Teklehaimanot et al. 1996; Henrique et al. 2006). The average number of roots per cutting was in the range 1 (NAA 5.37 mM) – 8 (IBA 4.92 mM). A maximum of 24.80 cm root length was observed with 2.85 mM IAA (Table 2.3). The better response in IBA is possibly due to stimulation of cambial activity resulting in mobilization of reserve food materials to the site of root initiation.

Higher doses (above than 7 mM) of all three auxins tested further inhibited sprouting. Inhibitions were more pronounced with NAA (8.05 mM), where only 8.90 %

sprouting was observed and no rooting response until 6 weeks of insertion. In general, there were significant differences between the treatments with regard to sprouting. IBA at 4.92 mM concentration was the best and produced maximum number of shoots (Fig. 2.4A) though not very significantly different from the control ($p<0.05$), but the average length of the shoots was thrice that of the control (Fig. 2.4B).

Varied combination and concentration of different hormones were also taken with the aim to evaluate their effect in stimulating the rhizogenesis of elite genotype of *Pongamia* cuttings. With the two-component and three-component mixture, the percentage increase in rooted cuttings was in range of 20.0 % to 73.33 % and 86.67 % respectively (Table 2.3). A higher yield of rooted cuttings was achieved in three component combinations (NAA 1.34 mM + IAA 1.42 mM + IBA 4.92 mM) enhancing their yield up to 4 times as compared to control. IBA in combination exerted a synergistic action on the effectiveness of rooting, which became evident not only in a higher yield of rooted cuttings but also in an improved root system of stimulated cuttings (Table 2.3). Auxins in combination also had stimulating effect on shooting particularly in the number of cuttings showing sprouting, although the total number of shoots was not significantly different from control as far as three-component combination was concerned. Similar results of synergism between IBA and synthetic auxins were reported in woody trees (Tantos et al. 2001; Henselova, 2002). Interestingly flowering was also observed in three-component mixture by the end of three month period and may be due to the availability of larger amount of reserve food materials in thicker cuttings (Fig. 2.5). The maximum sprouting (86.67 %) in *P. pinnata* was also observed in this mixture, although the maximum number of roots and maximum length of root was slightly less than that in IBA at 4.92 mM. This marginal reduction in rooting might be due to the plant's response to flowering. The negative influence of flowering on root formation appears to be due to the photoperiodic induction of the flowering stimulus rather than a direct competition for resources between flowers and developing roots. The flowering process in stem cuttings is strongly influenced by temperature, light intensity and genotype (DeVier & Geneve, 1997; Roh et al. 2005; Thiele et al. 2009).

The relatively poor rooting with IAA treated stem cuttings of *P. pinnata* (6.67 %) in comparison to IBA could be explained by the sensitivity of IAA to light (Hartmann et al. 2002)

Table 2.3. Effect of auxins on rooting of stem cuttings of *P. pinnata*

Treatments (mM)	No. of cuttings showing rooting	% of cuttings showing rooting	No. of Primary Roots*	Max. no of primary roots	Length of the roots (cm)*	Max. length of the primary root (cm)
Control (0)	3	20.00	3.25	3.33	4.88	9.23
IBA (1.23)	2	13.33	7.85	9.66	11.50	20.20
IBA (2.46)	3	20.00	8.30	11.00	14.47	17.66
IBA (4.92)	**10**	**66.67**	**8.44**	**11.66**	**11.16**	**20.50**
IBA (7.38)	1	6.67	2.75	3.00	13.23	13.90
NAA (1.34)	6	40.00	5.70	10.33	7.14	7.80
NAA (2.68)	3	20.00	2.12	2.66	9.20	11.20
NAA (5.37)	2	13.33	1.50	1.66	4.94	7.50
IAA (1.42)	4	26.67	2.46	2.66	9.75	11.43
IAA (2.85)	3	20.00	2.00	2.60	12.03	24.80
IAA (5.70)	1	6.67	3.75	4.33	3.87	6.16
NAA (1.34) + IBA (1.23)	6	40.00	4.15	6.33	3.97	5.03
NAA (1.34) + IBA (2.46)	9	60.00	4.50	8.33	8.03	15.30
NAA (1.34) + IBA (4.92)	**11**	**73.33**	**7.81**	**10.33**	**7.07**	**15.83**
NAA (1.34) + IAA (2.85)	4	26.67	1.45	2.00	1.05	1.26
NAA (1.34) + IAA (5.70)	7	46.67	3.19	4.33	5.95	9.96
IAA (1.42) + IBA (1.23)	3	20.00	3.70	4.66	7.04	9.23
IAA (1.42) + IBA (2.46)	7	46.67	4.19	5.66	7.29	12.30
IAA (1.42) + IBA (4.92)	9	60.00	8.89	15.66	7.65	15.20
IAA (1.42) + NAA (2.68)	6	40.00	2.75	4.00	10.12	10.53
IAA (1.42) + IBA (4.92) + NAA (1.34)	**13**	**86.67**	**8.87**	**11.00**	**7.14**	**16.23**
α	0.05		0.05	0.05	0.05	0.05
Error d.f.	42		42	42	42	42
Error Mean Square	0.47		1.05	0.77	0.92	0.72
Critical Value of *t*	2.01		2.01	2.01	2.01	2.01

Each value = Average of 3 replicates (each replicate contains 15 cuttings)

Bold indicates best responses

* Subsampling data

Figure 2.3. Effect of IBA (**4.92 mM**) on the rooting of cuttings of candidate plus tree *P. pinnata*.

T= Treated; C= Control

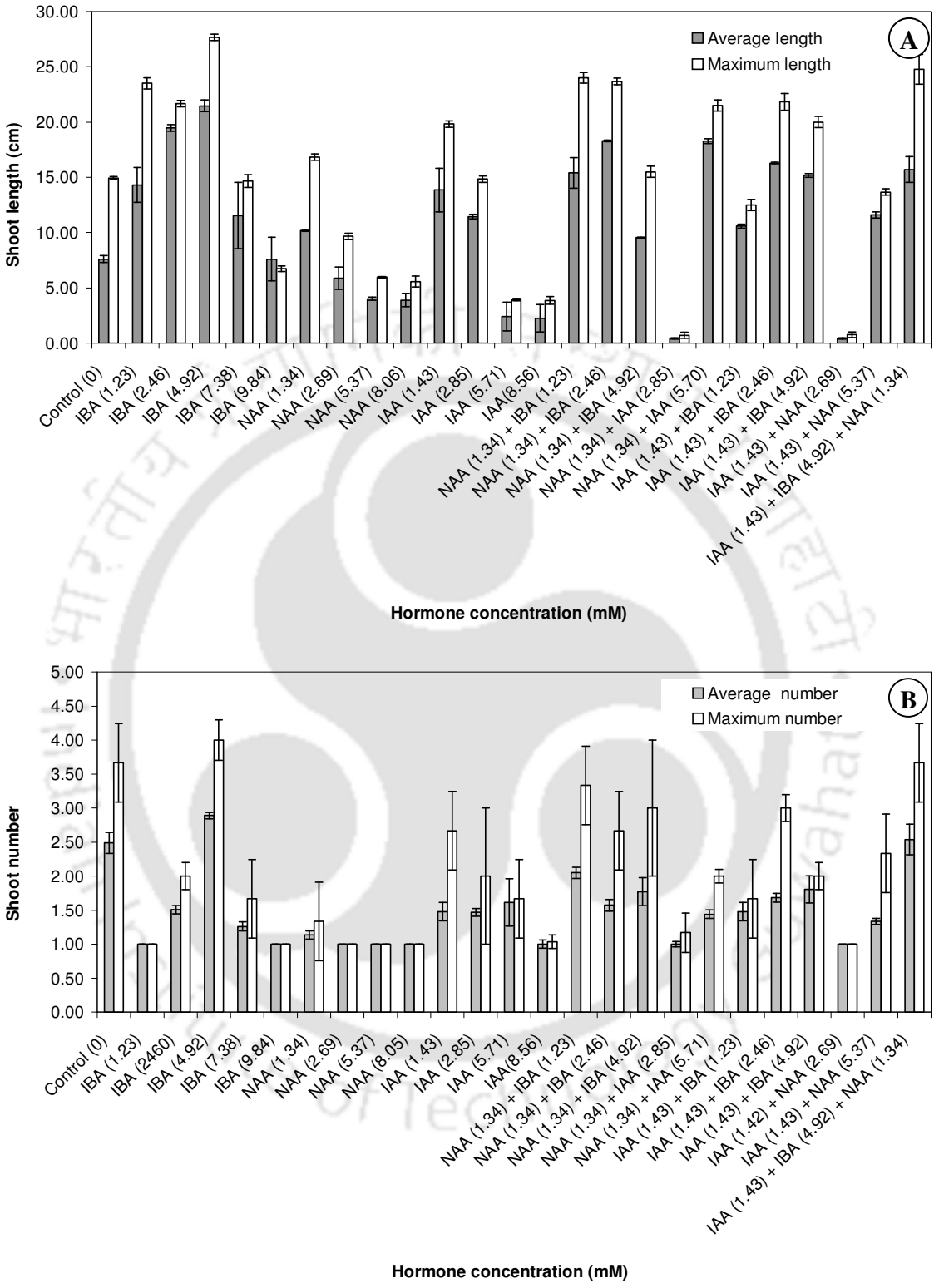


Figure 2.4. Effect of auxins on sprouting.

A. Shoot number; **B.** Shoot length. . Each value= Mean ±SD.

Figure 2.5. Flowering in rooted cuttings of *P. pinnata* in three component combination maintained in mist chamber.

(NAA 1.34 mM + IAA 1.42 mM + IBA 4.92 mM)

and production of more ethylene which is known to inhibit the root production (Mullins, 1972). Further, it has been found that enhanced ethylene synthesis negatively effect the lateral root formation in *Arabidopsis* (Negi et al. 2008). The growth of lateral roots was restored on application of sliver nitrate which is a strong antagonist to ethylene synthesis, clearly indicating that ethylene production suppresses the root formation. In contrast, combinatorial treatment of ethephon (derivative of ethylene) and IBA has been reported to have positive impact on adventitious root formation in *P. thunbergii* (Mori et al. 2008). Evaluating the total effectiveness of all the mixtures tested, the three-component combinations that had synergistic effect in enhancing rooting response were found to be the best.

Death of some cuttings commenced from the fifth week of propagation and by the 11th week of recording, the sprouted shoots had already senesced and eventually died. Cutting death was 33.3 % in NAA at 1.34 mM and 42.7 % with IAA at 1.42 mM respectively. Death was more pronounced in two component mixture. Nearly about 58 % of shoot death were recorded in IAA (1.42 mM) + IBA (2.46 mM) and 83 % in IAA (1.42 mM) + IBA (1.23 mM). In all experiments, cutting death was largely due to water deficits incurred as a result of physiological shock while the cuttings are taken. The result emphasizes the need to maintain high relative humidity around the cuttings during propagation, in order to minimize the water loss (Newton & Jones, 1993a, b; Nketiah et al. 1998). It is accepted that a positive effect of stimulators on the rooting process in plants is achieved if other conditions are adhered to- such as optimum temperature, humidity and also the type of cuttings (Henselova, 2002).

Many reports exist for differences in rooting frequency depending on the exogenous auxin or combination of auxins being used (Henselova, 2002; Blythe et al. 2004). It may be stated from the results obtained that the concentration of IBA *per se*, as well as its mixture with lower concentrations of IAA and NAA has an essential impact on the percentage yield of rooted cuttings. The differences in rooting success could also be due to physiological, anatomical and phenological differences among cuttings reflecting a number of factors, such as maturation, photoperiod, temperatures, carbohydrate reserves etc in the donor plant.

2.4.1.1 Effect of maturation on rooting in CPT, NGPP 46

It has been reported in woody tree species that the rooting potential of the cuttings is a juvenile characteristic and that the rooting capacity declines after maturation (House et al. 1996; Kibbler et al. 2004). Stem cuttings from 2 months old raised seedling of elite genotype NGPP 46 were also subjected to exogenous application of IBA at 4.92 mM that exerted the best stimulating action on plant rhizogenesis. The results indicate that there are differences in rooting percentage between the seedling raised in nursery bed and the cuttings obtained from field-grown trees. The cuttings obtained from young seedlings in nursery bed had a high rate of rooting (almost 80 %) in comparison with cuttings raised from the field (66.67 %) (Table 2.4). Similar observations were reported in *P. cineraria* which showed better rooting, up to 60 % in cuttings taken from 6 month old plants in comparison to 8 year old cuttings which showed a maximum rooting of only 35 % (Arya et al. 1994). It was observed that there was a decrease in rooting percentage with the increase in age of the parent plant and our findings in *P. pinnata* are in conformation with reports for other species where a decline in rooting was observed with age of the parent plant (Abramovich et al. 1980; Oduol & Akunda, 1988). There was no difference recorded between cuttings taken from the base of the mature plants propagated from cuttings and those taken from the apex indicating that juvenility (age of the material) and not the position of the cutting on the stock plant affects rooting capacity. Similar observations were recorded in recalcitrant woody tree species (Negash, 2002; Kibbler et al. 2004). The cuttings raised through seedlings were found to induce multiple stems when their tips were cut and the resultant multiple shoots could be used for rooting. Profuse rooting of cuttings is of practical importance especially when planting in denuded and marginal areas. The fast developing root system will lead to successful establishment of the plants which was the case in the present study.

Table 2.4. Effect of 4.92 mM IBA on rooting of juvenile and mature cuttings of *P. pinnata*.

Source	No. of explants showing rooting	% of cuttings showing rooting	No. of Primary Roots*	Max. no of primary roots	Length of the roots (cm)*	Max. length of the primary root (cm)
Mature field grown cuttings	10	66.67	8.44	11.66	11.16	20.50
2 month old nursey raised cuttings	12	80.00	6.06	8.33	8.17	12.05

2.4.2 Effect of genotypes and auxins on adventitious rooting from stem cuttings of CPTs

Auxins in addition to enhancing the rate of adventitious roots development has also been found to increase the number of roots initiated per cutting in a variety of species (Tchoundjeu et al. 2004; Atangana et al. 2006). The best responding auxin concentrations that have been reported by us in our previous study on effect of auxins on adventitious rooting from stem cuttings of NGPP 46 (Kesari et al. 2009a) were used in current study to see the effect of genotype and auxin treatments on rooting response in stem cuttings of 10 CPTs. There were significant differences in rooting and sprouting percentage between auxin-treated cuttings and control among the CPTs.

Mature stem cuttings from all the 10 CPTs starts sprouting in 15-30 days time. Percentage sprouting (86.67 %) and average number of the main shoots (>2.0) was recorded to be maximum in NGPP 26 and NGPP 46 in three-component mixture (IAA 1.42 mM + IBA 4.92 mM + NAA 1.34 mM). Highly significant differences were observed in sprouting percentage (26 - 86.67 %) across 10 CPTs and between auxin treatments at the end of 9 weeks (Fig. 2.6A). The mean number of shoots and mean shoot length per rooted cuttings varied from 1 (NGPP 49) to 2.32 (NGPP 46) and 4.53 cm (NGPP 49) to 22.23 cm (NGPP 26) respectively for the best auxin treatments used in the current experiment. There was a differential response among 10 CPTs of *Pongamia* to auxin application with regard to the percentage sprouting and shoot length of the cuttings. However, auxin application did not significantly affect the shoot number developed in treated CPTs and their control cuttings. Similar findings were reported in the cuttings of *Swietenia macrophylla, Chickrassia velutina* and *Baccaurea sapida* (Hossain et al. 2004; Abdullah et al. 2005). Comparisons with respect to percent sprouting; mean shoot number and mean shoot length in three different hormone combinations tried is shown in Fig. 2.6A.

IBA at 4.92 mM induced rooting in all the 10 genotypes which varied from 56.67 % (NGPP 49) - 93.33 % (NGPP 46) respectively, higher than that of control plants (13.33-33.33 %). IBA at 4.92 mM was found to be the best auxin which resulted in a significant

induction and growth of adventitious roots in cuttings (Kesari et al. 2009a). IBA in combination also exerted a synergistic action on the effectiveness of rooting, which became evident not only in a higher yield of rooted cuttings, but also in an improved root system of stimulated cuttings. With the two components and three-component mixture, the percentage increase in rooted cuttings was in the range of 66-96 % respectively (Fig 2.6B). Auxin application significantly increased degree of rooting response of stem cuttings and the effect was more in three-component mixture for all the 10 CPTs, indicating that IAA (1.42 mM) + IBA (4.92 mM) + NAA (1.34 mM) was more effective over IBA at 4.92mM treatment in *Pongamia*. Rooting after nine weeks was more than 96 % for the cuttings obtained from NGPP 26, NGPP 27 and NGPP 46, in three component mixtures (IAA 1.42 mM + IBA 4.92 mM + NAA 1.34 mM) and was approximately 4 times higher than that of control. Number of roots and root length per rooted cuttings increased due to auxin treatments in treated stem cuttings in comparison to control cuttings (Fig. 2.7A). Highest mean number of roots per rooted cuttings among the 10 CPTs studied was observed in NGPP 46 (13.23) in three-component mixture that was four fold higher than control (Fig. 2.7B), closely followed by NGPP 26. Difference in rooting response of the 10 CPTs may be due to difference in physiological state of the individual during collection and endogenous hormone levels (Krisantini et al. 2006). The interaction among auxin and month of collection was also found to be significant on root induction and differentiation. Seasons had a marked effect on rooting in stem cuttings (Singh et al. 2004). In *Pongamia*, stem cuttings collected during the month of January rooted better in terms of percentage response, average root number and average root length than those collected during October. However there was only marginal difference in sprouting response of the stem cuttings (86 % in the month of January and 91 % in the month of October). Key observation recorded was that sprouting % in January was lesser than rooting % which is reverse to that observed in October. This variation in seasonal rooting response may be attributed to the physiological condition of the plant cuttings. Cellular activities during root initiation require availability of sugars which are synthesized due to activity of various hydrolytic enzymes (Nanda, 1975).

The activity of these enzymes might have been at the highest level during monsoon and post monsoon months. The failure of cuttings to produce good root system in non-

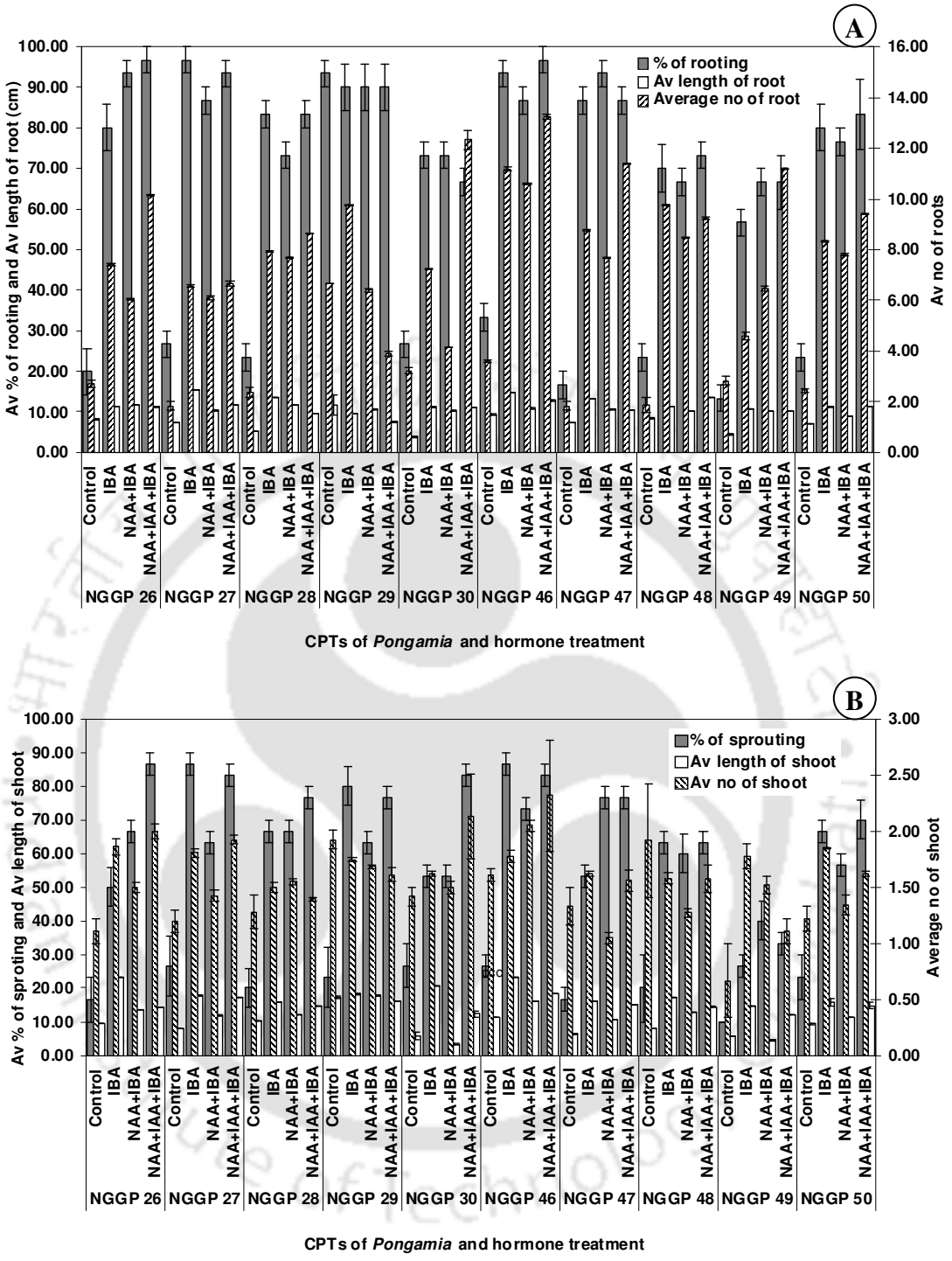


Figure 2.6. Effect of auxins on sprouting and rooting of mature stem cuttings from 10 CPTs of *P. pinnata*.

A. Sprouting percentage, shoot number and shoot length;

B. Rooting percentage, root number and root length. Each value= Mean ±SD.

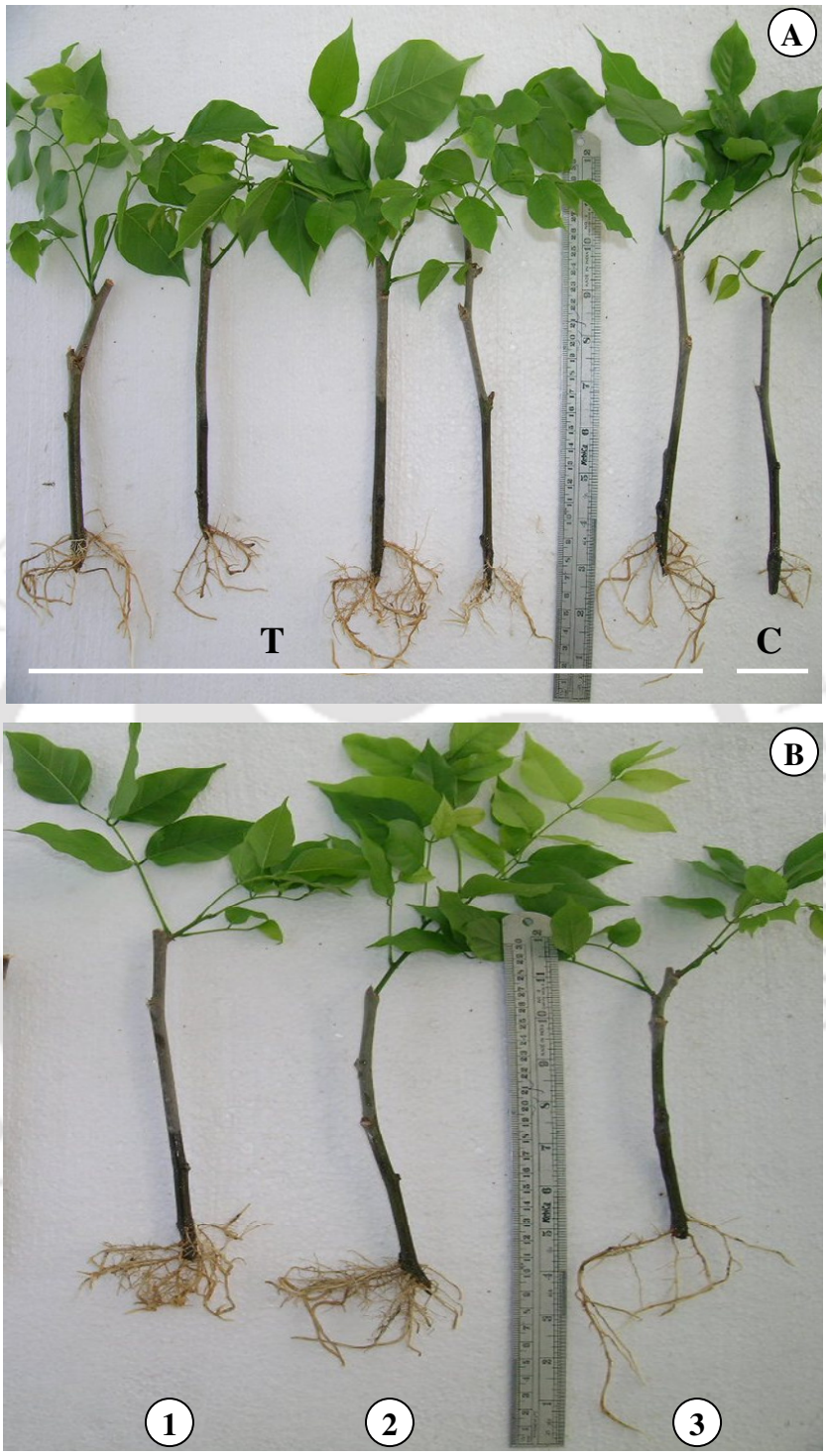


Figure 2.7. Effect of auxins on the rooting of cuttings from CPT of *P. pinnata* (NGPP 46).

A. T= Treated, (IAA 1.42mM + IBA 4.92mM + NAA 1.34mM); C= Control

B. 1, IAA 1.42mM + IBA 4.92mM + NAA 1.34mM;

2, NAA 1.34mM + IBA 4.92mM; and **3**,IBA 4.92mM.

monsoon months may be due to a high rate of metabolism, low enzymatic activity and increased inhibitor promoter ratio (Eganathan et al. 2000). Results on propagation by cuttings of 10 CPTs of *Pongamia* showed that they reacted quite differently as to their regenerative ability, and this depends on many factors besides genotype. Moe & Andersen (1988) and Menzies (1992) reported that the physiological status of the stock plant has been shown to exert a strong influence on subsequent root formation in stem cuttings. This difference in rooting ability may be explained by different physiological state of 10 CPTs. Mature stem cuttings which have a high level of auxin and carbohydrates are especially suitable for adventitious rooting. A number of researchers have gone in to show that rooting is facilitated when the carbohydrate reserve foods are in abundance (Haissig, 1974; Eganathan et al. 2000). The auxin induced effect on rooting of cuttings is presumed to be mediated through its effect in mobilizing the reserve food material by enhancing the activity of hydrolytic enzymes (Nanda, 1975).

2.4.3 In vitro propagation in CPT

Establishment of the *in vitro* cultures in *P. pinnata* posed considerable problems with contamination in primary cultures, which reappeared even after repeated subculturing. The problem was overcome by treating the explants with 0.1 % (w/v) Bavistin, a systemic fungicide (BASF, India) for 2 h and then with 70 % ethanol for 2 min before surface-sterilizing with 0.1 % (w/v) mercuric chloride for 7 min, followed by five rinses with sterile, double-distilled water. In field-grown trees, contamination is a major problem during the establishment of aseptic cultures (Bhatt & Dhar, 2004, Chabukswar et al. 2006). It was observed that for *P. pinnata,* the percentage of explants showing contamination was highly dependent on the season during which the material was collected. Spring (February - May), when plants are actively growing, was found to be the most favourable season for initiation of culture; 68.89 ± 1.0 % of the cultures developed adventitious shoots and rate of contamination was also less. Cultures collected during the rainy season did not survive due to heavy fungal and bacterial contamination. The seasonal effect on *in vitro* bud growth has been reported in many tree species, including *Myrica esculenta* (Bhatt & Dhar, 2004) and *Garcinia indica* (Chabukswar et al. 2006).

2.4.3.1 Direct organogenesis and genetic stability assessment

2.4.3.1.1 Initiation and multiplication from in vitro raised explants

In vitro-raised explants (nodal and epicotyl segments) responded to induced multiple shoots in different degrees in two different media tried *viz.* MS and WP media with varying concentrations and combinations of cytokinin and auxin as given in Table 2.5. Explants on MS medium and WP with either basal salts only or supplemented with BA (2.2 µM) or Kn (2.2 µM) sprouted or regenerated but they did not produce multiple buds. Nodal and epicotyl segments, when cultured on WP and MS based media containing BA (4.4 - 31.1 µM) and Kn (4.4 - 31.1 µM) started to proliferate, producing new shoots after 3 weeks of culturing. The percentage response of nodal and epicotyl segments producing multiple shoots on combination of BA and Kn is presented in Table 2.5.

The greatest response for enhanced induction of nodal explants was recorded after 8 weeks on MS (93.33 %) and WP (97.78 %) supplemented with 22.2 µM BA + 2.3 µM Kn. The highest mean number of buds per explant was also recorded on the same medium (Table 2.5). However, the mean length of the longest shoots was recorded as 3.33 ± 0.07 cm in the WP supplemented with 13.3 µM BA + 4.6 µM NAA. In epicotyl explant, the highest percentage of cultures that regenerated shoots was 75.56 % and 82.22 % in MS and WP, respectively, as shown in Table 2.5. The highest mean number of shoots per culture recorded was 7.81 ± 0.19 in WP medium supplemented with 22.2 µM BA + 2.3 µM Kn showing better response than MS medium having same hormone concentrations (Figure 2.8A). The mean length of the longest shoots in WP was recorded 3.02 ± 0.04 supplemented with 13.3 µM BA + 4.6 µM NAA. Basal swelling and proliferation of callus could be observed from explants in response to all the plant growth hormones and media types used. Along with callus proliferation, induction of lateral multiple buds started in 2 week old cultures. WP containing 22.2 µM BA and 2.3 µM Kn was, therefore, considered optimal for shoot multiplication.

BA was found to be a more effective growth hormone than Kn for multiple shoot regeneration at concentration (22.2 µM) when used alone in both types of media but the

Table 2.5. Percentage response and multiple shoot formation from *in vitro* raised seedlings in *P. pinnata* (after 8 weeks of culture)*.

Growth Regulators	Concentrations (µM)	Nodal explant						Epicotyl explant					
		MS medium			WP medium			MS medium			WP medium		
		% of explants responded	Mean number of buds per explant	Mean length of the longest shoot (cm)	% of explants responded	Mean number of buds per explant	Mean length of the longest shoot (cm)	% of explants responded	Mean number of buds per explant	Mean length of the longest shoot (cm)	% of explants responded	Mean number of buds per explant	Mean length of the longest shoot (cm)
BA	00	60.00 ± 2.22	1.00 ± 0.11a	2.03 ± 0.07 cd	71.11 ± 1.18	1.00 ± 0.09 a	2.30 ± 0.11 ef	31.11 ± 1.78	1.00 ± 0.00 a	1.06 ± 0.10 c	44.44 ± 1.49	1.00 ± 0.00 a	1.25 ± 0.05 c
	4.4	66.67 ± 2.11	2.00 ± 0.12 b	2.68 ± 0.06 g	80.00 ± 1.92	2.17 ± 0.12 b	2.76 ± 0.05 h i	46.67 ± 2.52	1.71 ± 0.10 bc	2.01 ± 0.09 ef	48.89 ± 1.42	1.77 ± 0.09 bc	2.40 ± 0.07 h
	13.3	86.67 ± 1.85	5.95 ± 0.14 g	2.87 ± 0.04 d	93.33 ± 1.78	7.86 ± 0.23 g	2.94 ± 0.06 i	71.11 ± 1.18	4.94 ± 0.18 f	2.64 ± 0.09 g	75.56 ± 1.14	5.00 ± 0.20 e	2.87 ± 0.04 jk
	22.2	88.89 ± 1.05	7.28 ± 0.15 i	2.19 ± 0.06 h	95.56 ± 1.02	8.21 ± 0.21 i	2.45 ± 0.07 fg	73.33 ± 2.01	6.03 ± 0.15 g	1.54 ± 0.08 e	77.78 ± 1.13	6.17 ± 0.24 f	2.01 ± 0.05 f
	31.1	55.56 ± 1.33	1.88 ± 0.15 b	1.00 ± 0.08 ad	66.67 ± 2.11	1.97 ± 0.14 b	1.46 ± 0.14 b	33.33 ± 2.98	1.20 ± 0.11 ab	0.69 ± 0.08 b	48.89 ± 1.42	1.41 ± 0.11 ab	0.96 ± 0.05 b
Kn	4.6	62.22 ± 1.26	1.04 ± 0.10 a	1.59 ± 0.07 b	73.33 ± 2.01	1.24 ± 0.11 a	1.89 ± 0.10 c	35.56 ± 1.67	1.25 ± 0.11 ab	0.93 ± 0.06 c	51.11 ± 1.39	1.35 ± 0.10 ab	1.14 ± 0.03 c
	13.9	68.89 ± 1.20	2.16 ± 0.13 b	2.05 ± 0.06 c	75.56 ± 1.14	3.35 ± 0.23 b	2.11 ± 0.06 de	42.22 ± 1.53	1.95 ± 0.18 c	1.26 ± 0.04 d	57.78 ± 1.31	2.12 ± 0.11 c	1.49 ± 0.07 d
	23.2	71.11 ± 1.18	2.94 ± 0.16 c	1.89 ± 0.06 c	77.78 ± 1.13	4.77 ± 0.16 c	1.96 ± 0.06 cd	46.67 ± 2.52	2.52 ± 0.11 d	0.87 ± 0.05 c	60.00 ± 2.22	2.96 ± 0.12 de	0.93 ± 0.04 b
	32.5	42.22 ± 1.53	1.80 ± 0.21 b	0.96 ± 0.07 a	55.56 ± 1.33	1.96 ± 0.15 b	0.96 ± 0.05 a	20.00 ± 3.85	1.22 ± 0.15 ab	0.49 ± 0.06 a	31.11 ± 1.78	1.29 ± 0.13 ab	0.57 ± 0.05 a
BA + Kn	13.3 + 2.3	88.89 ± 1.05	6.78 ± 0.17 h	2.63 ± 0.05 g	95.56 ± 1.02	9.44 ± 0.24 h	2.70 ± 0.05 h	71.11 ± 1.18	6.13 ± 0.15 g	2.12 ± 0.06 f	77.78 ± 1.13	6.97 ± 0.21 g	2.74 ± 0.04 ij
	13.3 + 4.6	91.11 ± 1.04	6.70 ± 0.19 h	2.59 ± 0.04 fg	95.56 ± 2.03	9.00 ± 0.17 h	2.69 ± 0.05 h	73.33 ± 2.01	6.18 ± 0.16 g	2.03 ± 0.04 f	80.00 ± 1.92	6.94 ± 0.20 g	2.69 ± 0.04 i
	22.2 + 2.3	93.33 ± 1.78	8.14 ± 0.17 j	2.00 ± 0.05 c	97.78 ± 1.01	11.05 ± 0.21 j	2.23 ± 0.07 ef	75.56 ± 1.14	6.94 ± 0.15 h	1.37 ± 0.05 ed	82.22 ± 1.10	7.81 ± 0.19 h	1.79 ± 0.06 e
	22.2 + 4.6	91.11 ± 1.04	8.05 ± 0.17 j	1.99 ± 0.05 c	97.78 ± 1.01	10.98 ± 0.17 j	2.14 ± 0.07 de	75.56 ± 1.14	6.85 ± 0.20 h	1.00 ± 0.02 c	82.22 ± 1.10	7.76 ± 0.16 h	1.75 ± 0.04 e
BA + NAA	13.3 + 2.3	86.67 ± 1.85	4.87 ± 0.13 e	3.01 ± 0.06 hi	91.11 ± 1.04	6.98 ± 0.18 e	3.24 ± 0.07 j	68.89 ± 1.20	4.42 ± 0.17 ef	2.89 ± 0.05 h	75.56 ± 1.14	4.88 ± 0.18 e	2.96 ± 0.05 k
	13.3 + 4.6	86.67 ± 1.85	4.37 ± 0.16 d	3.13 ± 0.06 i	91.11 ± 1.04	6.56 ± 0.17 d	3.33 ± 0.07 j	64.44 ± 1.24	4.32 ± 0.19 e	2.96 ± 0.04 h	73.33 ± 2.01	4.58 ± 0.16 e	3.02 ± 0.04 k
	22.2 + 2.3	84.44 ± 1.08	5.38 ± 0.15 f	2.40 ± 0.04 e	93.33 ± 1.78	7.36 ± 0.23 f	2.60 ± 0.06 gh	68.89 ± 1.20	4.77 ± 0.22 ef	1.53 ± 0.05 e	75.56 ± 1.14	5.03± 0.17 e	2.17 ± 0.09 fg
	22.2 + 4.6	84.44 ± 1.08	5.03 ± 0.11 ef	2.44 ± 0.04 ef	93.33 ± 1.78	7.26 ±0.16 ef	2.63 ± 0.07 gh	68.89 ± 1.20	4.74 ± 0.24 ef	1.97 ± 0.03 f	73.33 ± 2.01	5.00 ± 0.13 e	2.24 ± 0.05 gh

* Results are for 15 replicates repeated three times ± standard error.

Treatment means followed by the same letters in each column are not significantly different at P<0.05 according to Duncan's multiple range test (DMRT).

presence of Kn in low amounts (2.3 µM) supplemented with BA had a significant positive effect on shoot production. The greatest response for enhanced multiplication was recorded on WP supplemented with BA and kinetin. An average of 11 shoots, 3 cm in length, was observed with the above combination (Figure 2.8B). Subsequent subculturing on the optimal multiplication medium repeatedly for two or three cultures increased the multiplication rate. However, if explants kept on media containing a higher concentration of cytokinin for a longer time it suppressed shoot elongation. Traditionally, WP has been used for the propagation of tree species (Mascarenhas et al. 1982). When WP was used as a basal medium, the responses were greater for all the explant types. MS basal medium with a suitable hormone combination for shoot induction and multiplication did not yield responses as good as with WP. A possible explanation for this response could be the difference in mineral salt composition; WP had a lower concentration of mineral salts. Earlier reports suggest that weaker salt formulations promote axillary bud development in forest trees (McCown & Sellmer, 1987; Bhatt & Dhar, 2004). It is apparent that the presence of exogenous cytokinin at a particular threshold level is important for shoot induction and growth in *P. pinnata*, as single shoots were produced in the absence of cytokinin. The importance of cytokinins has also been demonstrated for *in vitro* shoot production of other members of the family Fabaceae such as in *Clitoria ternatea* (Barik et al. 2007), *Pterocarpus marsupium* (Anis et al. 2005). Cytokinins promote axillary shoot formation by inhibiting apical dominance.

Cytokinins were not effective for the sprouting of axillary buds; hence combinations of cytokinin and auxin were used. BA was far more effective than Kn for inducing proliferation of axillary buds, in contrast to the result reported earlier in *Bauhinia vahlii*, a member of Fabaceae, where Kn was found to be more favourable for multiple shoot induction (Dhar & Upreti, 1999). Explant type, growth medium, and endogenous hormone levels are known to influence shoot induction and these factors may contribute toward the variation observed in the present study. BA has been found to be more favourable than Kn in multiple shoot induction in other woody tree species, *Pterocarpus marsupium* (Anis et al. 2005). The most effective concentration was chosen for the subsequent experiments that aimed at investigating the synergistic effects of BA and low concentrations of Kn or NAA on shoot initiation, multiplication, and elongation. Supplementing lower auxin (NAA) concentration with

cytokinin did not have significant effect on shoot bud multiplication, but it did lead to the formation of large amounts of basal callus. As a result, the chances of explant getting contaminated increased drastically. Incorporation of lower concentration of Kn with best concentration of BA induced a significant increase in induction within two weeks.

2.4.3.1.2 Production of shoots from nodal segments

In case of explants collected from field-grown plants, the period of collection during a particular season proved significant with respect to bud breaks and explant establishment from nodal segments. In the spring, the maximum number of explants survived shoot initiation and the establishment, as compared to those collected during rainy and winter seasons. The rate of contamination was highest during the rainy season. The percentage of nodal segments producing multiple shoots on combination of BA and Kn is shown in Fig. 2.9A. There was no significant variation in multiple bud induction of the explants irrespective of medium type or when supplemented with BA alone or BA in combination with NAA. Furthermore, the amount of basal callus increased as the level of NAA concentration increased from 2.3 to 4.6 µM. Explants when cultured on media containing higher concentrations of cytokinin (31.1 µM BA or Kn) resulted in lower response regeneration frequency than that observed in the control. When the explants were cultured on cytokinin containing media with a concentration of NAA (2.3 µM) elongated shoots were smaller (Fig. 2.8C), and the number of multiple buds were lower (Fig. 2.9B). Maximum elongation (4 cm) was observed when the concentration of growth regulator (BA) used was lower (4.4 µM) than in the shoot-multiplication media. Trimming of shoots after initiation helped to overcome apical dominance and also enhanced the induction of multiple shoots.

2.4.3.1.3 Rooting and acclimatization

Single shoots >1.5 cm were excised from shoot clusters and transferred to a ½-strength MS basal medium with varying concentrations of auxins (NAA, IAA and IBA) for rooting as shown in Table 2.6. Multiplied shoots begin producing roots in the multiplication medium itself which contained both BA and NAA. The number of roots produced in this medium was

limited, and the proliferation was also slow. Attempts to harden rooted plants were not successful. When the multiplied shoots were put on optimal rooting media, the rooting and proliferation response was greater. Shoots started to root after 15 days of culture in the rooting medium, but an additional two weeks of growth were necessary for sufficient development to acclimatize plants in the greenhouse. After two weeks of treatment, the maximum rooting 82.22 ± 2.90 % was observed with 2.5 µM IBA followed by 75.56 ± 3.02 % in 2.5 µM NAA. IAA at concentration 2.5 µM gave only 51.11 ± 3.68 % of rooting. Higher auxin concentration reduced rooting percentage and root number, as shown in Table 2.4. The type and concentration of auxins influenced the average number of roots produced per explant, as well as the mean length of the roots. IBA was found to be more effective in adventitious root formation (Fig. 2.8D). The optimum length of root was observed with 2.5 µM of IBA (Table 2.6).

Table 2.6. Effect of auxin treatment on *in vitro* rooting in shoots of *P. pinnata* cultured in half- strength MS medium*,z

Supplements (µM)	% of explants rooted± SE	Mean no of roots per shoot ± SE	Mean length of the longest root (cm) ± SE
1/2 MS	0.00	0.00	0.00
1/2 MS +0.5 (NAA)	46.67 ± 4.36	1.32 ± 0.06 a	2.07 ± 0.02 a
1/2 MS +1 (NAA)	71.11 ± 1.18	3.78 ± 0.08 e	2.42 ± 0.03 b
1/2 MS +2.5 (NAA)	75.56 ± 3.02	4.79 ± 0.04 f	3.21 ± 0.02 d
1/2 MS +5 (NAA)	60.00 ± 2.22	3.43 ± 0.13 de	2.73 ± 0.08 c
1/2 MS +0.5 (IBA)	66.67 ± 2.11	3.11 ± 0.10 d	2.53 ± 0.05 b
1/2 MS +1 (IBA)	75.56 ± 1.14	4.72 ± 0.12 f	4.03 ± 0.02 e
1/2 MS +2.5 (IBA)	82.22 ± 2.90	6.92 ± 0.09 g	5.14 ± 0.01 g
1/2 MS +5 (IBA)	73.33 ± 2.01	3.29 ± 0.10 de	4.28 ± 0.07 f
1/2 MS +0.5 (IAA)	40.00 ± 2.72	1.10 ± 0.04 a	1.96 ± 0.06 a
1/2 MS +1 (IAA)	46.67 ± 2.52	2.19 ± 0.21 bc	2.30 ± 0.06 b
1/2 MS +2.5 (IAA)	51.11 ± 3.68	2.54 ± 0.15 c	2.95 ± 0.09 c
1/2 MS +5 (IAA)	44.44 ± 1.49	1.85 ± 0.06 b	2.43 ± 0.08 b

* Results are for 15 replicates repeated three times; z Observation recorded after 6 weeks of culturing; Treatment means followed by the same letters in each column are not significantly different at P<0.05 according to Duncan's multiple range test (DMRT); SE = standard error.

Well rooted shoots were transferred in (poly bags containing soil and sand in the ratio of 1:4) for further acclimatization and establishment of the plantlets. Immediate transfer of plants from low light intensity and high humidity to controlled temperature and normal atmospheric humidity conditions caused death of regenerants. A gradual transfer procedure was found imperative. Established plants were transferred from poly bags to earthen pots and covered with polyethylene bags for four weeks to maintain high humidity in growth chamber maintained at 25 ± 2 °C (Fig. 2.8E). Covers were removed upon the appearance of new leaves. The plantlets acclimatized with 70 % success and upon transferring to the greenhouse, plants showed no morphological abnormalities.

2.4.3.1.4 Genetic stability assessment

Out of the 20 different RAPD primers tested, nine primers produced clear and scorable bands. Each primer pair generated a unique set of amplification products that were monomorphic across all the micropropagated plants. Overall, no changes in the amplified fragments were detected among all micropropagated plantlets with reference to donor plant, which confirmed the genetic stability of these plantlets derived *in vitro*. The number of the monomorphic fragments produced by primers ranged from 3 (OPA 03) to (OPAJ 19) 11 and were 0.3 to 2.0 kb in size. Representative banding pattern produced by the RAPD primers is shown in Fig. 2.10. There were no polymorphic DNA fragments among the micropropagated plants or mother plants. In SDS-PAGE analysis, 14 unique bands were observed and the protein patterns of both micopropagated plants and the mother plant exhibited relatively high degree of identity (Fig. 2.11). The molecular sizes ranged from 14 to 116 kDa. The difference among polypeptides was the relative intensity of the stained bands rather than their number. It was observed that the low molecular weight proteins (lower than 66 kDa) produced high intensity bands while the high molecular weight proteins were present in lower relative concentration.

The explant source and mode of regeneration (somatic embryogenesis, organogenesis, axillary bud multiplication) are known to play a major role in determining the presence or absence of variation. Bimolecular analysis using RAPD and SDS-PAGE of

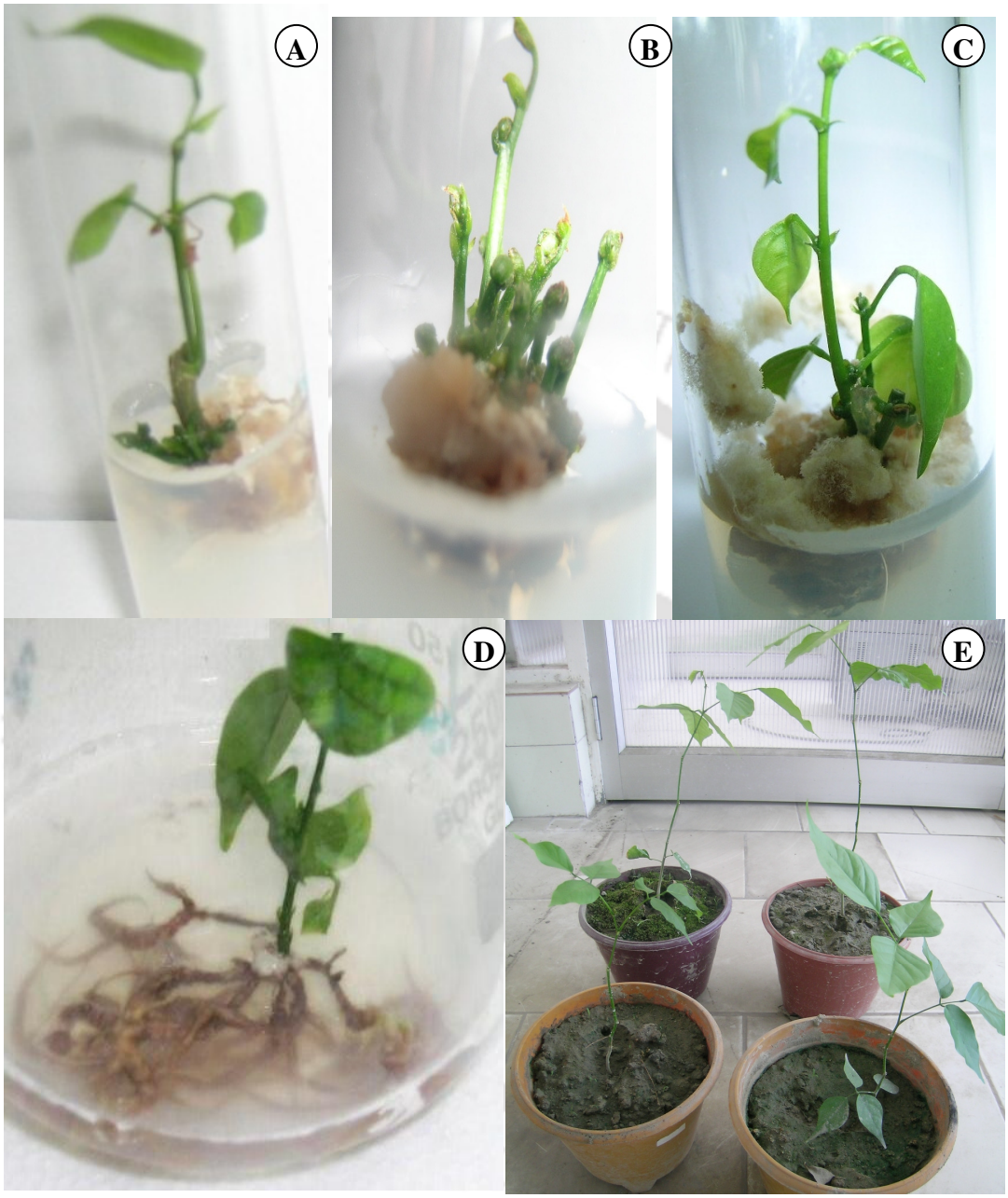


Figure 2.8. Plant regeneration in *P. pinnata.*

A. shoot induction and multiplication from epicotyl in WP medium supplemented with BA and kinetin after 4 weeks; **B.** multiple shoots formed on nodal explant in WP medium (22.2 µM BA + 2.3 µM Kn) after 6 weeks; **C.** elongation of shoot in WP medium (13.3 µM BA + 2.3 µM NAA) after 6 weeks; **D.** rooting of shoots in ½ MS + IBA (2.5 µM) after 4 weeks; **E.** 2- month old plants growing in pot.

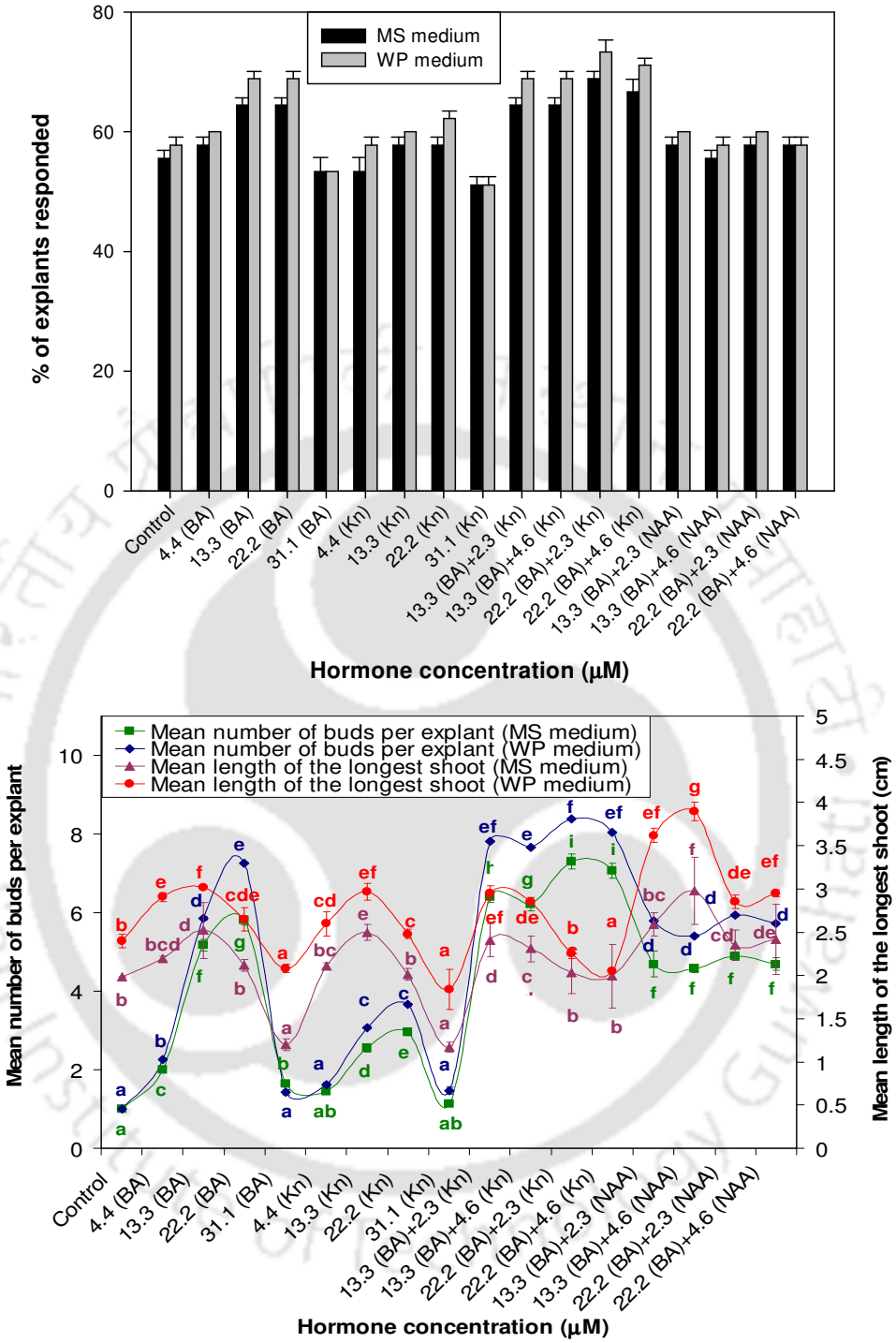


Figure 2.9. Effect of plant growth regulators on shoot production from nodal segments of *P. pinnata* after 8 weeks. Bars represent SE values.

A. percentage response in WP and MS-based media; **B.** mean number of buds/ explant and mean length of longest shoot in WP and MS-based media.

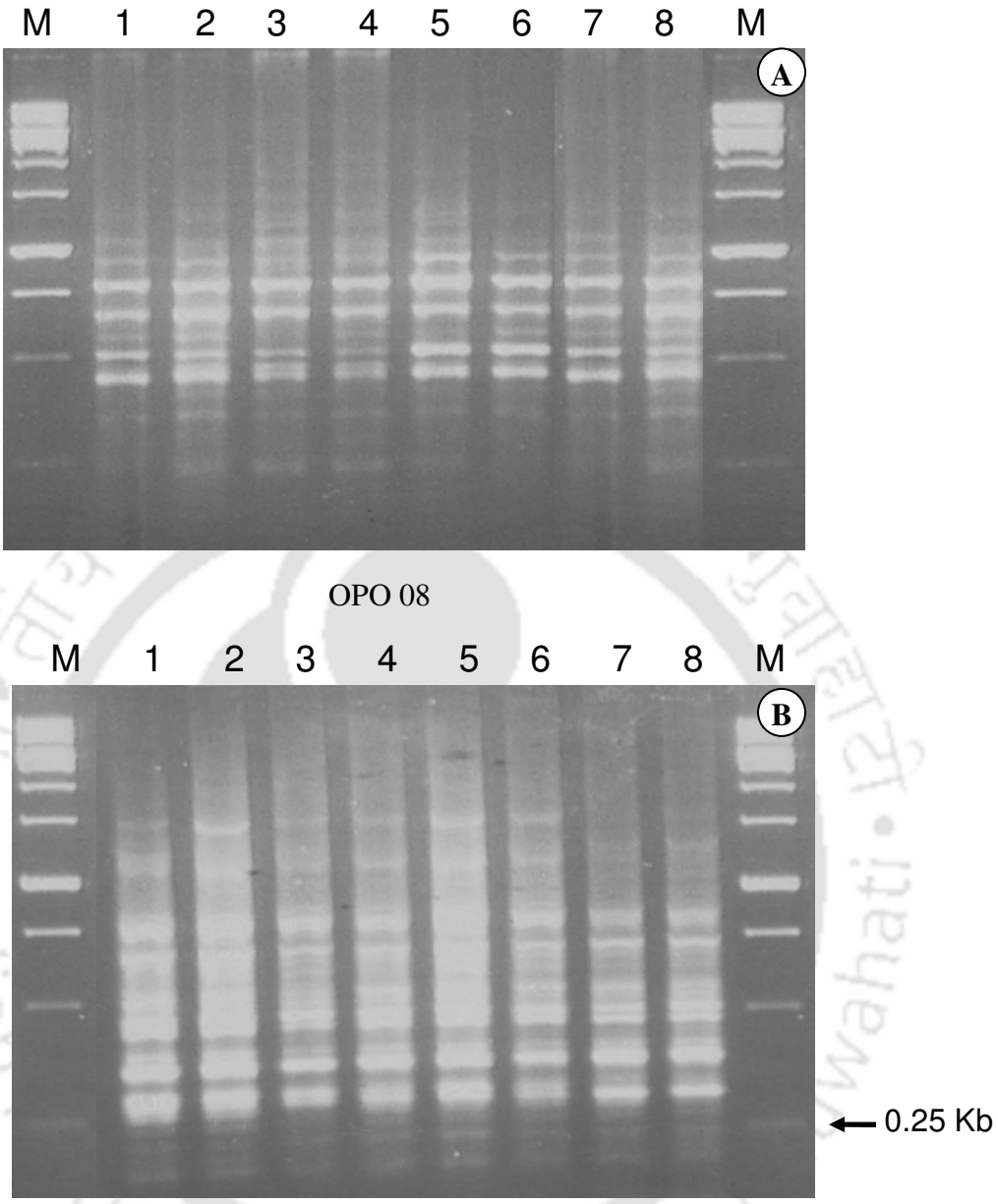


Figure 2.10. RAPD profiles of micropropagated plants from nodal and epicotyl explants of *P. pinnata* using the decamer primers.

A. OPO 08; **B.** OPAJ 19;

M-Marker, 1kb DNA ladder;

Lane 1, DNA from the field grown mother plant;

Lanes 2-8, DNA from micropropagated plants.

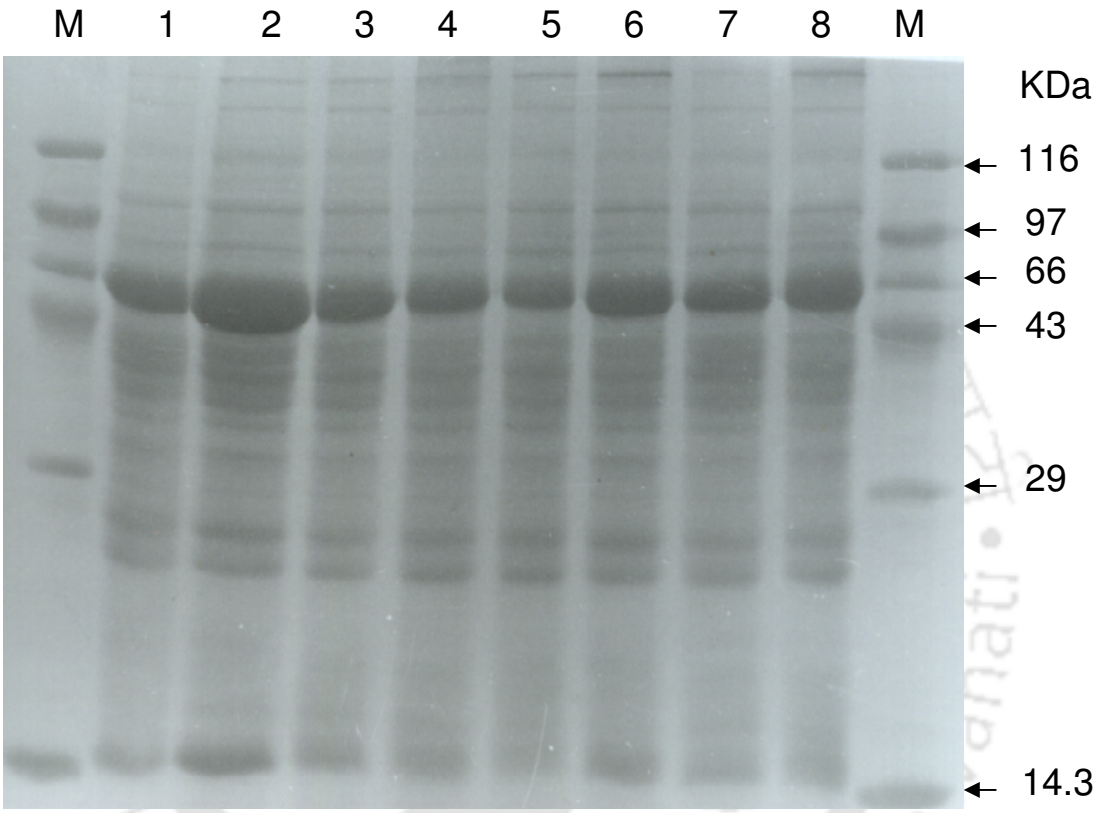


Figure 2.11. Polypeptide profiles from leaves of micropropagted plants of *P. pinnata* using SDS-PAGE analysis.

M- Molecular mass marker (14 – 116 KDa);

Lane 1, Total protein from the field grown mother plant;

Lanes 2-8, Total protein from micropropagated plants.

micropropagated plants of *P. pinnata* showed a profile similar to the mother plant indicating that no variation had occurred *in vitro* at DNA and protein level. RAPD-based assessment of genetic stability of *in vitro*-grown plants has been reported in many other plant species (Martins et al. 2004; Valladares et al. 2006) but no reports are available on SDS-PAGE analysis for *in vitro* grown plants. Axillary meristems were used as explants for micropropagation of *P. pinnata* because it lowers the risk of genetic instability. The results concur with earlier reports that the micropropagation through explants containing organized meristem is generally associated with low risk of genetic instability and are generally more resistant to genetic changes that might occur during cell division or differentiation under *in vitro* conditions (Ning et al. 2007) therefore in this section, *in vitro* regeneration of *P. pinnata* was attempted with the aim of developing a general and reproducible protocol that could be used for further crop improvement and plant breeding programmes.

2.4.3.2 Indirect organogenesis

2.4.3.2.1 Effect of media type on callus induction and regeneration

Many hormone combinations *viz.*: 2,4-D, BA and Kn (at various concentrations and combinations) in WP basal media were tried to induce callus and organogenic response. Callus induction were observed from leaf and root tissues on WP media supplemented with auxin 2,4-D alone and in combination with cytokinin (BA and Kn). The results are summarized in Table 2.7.

Basal media failed to initiate any callus response in both root and leaf. Callus induction was observed in almost all combinations when the medium was fortified with cytokinin (BA, 0.5-1 mg and Kn, 0.5-1 mg) along with 2,4-D (2.5 mg).Callus formation from the roots were observed within 2 weeks in all the concentrations and combinations of plant growth regulators (PGRs) whereas in leaves it was quite slow and takes 4-8 weeks for callus formation. Callusing were observed along the cut ends of the leaf discs and root segments (Fig. 2.12, A & B). With the increasing concentration of cytokinin: auxin ratio (3 mg BA+1 mg 2,4-D, 3 mg Kn+1 mg 2,4-D, 3 mg BA, 3 mg Kn) the cali turned greenish and

compact (Fig. 2.12, C & D). The callus was repeatedly subculture onto fresh set of media for monitoring and testing the organogenic capacity. The experimentation failed to evolve any organogenic or embryogenic response from the leaf disc or root calli. No organogenesis or embryogenesis was recorded.

Table 2.7. Effect of auxin and cytokinin on callus induction from leaf and root explants on WP media

Explant type	2,4-D mg	BA mg	Kn mg	Callus induction	Nature of callus	Texture of callus
Leaf	0	-	-	-	-	-
	1	-	-	++	Soft	Yellowish
	2.5	-	-	+++	Soft	Yellowish, friable
	5	-	-	+	Soft	Brown
	2.5	0.5	-	+++	Soft	Yellowish
	2.5	1	-	+++	Compact	Yellowish green
	2.5	-	0.5	+++	Soft	Yellowish
	2.5	-	1	+++	Compact	Yellowish green
	0.5	3	-	++	Compact	Green
	0.5	3	-	++	Compact	Green
	0.5	-	3	+	Compact	Greenish yellow
	0.5	-	3	+	Compact	Greenish yellow
Root	0	-	-	-	-	-
	1	-	-	++	Soft	Yellowish
	2.5	-	-	+++	Soft	Yellowish
	5	-	-	+	Soft	Brown
	2.5	0.5	-	+++	Compact	Yellowish
	2.5	1	-	+++	Compact	Yellowish
	2.5	-	0.5	+++	Compact	Yellowish
	2.5	-	1	+++	Compact	Yellowish
	-	3	-	+	Compact	Greenish yellow
	-	-	3	+	Compact	Greenish yellow
	1	3	-	+++	Compact	Greenish yellow
	1	3	-	+++	Compact	Greenish yellow
	1	-	3	++	Compact	Greenish yellow
	1	-	3	++	Compact	Greenish yellow

+, ++, +++ positive; - Negative.

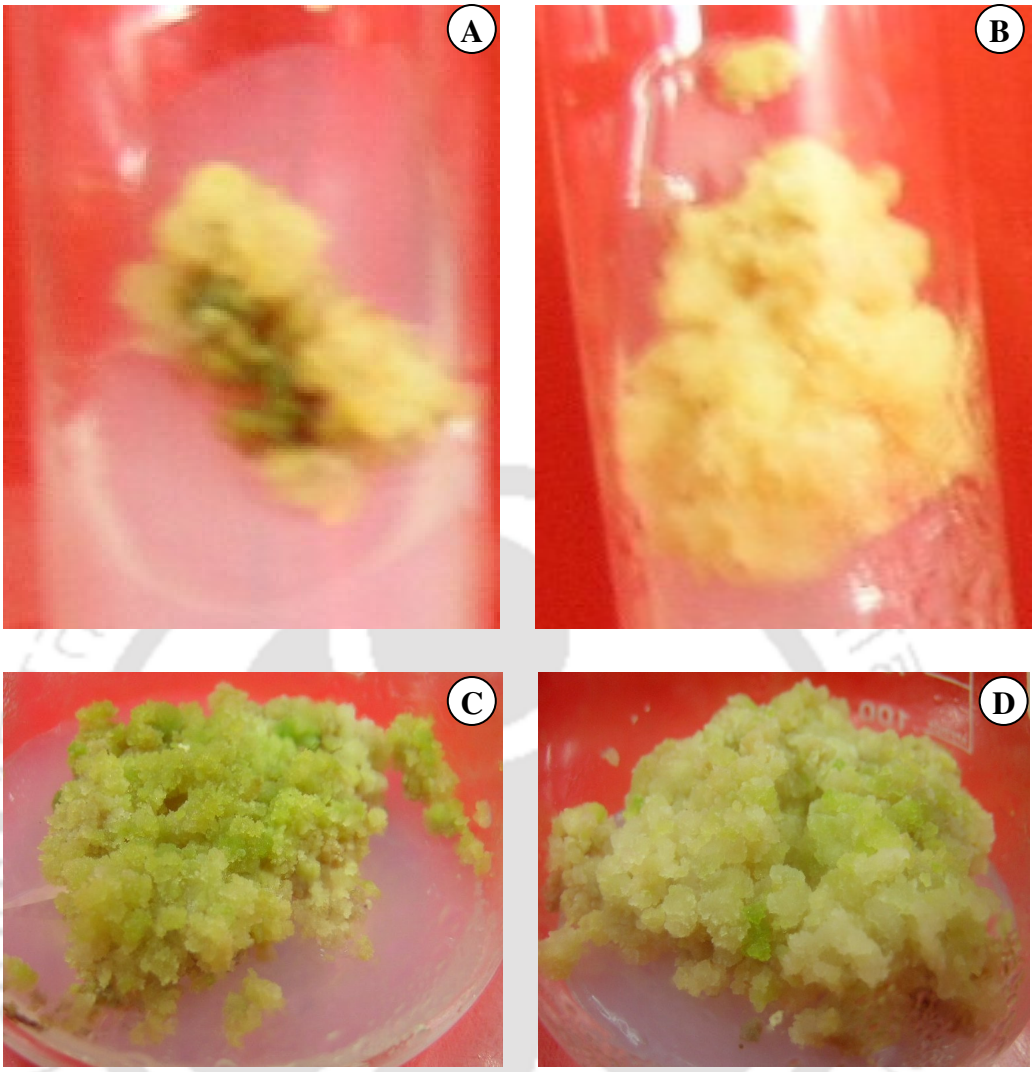


Figure 2.12. Callus induction from leaf and root explants of *P. pinnata*.

A. Callus induction in leaf (2.5 mg 2,4-D);

B. Callus induction in root (2.5 mg 2,4-D);

C. Proliferating leaf calli (3 mg BA+1 mg 2,4-D);

D. Proliferating root calli (3 mg BA+1 mg 2,4-D)

2.5 Conclusion

Today, with the growth of cities and suburbs, lands are no longer available for agriculture, let alone forestry. Thus trees will probably have to be planted on lands that are less than optimal with regard to fertility and water availability. Large scale plantations of CPTs of *P. pinnata*, non edible oil yielding tree species offers scope for rehabilitating degraded degraded lands and improves livelihoods in developing countries. Propagation through seeds leads to genetic variability and makes the crops prone to diseases, propagation through vegetative means offers an advantage in developing true-to-type, disease-free varieties of economically and commercially important plants for clonal multiplication. The result of chapter 2 revealed that the rooting ability of the *Pongamia* is linked to their genotype, in addition to the seasonal effects and other contributing factors. *Pongamia* plantations established by vegetative propagation (stem cuttings) can be considerably more profitable than those using conventional seed propagation techniques. The profitability of cuttings in terms of economic gains can be raised further if the cuttings were to obtain from an elite genotype due to its inherent positive characteristics. The chapter also describes the systematic study of the difference in rooting capacity and their effectiveness from 10 CPTs of *P. pinnata* trees. The procedure presented here provides a rapid mass multiplication by vegetative propagation technique for CPTs of *Pongamia* for raising populations of superior clones in an efficient and cost effective manner, especially in the tropical area where they are grown. Evaluating the total effectiveness of all the mixtures tested, the three-component combinations that had synergistic effect in enhancing the rooting response were found to be the best. This is the first study that shows the synergistic effect of phytohormones for adventitious root production from mature stem-cuttings of CPTs in *P. pinnata*. A more interesting observation is that shoots are formed earlier in CPT, NGPP 46 of *Pongamia* species than roots. Shoots thus formed earlier, due to reserve carbohydrates start producing auxins which moves downward, thereby accumulating in the lower portion of the cuttings. When the concentration reaches a threshold value, endogenous auxins at the extreme basal end start getting metabolized and signal the process of root initiation. The results of the present chapter also elucidated that genotype, exogenous application of auxins, season and juvenility are critical factors in the formation of roots from stem cuttings in CPTs of *P.*

pinnata. The formation of adventitious roots in *P. pinnata is* strongly influenced by genotype and the decline in rooting capacity after maturation of the stock plant. The results of current study on adventitious rooting from CPT varying different natural and synthetic auxins would encourage tree breeding and its commercialization in areas suitable for its growth. It also has important implications for germplasm utilization and may establish a case study of genetic enhancement of biodiesel species (Rangan, personal communication). With an overall rooting success of 40-60 % and with a reasonably good growth rate, production of planting material from stem-cuttings of *P. pinnata* through vegetative means offers opportunities for a cheaper, practically feasible and technically less demanding alternative means of propagation.

Rapid multiplication through micropropagation of organized meristems is now established practice for many other plants, especially horticultural crops and some tree species. Direct morphogenesis offers several advantages in application of biotechnological approaches for improvement of plant and also as an experimental system. The present investigation was initiated to test the feasibility of using this simple approach for inducing regeneration in a woody species like *P. pinnata*. Thus the development of a highly reproducible protocol for regeneration of shoot buds *in vitro* in CPT of *P. pinnata* is also dealt with in this chapter. Previous studies on *P. pinnata* emphasized nodal segments as explant for mass multiplication in MS media. In current study, both epicotyl and nodal explants were used from *in vitro*-raised seedlings and comparison drawn for the response on two different medium. The results demonstrated that for all the tissues used in the present study, WP is more effective than MS media for obtaining a large number of plantlets in a short time. The results clearly showed that addition of lower concentration of auxin along with optimum concentration of kinetin significantly improved the shoot bud formation from all the three explants of *P. pinnata* used in the current study. In other words, the study related to the *in vitro* regeneration underline the importance of auxin/cytokinin ratio in the culture medium. The results obtained suggests that *in vitro* shoot multiplication using axillary meristem in *P. pinnata* may be used for rapid clonal propagation and conservation with high genetic stability particularly for elite clones of this important biodiesel plant.

In Indian context, large scale plantation of CPT of *P. pinnata*, a potential biodiesel tree species which is the ultimate aim of this chapter would not only serve to reduce import of petrodiesel but also aid in generation of employment opportunities, accelerated rural development and meeting the environmental obligations such as reduction of green house gases, carbon sequestration, etc. Further large wasteland could be utilized for the cultivation of non-edible oil producing trees for production of biodiesel. The study reported here has applied significance. Standardization of the vegetative propagation techniques, *in vitro* propagation techniques and establishment of PGRC (*Pongamia* Genetic Resources Center) can be a timely step in this direction for large scale production of genetically superior saplings throughout the year. Further investigations will be needed to modify the *in vitro* protocol for gene transformation of this species.


भारतीय प्रौद्योगिकी संस्थान गुवाहाटी
Indian Institute of Technology Guwahati

Chapter 3

Chapter 3

Systematic characterization for genetic diversity studies in CPTs

3.1 Introduction

Pongamia pinnata is an outcrossing tree species, starts yielding at the age of 4-7 years. Recognition of the genetic variability is the base of plant development and evaluation programs and is suitable for selection of superior genotypes with desired traits. Systematic germplasm collection, characterization and evaluation programme is essential to identify superior planting material from the existing natural variations. Research at the molecular level on *P. pinnata* has lagged behind than that of other biodiesel crops such as *Jatropha*. No significant research has been undertaken to improve *Pongamia* for the desired traits. One of the important factors restricting the large scale production and development of high seed and oil yielding varieties/genotypes is that no information is available about genetic diversity in naturally growing populations of *P. pinnata* at inter or intra populations level. It is therefore essential that genetic variability in naturally growing *P. pinnata* populations be assayed in the context of total available genetic diversity for each individual. Considerable diversity exits among the naturally growing populations of *P. pinnata* as evident from chapter 1 that was based on morphometric (both vegetative and reproductive) traits (Kesari et al. 2008). This diversity of *P. pinnata* is a very important consideration for the establishment of biofuel cropping system and for the success of crop breeding programs (Kesari et al. 2009a). DNA marker technology has been rapidly developing and many techniques that seemed unfeasible before are now routinely used (Asif & Cannon, 2005). The DNA based markers significantly shortens the evaluation process and eliminates the problematic influence of external factors. The same DNA marker may also be used in identifying genotypes, determine the degree of genetic similarity and even selection of genotypes that deliver the most desirable attributes such as marker assisted selection (MAS). So far, the seeds which are rich source of triglycerides had very little attention from plant molecular biologists. Thus, the present chapter

was undertaken with the objective to screen the untapped genetic variability in CPTs of *P. pinnata* tagged from North Guwahati, Assam, using SDS PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) seed protein marker and PCR based DNA markers. The isolation of high quality DNA is a prerequisite for any molecular biological studies when using the seed source. Because the seed contain high amounts of many different substances, it is unlikely that just one nucleic acid isolation method suitable for all seed can ever exits. Standardized protocols for DNA isolation failed to yield high quality DNA from seeds of *Pongamia* that are rich source of triglycerides. To evaluate the genetic diversity in CPTs of *P. pinnata*, the objectives of this chapter encompasses: (i) standardization of an efficient protocol for isolating high quality PCR amplifiable genomic DNA from seeds of CPT of *P. pinnata*, (ii) to evaluate the level and pattern of genetic variability/relatedness among 10 CPTs of *P. pinnata* at the inter and intra-population level using different markers system (RAPD, ISSR and AFLP), (iii) to compare the informativeness of RAPD, ISSR and AFLP assays employed at the level of populations and individuals for analyzing the *P. pinnata* genome.

3.2 Review of Literature

Individuals of a population differ from each other at the morphological expression or genetic basis. In practice, distinctness or variability is required to differentiate between individuals at various steps of seed production, breeding and its further improvement for the desired traits. The effectiveness of tree breeding or improvement programme depends upon the nature and magnitude of existing genetic variability. Traditionally, morphological and agronomic traits have been used to measure genetic diversity but both these approaches have their limitations when it comes to precise positioning of the species in a dendrogram, since they are not always complete representative of the genetic structure (Noli et al. 1997). Conventional taxonomic techniques in conjunction with molecular biology tools may go a long way in providing accurate and powerful way of analyzing genetic relationship in the genus *Pongamia*. Despite the importance of *Pongamia* as a biodiesel legume plant and availability of appropriate molecular genetic tools, there have been few studies on the genetic structure and variation in this species (Caetano-Anollés et al. 1991; Lavin et al. 1998). Gross chromosome organization studies showed that *P. pinnata* is a diploid with 2n=20 or 2n=22 (Sarbhoy, 1977). The

characters used to evaluate systematic relationships and to discriminate between *Pongamia* individuals are traditionally and primarily morphological characters (quantitatively and qualitatively) being scored in field and seed laboratory tests (Kaushik et al. 2007; Kesari et al. 2008). Though such phenotypic evaluations are important, number of morphological traits is limited and is not understood at gene level. This is because most economic characters are polygenically inherited and their expression is influenced by environmental conditions (Reddy et al. 2002). The germplasm diversity evaluation has been tremendously empowered by invoking biomolecular analytical techniques like polypeptide and DNA polymorphism profiling, thus facilitating direct and reliable measurements of genetic divergence (Sangwan et al. 2003; Weising et al. 2005; Simmons et al. 2007; Li et al. 2008). Polypeptide has been more popularly applied to seeds analysis for diagnostics and estimating outcrossing rates (Ferreira et al. 2000). SDS PAGE of seed storage proteins is considered to be a practical and reliable method of genotype identification or differentiation because seed storage proteins are largely independent of environmental fluctuations (Javaid et al. 2004; Iqbal et al. 2005; Ghafoor & Ahmad, 2005).

Seed protein profiling studies have been done with leguminous species such as *Arachis* sp (Bianchi-Hall et al. 1993; Lanham et al. 1994). DNA based markers may extend and complement characterization based on morphological and polypeptide descriptions, providing more speed, accurate and detailed information. In addition, it is independent on the growth, stage, season, location and agronomic practices which gives them a high value (Lombard et al. 2001). Various molecular markers, based on genomic DNA nucleotide variations such as RFLPs (Hubbard et al. 1992), RAPDs (Williams et al. 1980; Wen et al. 2004), AFLPs (Zhang et al. 2001) and microsatellite markers such as SSRs (Esselink et al. 2003) and ISSRs (Zietkiewicz et al. 1994) have been used for analysis of genetic diversity and establishing relationships between individuals (Juchum et al. 2007; Li et al. 2008). Unlike RFLP, PCR-based DNA markers (RAPD, AFLP and ISSR) are technically simple, do not require hybridization and radioactive materials and have good throughput with relatively low cost and have been used extensively for assessing genetic variation within the species to measure the genetic diversity (Williams et al. 1990; Surekha & Larson, 2005).

A number of methods are available and are being developed for the isolation of nucleic acid from seeds and dehydrated tissues (Tomas & Tanksley, 1989; Chunwongse et al. 1993; Kang et al. 1998). It has been observed that increasing the ionic strength of the extraction buffer by the addition of NaCl lead to increased efficiency in the removal of polysaccharide contaminants and improves the DNA yield (Smith et al. 1991; Fang et al. 1992; Tibbits et al. 2006). Higher concentration of β-mercaptoethanol prevents oxidation of the secondary metabolites in the disrupted seed tissues. SDS an anionic detergent binds and denatures the proteins. Substituting ethanol for isopropanol has been shown to increase the yield and purity of DNA (Bult et al. 1992; Syamkumar et al. 2005). The seeds of plants contain exceptionally high amount of polysaccharides, polyphenols, and other secondary metabolites that can hamper DNA isolation, amplification, restriction digestion and subsequent molecular cloning (Webb & Knapp, 1990; Demeke & Adams, 1992; Jobes et al. 1995; Weishing et al. 1995; Porebski et al. 1997; Pirttila et al. 2001).

McDonald et al. (1994) reported use of DNA from dry seeds for varietal identification studies using RAPD analysis. Genetic diversity analysis by RAPD marker using seed bulk was also investigated in *Brassica oleraceaea* L. collection (Divaret et al. 1999). For inter and intra-population assessment with limited genetic variability, the molecular markers of choice must be very informative. Principle of RAPD and ISSR amplification are shown in Fig. 3.1. and steps involved in AFLP amplification are shown in Fig. 3.2. Although newer techniques like AFLPs, microsatellites (SSR and ISSR) are preferred due to their informativeness, RAPD analysis is still used because of its simplicity, low cost and lower infrastructure requirement (Williams et al. 1990; Caetano-Anollés et al. 1991). RAPD supports the simultaneous detection of polymorphism at many loci in the entire genome. Rout et al. (2009) investigated inter and intra-population variability of 111 individuals of *P. pinnata* occurring in different agroclimatic zones of Orissa using RAPD marker. The great advantages of ISSR analysis are accuracy, high polymorphism, genomic abundance and are randomly distributed throughout the genome. ISSR amplifies the DNA sequence between the two SSRs loci. ISSR primers are based on di-, tri-, tetra or pentanucleotide repeats with 5' or 3' anchored base(s). However, the greatest advantage of AFLP technique is its ability to reveal polymorphism at the DNA sequence level within the populations and

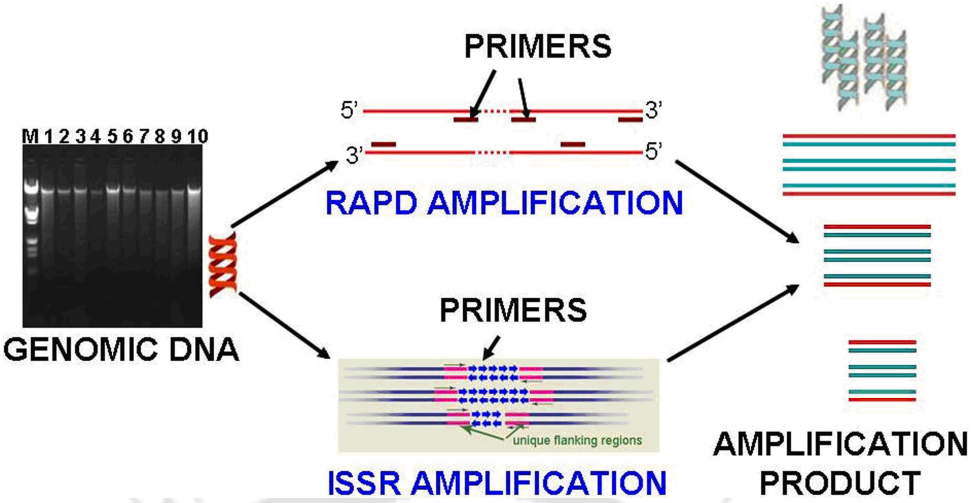


Figure 3.1. Principle of RAPD and ISSR amplification.

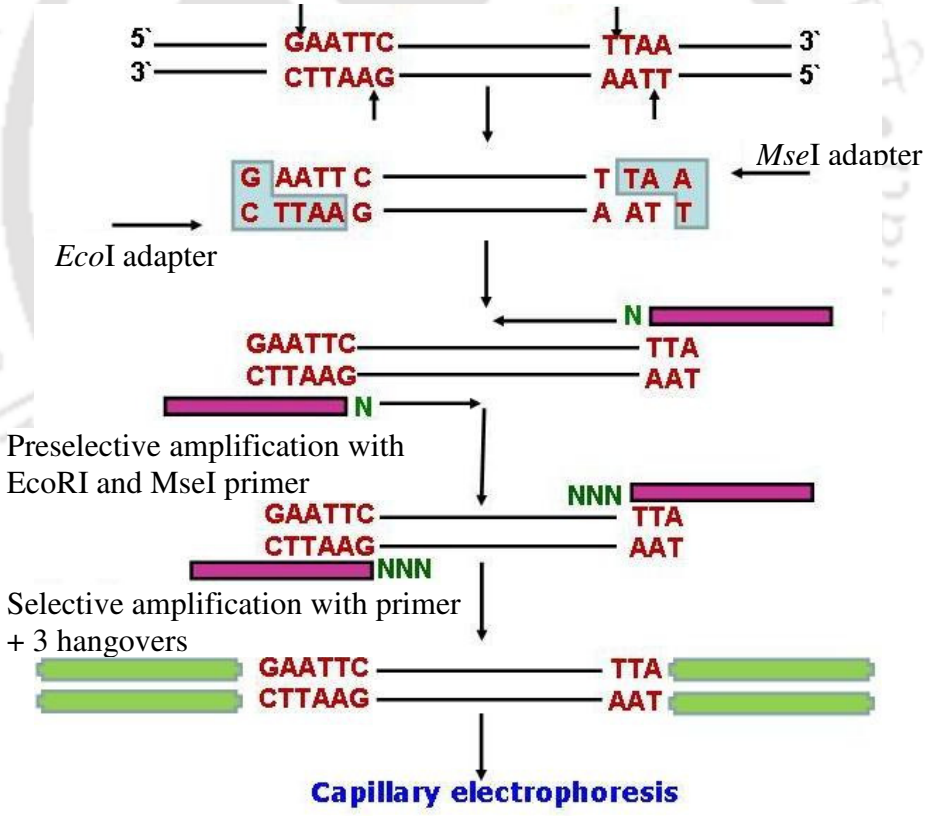


Figure 3.2. Schematic diagram showing the steps involved in amplified fragment length polymorphism (AFLP).

between individuals due to its potential to generate higher number of loci per assay and hence higher polymorphism rate (Surekha & Larson, 2005). These methods are adequate for describing population genetic diversity and effects of fragmentation, and are easy to implement when little or no molecular genetic research has been conducted for the species (Bartish et al. 2000; Nybom, 2004).

3.3 Methods

3.3.1 Genomic DNA isolation from seeds of CPT

Genomic DNA isolation from the seeds of CPT of *P. pinnata* was performed using CTAB method (Doyle & Doyle, 1989), SDS method (McCouch et al. 1988) and modified SDS method of McCouch et al. (1988).

3.3.1.1 Plant material

For DNA extraction healthy ripened seeds from mature trees were used (~5 g fresh weight). Seeds of CPT, NGPP 46 (approximately 2 in number) were collected in the month of April - May, 2007 (matured) (Sila Forest Range, North Guwahati, 26 °14'6" N, 91 °41'28" E, Assam). All the seed samples were appropriately stored at -20 ºC & -80 ºC for all future biomolecular analysis.

3.3.1.2 Reagents and solutions

1 M Tris Base (pH 8.0); 0.5 M EDTA (pH 8.0); 10 % sodium dodecyl sulphate (SDS); 5 M NaCl; β-mecaptoethanol; 5 M potassium acetate; 3 M sodium acetate (pH 5.2); Phenol:chloroform (1:1 v/v); Isopropanol, -20 ^{o}C; Absolute ethanol, -20 ^{o}C; 70 % (v/v) ethanol; TE: 10 mM Tris-HCl, pH 8; 1 mM EDTA, pH 8.0; 10 mg/mL RNase A (Sigma); Random decamer primers (Operon Technologies, Inc., USA); dNTPs (10 mM) (Finzymes); 1 % CTAB (Sigma); *Taq* DNA polymerase (5 U/µL) (Finzymes); *Taq* DNA polymerase buffer (Finzymes); *Eco*RI and *Bam*HI restriction enzymes (Bangalore Genei, India); Restriction endonuclease buffer (Bangalore Genei, India); DNA extraction buffer: 100 mM

Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 1.5 M NaCl, 1.25 % SDS and 5 % β-mercaptoethanol (v/v) added immediately before use.

3.3.1.3 DNA quantification and restriction digestion

DNA concentration was measured by obtaining the $A_{260/280}$ ratio with a spectrophotometer (Cary 100Bio; Varian). DNA quality and concentration was also checked by running 2 µL of DNA from each sample on a 1 % agarose gel containing 0.5 µg/mL of EtBr. Restriction digestion was done according to supplier's instructions. Around 2 µg of DNA was restricted with *Bam*HI and *Eco*RI and visualized on 1 % agarose gel.

3.3.1.4 PCR amplification by RAPD primer

PCR amplification of the isolated DNA was done using random primers (OPA 1-10) obtained from Operon Technologies, USA. PCR amplification was performed in 25 µL reaction volume (50 ng of DNA, 1 U *Taq* DNA polymerase, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 5 pmol of decanucleotide primers) using a DNA Thermal cycler (Applied Biosystems, USA) according to the procedure of Williams et al. (1990). Amplified products were loaded in 1.5 % agarose gel containing 0.5 µg/mL of EtBr and documented by a gel documentation system (Bio Rad, USA)

3.3.2 Genetic diversity studies in CPTs

3.3.2.1 Plant material

The germplasm used in this study for genetic diversity studies were seeds from identified CPTs of *P. pinnata* collected from each of the 10 different genotypes (NGPP 26-30 & NGPP 46-50) belonging to two different naturally growing populations viz. 6 and 10 from the study site. Seeds of *P. pinnata* were collected once during the month of September, 2007 (immatured) and another in the month of April - May, 2007 (matured). Storage conditions were same as mentioned in section in 3.3.1.1.

3.3.2.2 Protein extraction and SDS-PAGE

Seeds of two different stages (immatured and matured) for all the 10 CPTs were homogenized in Tris-EDTA buffer (pH 8.0) containing 10 mM Tris-HCl (pH 8.1), 10 mM EDTA (pH 8.0), 5 mM ß-mercaptoethanol and 0.1mg/mL pMSF (phenylmethanesulfonyl fluoride). The slurries after 15-20 min of homogenization were transferred into eppendorf vials and centrifuged at 12,000 *g* at 4 °C for 10 min. The supernatants were collected and protein concentrations were measured by Bradford (1976) method using BSA as a standard. Proteins were resolved using discontinuous 12 % SDS-PAGE according to Laemmli (1970). After electrophoresis, gels were stained overnight with 0.25 % CBB R250. In order to check the reproducibility of the method, two separate protein extraction were carried out and two separate protein gels were run under similar electrophoretic conditions.

3.3.2.3 Genomic DNA extraction

Genomic DNA extraction for genetic diversity studies was performed by SDS method of McCouch et al. (1988), CTAB method of Doyle and Doyle (1987) and modified SDS method of McCouch et al. (1988).

3.3.2.4 RAPD analysis

For RAPD fingerprinting, PCR amplification of the genomic DNA was carried out using 20 arbitrary decamer oligonucleotide primers (Table 3.1) (Operon Tech, USA). Each reaction mixture of 20 µl contained 50 ng/µl of template DNA, 1 x assay buffer (100 mM Tris sulfonic acid, pH 8.8, 15 mM $MgCl_2$, 500 mM KCl and 0.1 % gelatin), 0.2 mM each dNTPs (B'LGenei, India), 5 pM of each primer and 0.05 U of Taq polymerase (B'LGenei, India). The reaction was performed in 0.2 ml microfuge tubes (Dialabs). PCR amplification was carried out in a Mini Thermal Cycler (Applied Biosystems 9700) programmed for 35 cycles. The first amplification cycle consisted of initial denaturation step of 5 min at 94 ^{o}C. This was followed by 34 cycles of 45 sec at 94 ^{o}C, annealing for 1 min at 32 ^{o}C, and extension at 72 ^{o}C for 1 min 30 sec. An additional cycle of 5 min at 72 ^{o}C was used for primer extension. The amplification products were electrophoresed in 1.5 % agarose gels in 0.5 x TBE (10x stock contained 0.8 M Tris, 0.8 M boric acid, 0.5 M EDTA). The gels were photographed under a UV transilluminator.

3.3.2.5 ISSR analysis

PCR amplification was carried out for 20 ISSR primers. Table 3.2 lists each marker, repeat type and length, primer sequence and annealing temperature. The PCR composition was same as that used for RAPD analysis with a final volume of the reaction mixture being 20 µl. PCR conditions were: denaturation at 94 ^{o}C for 4 min, 35 cycles at 94 ^{o}C for 45 sec, annealing time was 1 min with varied temperatures as per the melting temperature of the ISSR primer used (Table 3.2), extension at 72 ^{o}C for 1 min 30 sec and final extension at 72 ^{o}C for 5 min. The amplified products were visualized in a 1.5 % agarose gel containing ethidium bromide and photographed for further analysis.

3.3.2.6 AFLP analysis

AFLP markers were obtained with the Plant Mapping Kit (Applied Biosystems) and assay was performed as per the manufacturer's protocol followed by Vos et al. (1995) with minor modifications. DNA (200 ng) was double digested with two restriction endonucleases *Eco*R1/*Mse*I for 2 h at 37 °C and heated at 70 °C for 15 min to inactivate the enzymes. The DNA fragments were then ligated to their respective adapters using ligation solution (*Eco*R1/*Mse*I adapters, 0.4 mM ATP, 10 mM Tris-HCl at pH 7.5, 10 mM Mg-acetate, 50 mM K-acetate) and 1µl T4 DNA ligase at 20 °C for 2 hrs. The diluted RE-ligation mixture (10 fold) was used for preselective amplification with preselective primers to reduce the overall complexity of the mixture by increasing concentration of the target sequences so that the double digested fragments become predominant. The pre-selective amplification cycles contained 20 cycles of denaturation (at 94 °C for 60 sec), primer annealing (at 56 °C for 60 sec) and primer extension (at 72 °C for 2 min). The preselective PCR product was diluted in a ratio of 1:50 with TE buffer (pH 8.0) and then used as a template for the selective amplification.

The selective amplification of specific target sequences was performed using selective primers available in the AFLP kit. Four primer combinations were used (Table 3.3). The *Eco*RI primers are labeled with different fluorescent dyes (6-FAM, JOE and NED), which make possible multiplexing of three primer pairs in one reaction. The selective amplification involved PCR amplifications with following thermal cycling conditions: denaturation at 94 °C for 2 min

followed by primer annealing at 65 °C for 30 sec and primer extension at 72 °C for 2 min. The annealing temperature was reduced by 1 °C every cycle till it reached 56 °C. At this annealing temperature, 20 more cycles of PCR amplifications were performed. This was followed by a final cycle of primer extension step at 60 °C for 30 min. Preselective and selective amplifications were carried out in a Gen Amp polymerase chain reaction (PCR) system 9700 thermocycler (Applied Biosystems) following the manufacturer's protocol.

Selectively amplified product (1µl) was mixed with 0.5 µl of the GeneScan 500 ROX internal size standard (Applied Biosystems P/N 402985) and 8.5 µl of Hi-Di Formamide (Applied Biosystems P/N 4311320). The PCR products were then denatured prior to separation by capillary gel electrophoresis on an automated DNA sequencer (ABI Prism 310, PE Applied Biosystems). The electrpherograms generated by the sequencer were interpreted with Gene scan software. Genotyper software was then used to create a list of fragments detected in each lane by fragment size. Fragments sized from 50 to 500 base pairs (bp) with a peak height >50 in the electropherogram were retained for subsequent analysis. Peakmatcher software (DeHaan et al. 2002) was used to convert the list of fragments detected in each sample into a binary table (1/0) for the presence or absence of each fragment in each sample. Peak matcher was set to retain markers with repeatability >90 %.

Table 3.1. Random oligonucleotide primers selected for amplification and polymorphism for 10 CPTs of *P. pinnata*.

S.No.	Primer	Sequence (5'-3')	S.No.	Primer	Sequence (5'-3')
1	OPC 07	GTCCCGACGA	11	OPA 12	TCGGCGATAG
2	OPL 11	ACGATGAGCC	12	OPAP 10	TGGGTGATCC
3	OPO 08	GCTCCAGTGT	13	OPAA 01	AGACGGCTCC
4	OPA 08	GTGACGTAGG	14	OPAB 01	CCGTCGGTAG
5	OPAH 15	CTACAGCGAG	15	OPAB 05	CCCGAAGCGA
6	OPAM 20	ACCAACCAGG	16	OPAB 14	AAGTGCGACC
7	OPAN 01	ACTCCAGGTC	17	OPAH 13	TGAGTCCGCA
8	OPAO 01	AAGACGACGG	18	OPAI 15	GAGACAGCCC
9	OPAP 20	CCCGGATACA	19	OPAJ 19	ACAGTGGCCT
10	OPAN 05	GGGTGCAGTT	20	OPX 20	CCCAGCTAGA

Table 3.2. ISSR oligonucleotide primers selected for amplification and polymorphism for 10 CPTs of *P. pinnata*.

S.No.	Primer	Sequence (5'-3')	S.No	Primer	Sequence (5'-3')
1	HB 12	CACCACCACGC	11	817	CACACACACACACACAA
2	HB 13	GAGGAGGAGGC	12	818	CACACACACACACACAG
3	HB 14	CTCCTCCTCGC	13	824	TCTCTCTCTCTCTCTCG
4	HB 15	GTGGTGGTGGC	14	$(CAG)_5$	CAGCAGCAGCAGCAG
5	P 6	CCACCACCACCACCA	15	807	AGAGAGAGAGAGAGAGT
6	P 8	CACCACCACCACCAC	16	825	ACACACACACACACACT
7	P 3	AGAGAGAGAGAGAGAGTG	17	826	ACACACACACACACACC
8	809	AGAGAGAGAGAGAGAGG	18	872	GATAGATAGATAGATA
9	811	GAGAGAGAGAGAGAGAC	19	$(AT)_8$	$(AT)_8$
10	816	CACACACACACACACAT	20	$(GATA)_4$	$(GATA)_4$

Table 3.3. AFLP primers combinations used for genetic diversity studies in 10 CPTs of *P. pinnata* belonging to populations 6 and 10 respectively.

Purpose	Oligonucleotide sequence
Adapters	
*Eco*RI- Adap seq 1	5'-CTCGTAGACTGCGTACC-3'
*Eco*RI- Adap seq 2	3'-CATCTGACGCATGGTTAA-5'
*Mse*I- Adap seq 1	5'-GACGATGAGTCCTGAG-3'
*Mse*I- Adap seq 2	3'-TACTCAGGACTCAT-5'
Primers	
Preamplification	
*Eco*RI- Primer	5'-GACTGCGTACCAATTCA-3'
*Mse*I- Primer	5'-GATGAGTCCTGAGTAAG-3'
Selective amplification	*Mse*I - CAA / *Eco*RI - ACT
	*Mse*I - CAC / *Eco*RI - ACA
	*Mse*I - CAG / *Eco*RI - AAC
	*Mse*I - CAT / *Eco*RI - ACC

3.3.2.7 Data analysis

For all the three types of marker systems, duplicate samples from each individual were tested and only clear, unambiguous, and reproducible bands amplified in both were considered for the scoring and data. To avoid taxonomic weighing, the intensity of bands was not taken into considerations, only the presence of band was taken as indicative. The numbers of polymorphic and monomorphic amplification products were determined for each primer for 10 genotypes. To compare the efficiency of primers polymorphic information content (PIC); as a marker discrimination power, was computed using the formula PIC = 1-$\sum p_i^2$, where p_i is the frequency of ith allele at a given locus (Anderson et al. 1993) and also marker index was calculated. The basic parameters for genetic diversity were calculated in the POPGENE application (Yeh et al. 1999). The polymorphism of amplification products (P), the number of observed alleles (n_a), the mean number of effective alleles (n_e), the mean Nei's gene diversity index (h), the Shannon index (I) and the level of gene flow (N_m) were determined. Within accession diversity (H_s), total gene diversity (H_t) and interpopulation differentiation (G_{st}) (Nei, 1973) were calculated within the species and within population using the POPGENE software.

Level of similarity among genotypes/ individuals was established as percentage of polymorphic bands, and a matrix of genetic similarity obtained by using the Dice's coefficient (1945) using the SIMQUAL program of NTSYS. Applying the UPGMA (Sneath & Sokal, 1973) method on this matrix using the SHAN subroutine through the NTSYS- pc (Numerical taxonomy system, 2.2 version) (Numerical taxonomy system, Applied Biostatistics, N.Y.) (Rohlf, 2002) a dendrogram generated representing the genetic relationship among 10 CPTs of *Pongamia*. The correlation between the original similarity indices and cophenetic values was calculated, and the Mantel's test (Mantel 1967) was performed using 250 permutations to check the goodness of fit of the CPTs to a specific cluster in the UPGMA cluster analysis to the similarity matrix on which it was used. Principal component analysis was undertaken for the families with modules STAND, CORR and EIGEN of NTSYS-pc (Rohlf, 2002) using the Euclidean distances derived from the standardized values using the software package NTSYS-pc-2.2.

3.4 Results and discussion

3.4.1 Genomic DNA isolation from the seeds of CPT and its PCR amplification

Seeds are storage organs and hence rich in proteins, lipids, polysaccharides, alkaloids and other secondary metabolites. These compounds can interfere with DNA isolation and successive amplification. While adapting the standard protocols of McCouch and CTAB method of Doyle & Doyle (1987), it was observed that the final DNA preparation was brown having lot of mucilage and was recalcitrant to restriction digestion and/or amplification. Hence the original protocol of McCouch was modified. DNA was successfully isolated from seeds of *P. pinnata* following SDS method of McCouch et al. (1988) after its modification as mentioned below.

One g of powdered tissue was homogenized with 5 mL of freshly prepared DNA extraction buffer (preheated to 65 ^{o}C) in a 15 mL polypropylene tube. Care was taken not to allow the powder to thaw at any point before homogenizing with extraction buffer. This was done by keeping the temperature below 0 ^{o}C so as to inactivate the oxidizing enzymes during the homogenization. The contents were mixed well by shaking the tube vigorously and incubating at 65 ^{o}C in a water bath for 20 min with occasional mixing. About 1.5 mL of 5 M potassium acetate (CH_3COOK) was added, agitated and incubated on ice for 20 min. Tubes were then centrifuged at 2795 *g* for 20 min at 4 ^{o}C. The aqueous clear phase was transferred to a fresh 15 mL centrifuge tube to which 2/3 volume of ice-cold 2-propanol was added and incubated at -20 ^{o}C for 2 hour to precipitate the DNA. Fibrous DNA settled as pellet can be separated from supernatant (containing impurities) by centrifugation at 2795 *g* for 20 min at 4 ^{o}C. The DNA pellet washed with 70 % ethanol (EtOH), vacuum dried for 10 min, is finally resuspended and dissolved in 1 mL Tris-EDTA (TE) buffer. RNase A (10 mg/mL) was added to each sample and incubated at 37 ^{o}C for 30 min to digest ribonuclease. Further extraction was done with equal volumes of phenol:chloroform to remove the unwanted impurities including proteins. The tubes were centrifuged at 11180 *g* for 10 min and the aqueous layer was transferred to a fresh 2 mL eppendorf tube. To this 1/10 volume of sodium acetate (CH_3COONa) and 2 volumes of absolute ethanol was added and incubated at -20 ^{o}C for an hour.

The fibrous DNA can be directly hooked out from the solution using an improvised hook (Pasteur pipette bent at the tip), washed with 70 % EtOH, vacuum dried to remove traces of EtOH and resuspended in TE buffer (amount of TE buffer used decided by the size of the DNA pellet). Alternatively if the DNA precipitate is not hookable from the salt solution, can be centrifuged at 2795 *g* for 10 min, washed with 70 % EtOH, air-dried and then resuspended in TE buffer. Care was taken not to over dry the DNA pellet making it difficult to dissolve in TE buffer. DNA samples were stored in 4 ^{o}C refrigerator for short-term use and in -20 ^{o}C freezer for long-term. The original SDS protocol of McCouch et al. (1988) employed for rice DNA extraction makes use of low salt concentration (0.5 M NaCl) and isopropanol for final DNA precipitation. Further the DNA preparation employing standard protocols was found to contain lot of mucilage and was also recalcitrant to restriction digestion and PCR amplification. The reason for this is that tree species often contain large amounts of polysaccharides and phenolic compounds that are difficult to separate from DNA (Mannerlöf & Tenning, 1997; Ostrowska et al. 1998) but are easily identified, because they make the DNA pellet sticky and gelatinous (polysaccharides) or often impart a brown colour (polyphenolics). Polysaccharides and polyphenols interfere with polymerases, ligases, and restriction enzymes (Mannerlöf & Tenning, 1997), and their removal is essential for reliable downstream molecular manipulations. Thus the critical change in modified DNA isolation procedure turned out to be the combined use of high concentration of NaCl and potassium acetate that significantly increased the efficiency of proteins, secondary metabolites and polysaccharide removal. Further the use of ethanol in contrast to isopropanol was found to be better in precipitating the final DNA yield in the solution. The yield of DNA was >3-fold over the method of McCouch et al. (1988) and Doyle & Doyle (1987) while maintaining the purity, as assessed by spectrophotometer and gel electrophoresis. Also, to prevent residual ribonucleosides from acting as primers during the thermal reaction (Porebski, 1997), used an extended RNase treatment of 30 min at 37 ^{o}C. This was sufficient to degrade RNA into small ribonucleosides that are not detectable by gel electrophoresis.

DNA purity is a concern in extraction procedures. Restriction endonuclease digestion requires fairly clean and large quantities of DNA (Doyle & Dikson, 1987). Random

amplified polymorphic DNA (RAPD) and related technologies require less DNA, but purity is necessary to ensure repeatability and confidence (Williams et al. 1990). Spectrophotometer measurements of DNA seeds samples of CPT, NGPP 46 of *P. pinnata* gave an absorbance ratio (A_{260}/A_{280}) of 1.33 for SDS method of McCouch etal. (1988) whereas absorbance ratio obtained were 1.78 and 1.97 for CTAB (Doyle & Doyle, 1987) and modified SDS method of McCouch et al. (1988) respectively indicating high purity DNA from the modified method (Fig 3.3). The quality of DNA was also checked by agarose gel electrophoresis. Conspicuous bands of high molecular weight RNA-free DNA about 30,000 bp with little shearing was observed. The DNA yields ranged from 20-25 mg kg^{-1} of seed for modified SDS method described above that is sufficient enough to carry 250-500 typical RAPD reactions whereas 15-20 mg kg^{-1} from SDS method of McCouch et al. (1988) and 5-6 mg kg^{-1} from CTAB method. When 2 µg of isolated DNA from modified SDS method of McCouch et al. (1988) was digested with 10 U of *Bam*HI and 10 U of *Eco*RI the digestion was complete as checked after 4 h of incubation in water bath at 37 ^{o}C (Fig 3.4). The DNA isolated with this method was successfully amplified by RAPD PCR and gene specific primer (Fig 3.5). These results are superior to those reported by McCouch et al. (1988) and Doyle & Doyle (1987), as the DNA extracted from the study material using the modified protocol reported in the current study could amplify the DNA fragments of <500 bp size by PCR. Furthermore, it has been confirmed that the high quality DNA thus extracted could be useful to screen the levels of genetic diversity using more advanced and sophisticated PCR techniques like ISSR and AFLP.

Although the experiments were carried out exclusively with *P. pinnata* a potential oil yielding tree species, it is believed that this modified method of McCouch et al. (1988) will be applicable to all types of oil yielding plants, though it is conceivable that some specimens will present their own specific purification challenges that might require modifications to the above mentioned procedure. In our laboratory, this method has been used to extract DNA from leaves and seeds of plants from Fabaceae, Euphorbiaceae, Zingiberaceae and Guttiferae. In addition, this procedure is affordable and does not require sophisticated equipment, making it a superior choice relative to expensive commercial kits for DNA extraction.

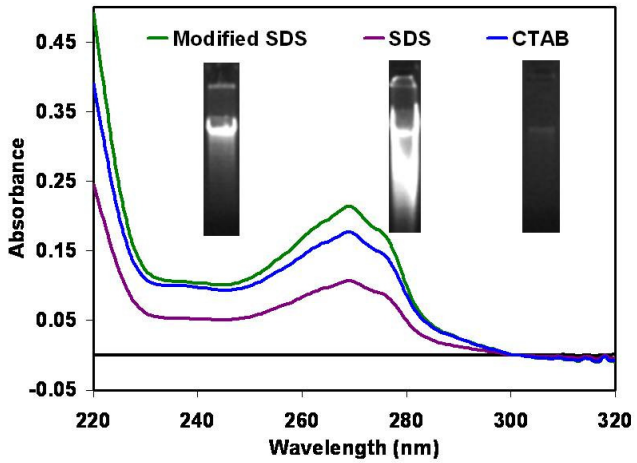


Figure 3.3. Purity index of genomic DNA from seeds of *P. pinnata* as shown by UV absorption spectra. Integrity and quality of the DNA was examined on a 0.8 % agarose-gel (inset). Extractions were repeated 3 times with similar results.

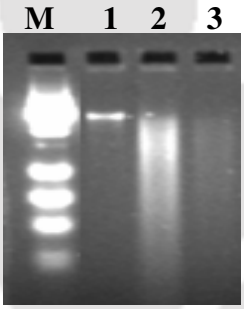


Figure 3.4. Agarose gel electrophoregram of digested DNA from *P.pinnata*. Lane 1, M: Lambda DNA *Hind*III marker; Lanes 2, No. 1: DNA undigested; Lanes 3, No. 2: digested with *Bam*HI; Lanes 4, No. 3: digested with *Eco*R I

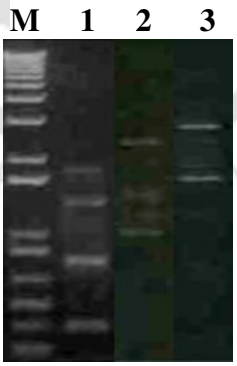


Figure 3.5. RAPD profile of DNA isolated using modified SDS method of McCouch et al. (1988) from seeds of *P. pinnata*. Lane 1, M: DNA Marker (100 bp ladder) Lane 2, No. 1: with OPA-01 (5' CAGGCCCTTC3'); Lane 3, No. 2: with OPA-03 (5' AGTCAGCCAC3'); Lane 4, No. 3: with OPA-05 (5' AGGGGTCTTG3').

3.4.2 Genetic diversity studies in CPTs

A wide range of variability exists in *Pongamia* with respect to seed and oil traits as shown in chapter 1. Genetic variability is essential to the long term survival of tree species to avoid the risk of extinction. The loss of genetic variation is thought to decrease both the short term and long term adaptability of populations in variable and changing environments. Morphological markers for identifying the individuals are limited in number and they do not often reflect genetic relationships because of interaction with the environment, epistasis and the largely unknown genetic control of the traits (Smith & Smith, 1989). In contrast, seed storage proteins and DNA markers are found in abundance and are not influenced by the environment or developmental stage of a plant, making them ideal for genetic relationships studies (Reddy et al. 2002). In addition, the effectiveness of the tree improvement programme depends upon the nature and magnitude of existing genetic variability and also on the degree of traits transmission or heritability (Zobel & Talbert, 1984; Rout et al. 2009) because genetic variation is the fundamental requirement for maintenance and long term stability of forest ecosystems. In the present study, genetic variability and structure of two local natural populations containing total of 10 CPTs of *P. pinnata* from NG, Assam were assessed using seed storage proteins and DNA (RAPD, ISSR and AFLP) markers. The study constitutes the first successful attempt at assessment of genetic variability using bio-molecular based characterization of the genus *Pongamia*.

3.4.2.1 Genetic diversity studies based on seed storage protein marker

In SDS PAGE analysis, 15-20 most prominent polypeptide bands stained with coomassie (CBB R250) were observed in immatured and matured stage among CPTs of *Pongamia* seeds. Their molecular size ranged from approximately 150 kDa (immatured stage) to 14 kDa (matured stage). Globulins are the main seed storage proteins in *Pongamia* that includes legumins (11S) and vicilins (7S), both of which are multisubunit proteins. In the present study no high molecular components are observed in the electrophoretic spectra. This is because of the presence of ß-mercaptoethanol that breaks disulphide-bonded monomers of 11S globulins to an acidic (40 kDa) and a basic (20 kDa) polypeptide joined via a disulfide

bond. There are a large number of reports in legumes which demonstrate that globulins represented by two main fractions: legumin like, legumins (MW 350-450 kDa) and vicilin-like, vicilins (MW 150-250 kDa) are the predominant polypeptides (Prakash & Rao, 1988; Santos et al. 1997; Lqari et al. 2004). Legumins exist as a mixture of trimers and hexamers comprising subunits of MW 50-60 kDa held together by non covalent interactions (Hayashi et al. 1988, Shotwell et al. 1988; Wang et al. 2001). The vicilin-like 7S globulins are typically trimeric proteins of MW 150-190 kDa, with subunit molecular mass in the range MW 40-80 kDa, joined together via weak interactions: hydrogen and hydrophobic bonds. No significant variation in protein profile amongst the 10 CPTs belonging to population 6 and 10 for a particular age of seed development was observed in current study. However the seed polypeptide banding pattern in immatured and matured stage showed differences in expression pattern of three main polypeptide bands (MW 50 kDa, 18 kDa and 14 kDa) for all the 10 CPTs (Fig 3.6A & B). There was occasional variation in the density or sharpness of a few bands, but this variation was not considered. In the current study, SDS PAGE of seed storage proteins showed no diversity. However, low level of intra-specific variation has been reported in various other legumes such as chickpea (Ghafoor et al. 2003b), groundnut (Sultan & Ghafoor, 2007), pigeon pea (Javaid et al. 2004), *Vigna* spp. (Rao et al. 1992) and black gram (Jha & Ori, 1996). Reports are available which shows that polypeptide marker could be detected at a very low frequency as diagnostic band, meaning thereby that few markers could be linked as a characteristic of the variety/genotype (Sangwan et al. 2003). In the case of pea, a considerable amount of variation was observed based on SDS-PAGE that indicated the valid utilization of seed protein markers for germplasm classification in pea (Ghafoor et al. 2003a; Ghafoor & Arshad, 2008). Genetic purity of sunflower plants was determined on the basis of electrophoretic spectrums of storage proteins (Aksyonov, 2005). Despite the morphological diversity amongst the 10 CPTs for the seed phenotypic traits and oil content as reported by Kesari et al. (2008), no change in protein profiles observed within a similar age of seed development. Odeigah & Osanyinpeju (1998) have reported that small differences between the cultivars of same species exist. In *Pongamia*, SDS PAGE of seed storage proteins can not be used for discriminating the 10 CPTs as no variation was observed as reflected in banding pattern. There may be possibility that differences may present at a finer level which

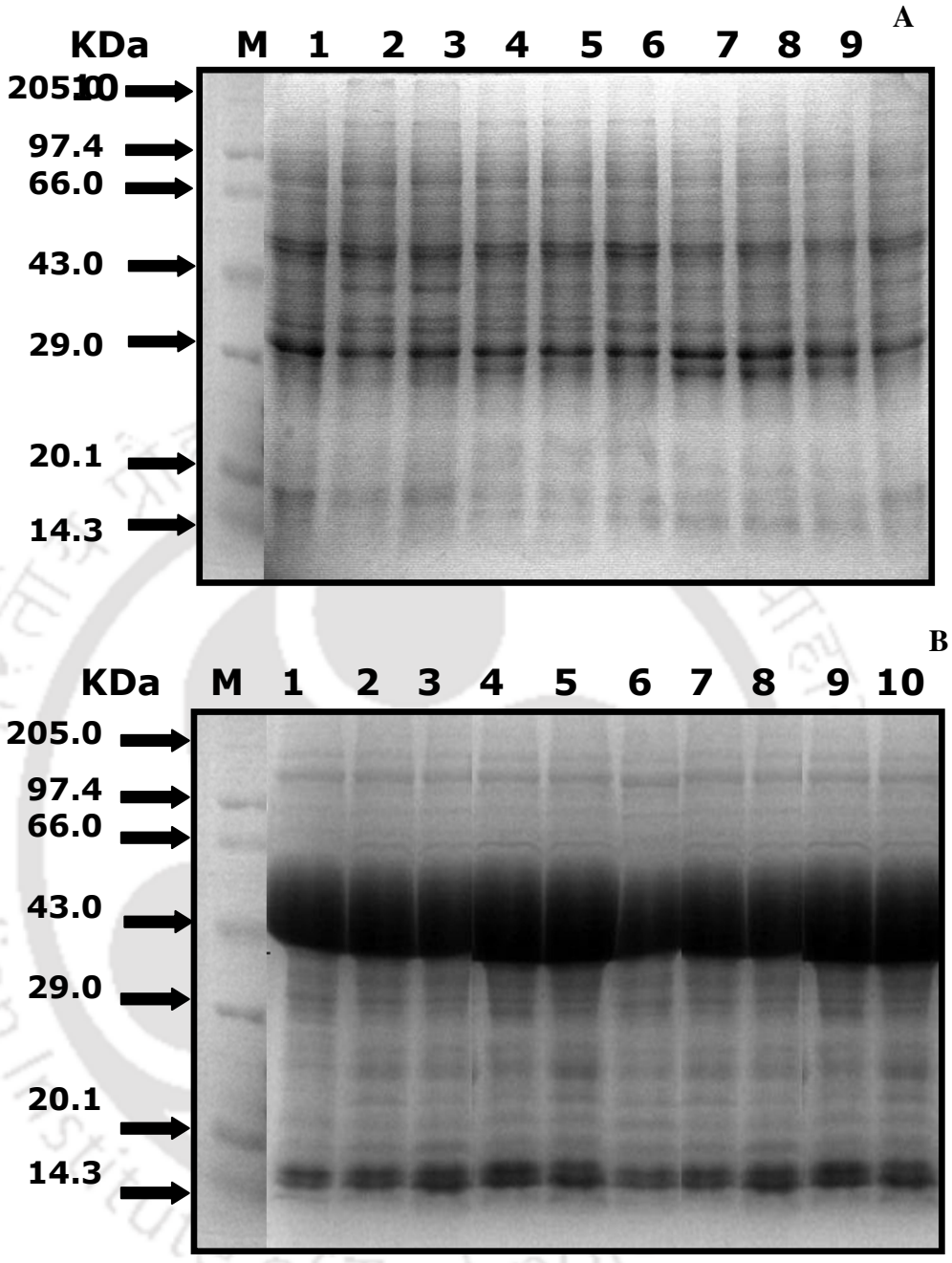


Figure 3.6. SDS-PAGE electrophoretic spectra of reduced total soluble seed protein profile of 10 CPTs of *P. pinnata* from North Guwahati, Assam showing monomorphic bands.

A. Immatured seeds; **B.** Matured seeds

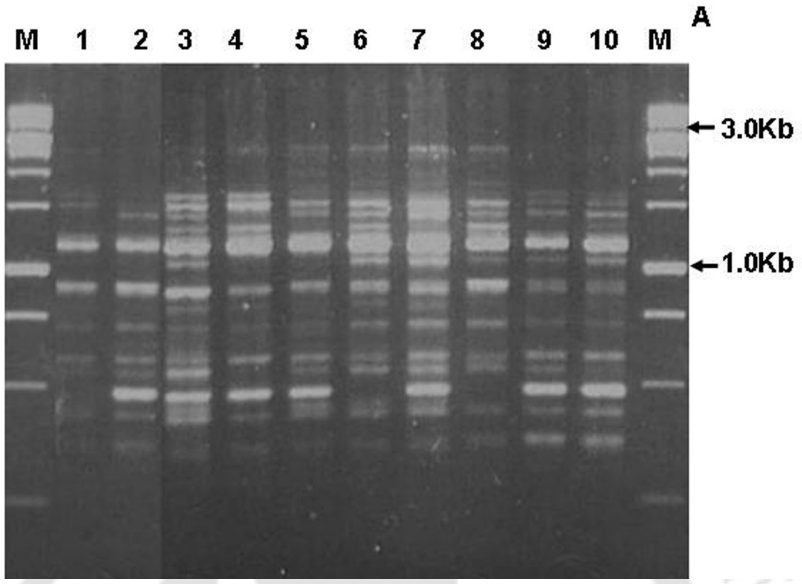
M- Molecular mass marker (14 – 116 KDa); Lane 2-6, Total seed protein from NGPP 26 - NGPP 30; Lanes 7-11, Total seed protein from NGPP 46 - NGPP 50

SDS-PAGE could not detect. Genotypes with similar banding patterns are suggested to be studied for detailed agronomic and biochemical analysis, including 2D electrophoresis and DNA markers for better management of the gene bank (Celis & Bravo, 1984; Beckstrom-Sternberg, 1989). Another option could be the use of larger scale gels or gradient gels, for finer resolution of genetic diversity. SDS-PAGE of the seed proteins of *Pongamia* did not show any definite correlation between any polypeptide and seed characteristics.

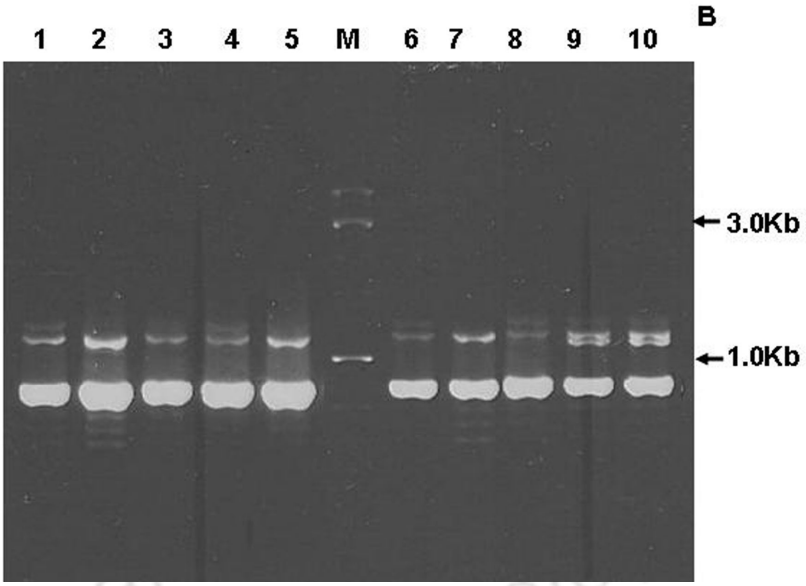
3.4.2.2 PCR fingerprinting analysis

3.4.2.2.1 RAPD analysis

Following preliminary screening, 18 primers were chosen for the evaluation of the genetic diversity among CPTs of *P. pinnata*. The remaining tested primers did not generate any amplification products or stable band patterns. Specific DNA banding pattern were observed with all the 18 primers used. The number of amplification products and the number of polymorphic fragments is given in Table 3.4. A comparison of all the 10 CPTs analyzed showed a total of 210 amplified products and the polymorphism index of all amplification products (P) reached 10.63 % for all studied genotypes (Table 3.4). Primers varied in their ability to detect variation both within and between populations as shown in Table 3.4. Of the 18 RAPD primers 8 of them produce monomorphic fragments for 10 CPTs (OPO 08, OPAM 20, OPAN 05, OPAA 01, OPAB 05 OPX 20, OPAJ 19 and OPAF 02). Band number per primer ranged from 5 (OPAB 05, OPAF 02) to 21 (OPX 20) with an average of 11.67 bands per primer. The RAPD polymorphism for these 10 CPTs ranged from a maximum of 3/primer for OPC 07, OPAN 01, OPAO 01 and OPAH 13 to a minimum of 1/primer for OPL 11, OPAP 10 with a mean of 1.2 markers (Table 3.4). Typical PCR amplification products generated by the representative RAPD primer are presented in Fig 3.7. The amplified product ranged between 0.35-1.8 kb in size. The PIC values for polymorphic RAPD primers ranged from 0.32 (OPL 11) to 0.49 (OPAO 01), whereas marker indices ranged between 2.91 (OPL 11) to 11.38 (OPAO 01). All the selected polymorphic primers had PIC values greater than 0.32 and the average PIC value for the amplification products were 0.43.



RAPD (OPAH 13)



RAPD (OPAB 05)

Figure 3.7. RAPD profiles of CPTs of *P. pinnata* DNA from study site.

A. Polymorphic marker (OPAH 13); **B.** Monomorphic marker (OPAB 05).

M, 1Kb DNA ladder; No. 1-5 NGPP 26-NGPP 30; No. 6-10 NGPP 46-NGPP 50

3.4.2.2.2 ISSR analysis

Amplification of ISSR markers was performed using 20 primers of which 12 primers gave reproducible and good quality banding patterns. The 12 ISSR primers produced total of 129 fragments of sizes ranging from 0.2-0.85 kb. Out of 129 ISSR loci, 14 bands were polymorphic among 10 CPTs by six primers. The percentage of ISSR polymorphism for these 10 CPTs is thus a maximum of 10.9 %. Of the 12 ISSR primers 6 of them produce monomorphic fragments for 10 CPTs (HB 14, HB 15, P 6, P 8, 809, 824). The average number of bands per primer was 10.75 and the average number of polymorphic bands per primer was 1.2. A survey of different ISSR primers used (Table 3.4) indicates that for each primer the number of visible bands ranged from 6-17 obtained by primer 824 and HB 12 respectively. Primers 825 (ACACACACACACACACT) and 826 (ACACACACACACACACC) exhibited the highest level of variability and the percentage of polymorphic bands was 25 % by both cases. Typical polymorphic and monomorphic ISSR fingerprints using the primer 825 and HB 12 are shown in Fig. 3.8 A & B respectively. To evaluate the effectiveness of microsatellite marker (ISSR) for the study of genetic relationship in CPTs genetic similarity coefficient was calculated which varied from 0.267 between NGPP 28 and NGPP 49 to 0.875 between NGPP 47 and NGPP 48. The wide variation in genetic similarity among the 10 CPTs reflected a high level of polymorphism at the DNA level. The PIC values for polymorphic ISSR primers ranged from 0.18 (818) to 0.50 $(CAG)_5$ with an average of 0.39, whereas marker indices ranged between 2.25 (818) to 12.33 (825).

3.4.2.2.3 AFLP analysis

DNA fingerprinting profiles of the 10 CPTs of *P. pinnata* exhibited a high degree of polymorphism with AFLP marker. The number of polymorphic fragments generated by each primer pair combinations in AFLP varied from 26 (*Mse*I - CAC / *Eco*RI - ACA) to 124 (*Mse*I - CAT / *Eco*RI - ACC), with an average of 62.25 fragments (Table 3.5). A total of 249 bands were produced with size of amplified products ranging from 50 to 545 bp and all were polymorphic (100 %). The genetic similarity value varied from a maximum of 0.714

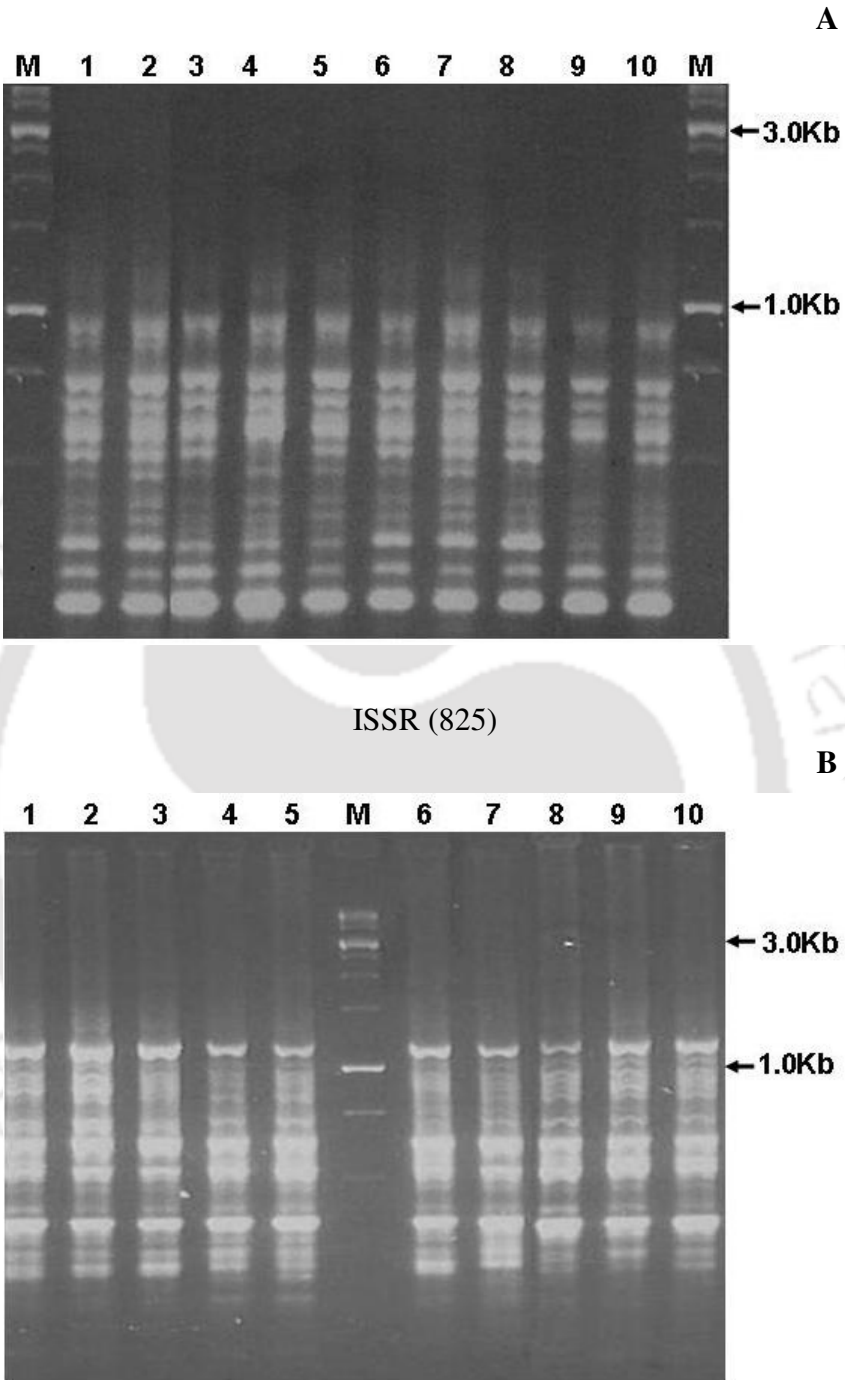


Figure 3.8. ISSR profiles of 10 CPTs of *P. pinnata* DNA from study site.

A. Polymorphic marker (825); **B.** Monomorphic marker (HB)

Lane 1, 1kb DNA ladder; Lane 2-6, NGPP 26 - NGPP 30; Lanes 7-11, NGPP 46 - NGPP 50

Table 3.4. Degree of polymorphism and polymorphic information content for RAPD and ISSR primers applied to 10 CPTs of *P. pinnata* from study site.

Markers	Primer code	Total number of bands	Number of polymorphic bands	POL (%)	PIC	MI
RAPD	OPC 07	14	3	21.43	0.42	9.00
	OPL 11	11	1	9.09	0.32	2.91
	OPO 08	13 (M)	0	-	-	-
	OPAH 15	9	2	22.22	0.42	9.33
	OPAM 20	3 (M)	0	-	-	-
	OPAN 01	19	2	10.53	0.46	4.84
	OPAO 01	13	3	23.08	0.49	11.38
	OPAP 20	8	2	25.00	0.33	8.25
	OPAN 05	8 (M)	0	-	-	-
	OPAP 10	15	1	6.67	0.48	3.20
	OPAA 01	8 (M)	0	-	-	-
	OPAB 01	13	2	15.38	0.45	6.92
	OPAB 05	5 (M)	0	-	-	-
	OPAB 14	13	2	15.38	0.45	6.92
	OPAH 13	14	3	21.43	0.45	9.58
	OPAF 02	5 (M)	0	-	-	-
	OPAJ 19	18 (M)	0	-	-	-
	OPX 20	21 (M)	0	-	-	-
	Total	210	22	10.63	-	-
	Mean	11.67	2.2	-	-	-
	Range	5 - 21	1 - 3	6.67 - 21.43	0.33 - 0.49	3.20 - 11.38
ISSR	HB13	12	2	16.67	0.49	8.17
	HB14	8 (M)	0	--	--	--
	HB15	8 (M)	0	--	--	--
	P8	9 (M)	0	--	--	--
	824	6 (M)	0	--	--	--
	825	12	3	25.00	0.49	12.33
	826	8	2	25.00	0.25	6.25
	P6	10 (M)	0	--	--	--
	809	10 (M)	0	--	--	--
	818	16	2	12.50	0.18	2.25
	HB12	17	3	17.65	0.41	7.29
	$(CAG)_5$	13	1	7.69	0.50	3.85
	Total	129	13	10.08	-	-
	Mean	10.75	2.17	-	-	-
	Range	6-17	1-3	7.69-25.00	0.18-0.49	3.85-12.33

POL – Polymorphism; M - Monomorphic marker; PIC - Average polymorphic information content for polymorphic bands; MI - Marker Index = POL (%) x PIC

PIC values for four AFLP primer combinations ranged from 0.26 (*Mse*I - CAC / *Eco*RI - ACA) to 0.34 (*Mse*I - CAA / *Eco*RI - ACT) with an average of 0.31, whereas marker indices ranged between 26 (*Mse*I - CAC / *Eco*RI - ACA) to 34 (*Mse*I - CAA / *Eco*RI - ACT).

Table 3.5. Degree of polymorphism and polymorphic information content for AFLP primers applied to 10 CPTs of *P. pinnata* from study site.

Primer combinations	Total number of bands	POL		PIC	MI
		Number	%		
*Mse*I - CAA / *Eco*RI - ACT	50	50	100	0.34	34
*Mse*I - CAC / *Eco*RI - ACA	26	26	100	0.26	26
*Mse*I - CAG / *Eco*RI - AAC	49	49	100	0.30	30
*Mse*I - CAT / *Eco*RI - ACC	124	124	100	0.33	33

POL - Polymorphism

PIC - Average polymorphic information content for polymorphic bands

MI - Marker Index = POL (%) x PIC

3.4.2.2.4 Gene diversity

Moderate levels of genetic diversity within and between populations were observed based on RAPD and ISSR whereas higher extent of genetic diversity observed based on AFLP for 10 CPTs of *P. pinnata*. At the population level, the average values of n_a, n_e, h_{pop} and percentage of polymorphism for all the populations were ranged from 1.054-1.745, 1.042-1.370, 0.023-0.229 and 5.43-74.50 % respectively using three molecular markers (RAPD, ISSR and AFLP) (Table 3.6). Similarly, at the species level n_a, n_e, h_{sp} and percentage of polymorphism were ranged from 1.101-1.992, 1.064-1.389, 0.038-0.247 and 10.08-100 % respectively. Heterogeneity was calculated and data demonstrated that inter-population diversity (h_{sp}) was highest as compared to average intra-population diversity (h_{pop}) based on all three molecular markers used in the current study. These trends have been observed in other crop species such as wild species of almond (Blair et al. 2006; Sorkheh et al. 2007). The mean Shannon's indexes (I) of populations (POP 1 and POP 2) and at the species level based on RAPD and ISSR displayed similar estimates of 0.035-0.033 and 0.057-0.061

respectively (Table 3.6). The highest level of mean Shannon's indexes at the population level (0.356) and at the species level (0.395) was obtained based on AFLP variation (Table 3.6). The data of AFLP revealed high levels of genetic diversity among and between populations according to Shannon index. On an average, over all the three makers used in the current study, POP 2 exhibited lowest level of genetic variation based on Nei's genetic diversity. The highest average values for genetic data of populations were obtained for AFLP marker and the lowest was observed generally for ISSR with few exceptions.

Genetic variation and it's partitioning among and within populations of a plant species are determined by a number of factors, of which reproductive biology system (outcrossing vs. selfing) is the most important (Hamrick & Loveless, 1989). In the present study, the average coefficient of genetic differentiation (G_{st}) were 0.377, 0.452 and 0.074 for RAPD, ISSR and AFLP respectively among two populations of *P. pinnata* (Table 3.7). Genetic differentiation among the population was very low suggesting extensive gene flow among them. The significant differentiation between populations of *P. pinnata* was also revealed in the estimates of gene flow (N_m). A value of Nm obtained was lowest for ISSR (0.607) to a high value of 6.2874 for AFLP whereas N_m was of a moderate value for RAPD 0.8275 (Table 3.7). Thus the RAPD and ISSR data indicated moderate gene flow between the two populations of *P. pinnata* studied from North Guwahati, Assam. However, AFLP data showed higher level of gene flow between populations. The data obtained in the current study are in contrary to the study of Rout et al. (2009) in *Pongamia* from Orissa which reports Nm value of 0.3 based on RAPD analysis. The outcrossing habit of *P. pinnata* may play a key role in the maintenance of genetic diversity of the species.

3.4.2.2.5 Genetic diversity analysis as revealed by RAPD, ISSR and AFLP

Finally, 10 RAPD (dominant marker), 6 ISSR (co-dominant marker) and 4 AFLP (dominant marker) primer combinations were selected for cluster analysis through UPGMA dendrograms constructed using SHAN neighbor-joining tree separately. The genetic relationships among 10 CPTs of *P. pinnata* belonging to 2 populations were analyzed on the

basis of Dice genetic distance (Dice, 1942). The UPGMA clustering algorithm from RAPD grouped the genotypes into two major clusters at a similarity index value of 0.26 (Fig. 3.9A). CPTs, NGPP26 and NGPP 50 were the two extremes in the dendrogram. Cluster I consists of individuals belonging to population 6 (NGPP 26-30) whereas individuals belonging to population 10 (NGPP 46-50) grouped in cluster II. Within cluster I, two subgroups were evident, one containing NGPP 26, 27 and 28 genotypes while the other included NGPP 29 and 30 respectively. Cluster II consisted of 3 subgroups having NGPP 46, 47 and 50 grouped together and NGPP 48 and 49 clustered independently. The UPGMA cluster from ISSR marker analysis separated the 10 CPTs into two major groups at a similarity index value of 0.55 (Fig. 3.9B), which was similar to RAPD analysis. However unlike the grouping with RAPD analysis in which NGPP 26 and NGPP 28 in cluster I and NGPP 46 and NGPP 47 were most closely related, in ISSR analysis NGPP 28 and NGPP 29 in cluster I were most closely related. According to the cluster analysis on the basis of RAPD and ISSR markers, 10 CPTs were classified into 2 major clusters, but the genotypes intra-positioning in the dendrogram were different. Thus RAPD and ISSR markers showed a high degree of similarity in dendrgram topologies, although with some differences in the positioning of some genotypes. Relationships inferred from a neighbor-joining tree generated from RAPD and ISSR bands were similar to results obtained from morphology but somewhat better resolutions were achieved. RAPD and ISSR aim to amplify a different region of genome. Thus it is reasonable that there are some fine differences between the two dendrograms based on an individual data set. The similar structure of dendrogram also suggests that both techniques are suitable for genetic polymorphism research on *Pongamia*. In the cluster some genotypes shared great similarity, suggesting that they are close to each other in genetic relation, and may have come from single parents. The relationships among individual CPTs belonging to two populations are also visualized in the UPGMA dendrograms generated from AFLP analysis (Fig. 3.9C). Five clusters were formed in AFLP dendrogram. The AFLP discriminated most genotypes and grouped individuals together though belonging to the two different populations (such as NGPP 26, 48; NGPP 28, 46 and NGPP 49, 30 more closely related) hinting that interpopulation differences were more significant compared to intrapopulation ones (Fig. 3.9C). Ozkan et al. (2005) concluded from their studies on *Hordeum spontaneum* that grouping based on genetic parameters was

not related to the geographic origin of sampling regions. AFLP results showed that the genetic variability was not according to the population distribution.

The first three most informative principal coordinates accounted for 66.52, 71.10 and 57.7 % of the genetic similarity variance based on RAPD, ISSR and AFLP respectively. The PCo analysis supported the results of clustering. Finally, the phenogram and principle-coordinate maps (Fig. 3.10 A, B & C) shows the groups found with both methods and were comparable. PCo based on RAPD and ISSR also indicates that samples from the same population are genetically much closer than samples from different populations. The Mantel method used for comparing the similarity matrixes produced correlation coefficients that were statistically significant for each of the three marker systems used independently viz., RAPD, ISSR and AFLP. The cophenetic correlation coefficient between dendrogram and the original similarity matrix were large and significant for RAPD (r=0.84), ISSR (r=0.73) and AFLP (r=0.98), giving a good degree of confidence in the association obtained for the CPTs.

Two aspects of the performance of the three markers (RAPD, ISSR and AFLP) were considered: overall efficiency in detecting polymorphism within germplam collection, and overall utility of the marker in detecting variation in order to infer genetic diversity among CTP s of *P. pinnata*. DNA based markers, along with appropriate statistical procedures, are suitable for genetic variation analyses at both intra and inter-population levels (e.g. Chalmers et al. 1992; Stewart & Excoffier, 1996; Martin et al. 1997; Bussell, 1999). The level of polymorphism for each of the three marker system was quite variable, ranging from 10.07 % to 100 %. AFLP analysis revealed a high level of genetic diversity (100 %) in natural populations of *P. pinnata* while only a small amount of variation (approx. 10 %) was detected based on both RAPD and ISSR markers in CPTs of *P. pinnata*. Kuashik et al. (2007) also reported that the distribution of genetic variability is limited in *P. pinnata* on the basis of their morphological characteristics such as pod and seed traits. However higher level of polymorphism (94.3 %) was reported in seven populations of *P. pinnata* using few RAPD markers, but the samplings were done from three different eco-geographical regions of Orissa (Rout et al. 2009). Other woody perennial species such as *Populus tremuloides* and *Isotoma petraea* display lower levels of genetic variation detected with the same RAPD

methodology (Yeh et al. 1995; Bussell, 1999). Using RAPD markers, Chen et al. (2004) also showed that the diversity within the population of *I. sinensis* in China has a mean value of only 5.04 %, ranging from 0.8 % to 12.9 %. Genetic drift may eventually reduce genetic variation in isolated populations (Frankham, 1996). Furthermore, it has been confirmed in many

Table 3.6. Mean genetic data of populations of CPT of *P. pinnata* collected from North Guwahati, Assam.

Markerss	Populations	n_a	n_e	h	I	p	% P
RAPD	POP 1	1.087±0.283	1.068±0.228	0.038±0.124	0.054±0.177	18	8.7
	POP 2	1.039±0.193	1.016±0.092	0.010±0.056	0.017± 0.087	8	3.86
	Mean	1.063±0.238	1.042±0.160	0.024±0.090	0.035±0.132	13	6.28
	At species level	1.105±0.307	1.064±0.206	0.038±0.116	0.057±0.171	22	10.63
ISSR	POP 1	1.054±0.227	1.046±0.188	0.024±0.101	0.034±0.144	7	5.43
	POP 2	1.054± 0.227	1.040±0.178	0.022±0.096	0.032±0.137	7	5.43
	Mean	1.054±0.227	1.042±0.183	0.023±0.098	0.033±0.141	7	5.43
	At species level	1.101±0.302	1.076±0.241	0.042±0.129	0.061±0.185	13	10.08
AFLP	POP 1	1.807±0.395	1.399±0.325	0.248±0.165	0.385±0.229	201	80.72
	POP 2	1.683±0.466	1.340±0.334	0.210±0.177	0.326±0.253	170	68.27
	Mean	1.745±0.431	1.370±0.330	0.229±0.171	0.356±0.241	186	74.50
	At species level	1.992±0.089	1.389±0.308	0.247±0.149	0.395±0.183	249	100

n_a , observed number of alleles; n_e, effective number of alleles; h, Nei's (1973) gene diversity; I, Shannon's Information index; P, number of polymorphic bands; % P, percentage polymorphism

Table 3.7. Genetic population structure and estimate of gene flow within the populations of CPT of *P. pinnata* collected from study site.

	H_t (H_{sp})	H_s (H_{pop})	G_{st} (1-H_{pop}/H_{sp})	N_m
RAPD	0.038±0.0137	0.024±0.0066	0.377	0.828
ISSR	0.042±0.0166	0.023±0.0070	0.452	0.607
AFLP	0.247±0.0222	0.229±0.0202	0.074	6.287

H_s Total variability; H_{pop}, variability within population; G_{st}, inter population differentiation; Nm, estimate of gene flow from G_{st} or G_{cs}. E.g., $Nm = 0.5(1 - G_{st})/G_{st}$

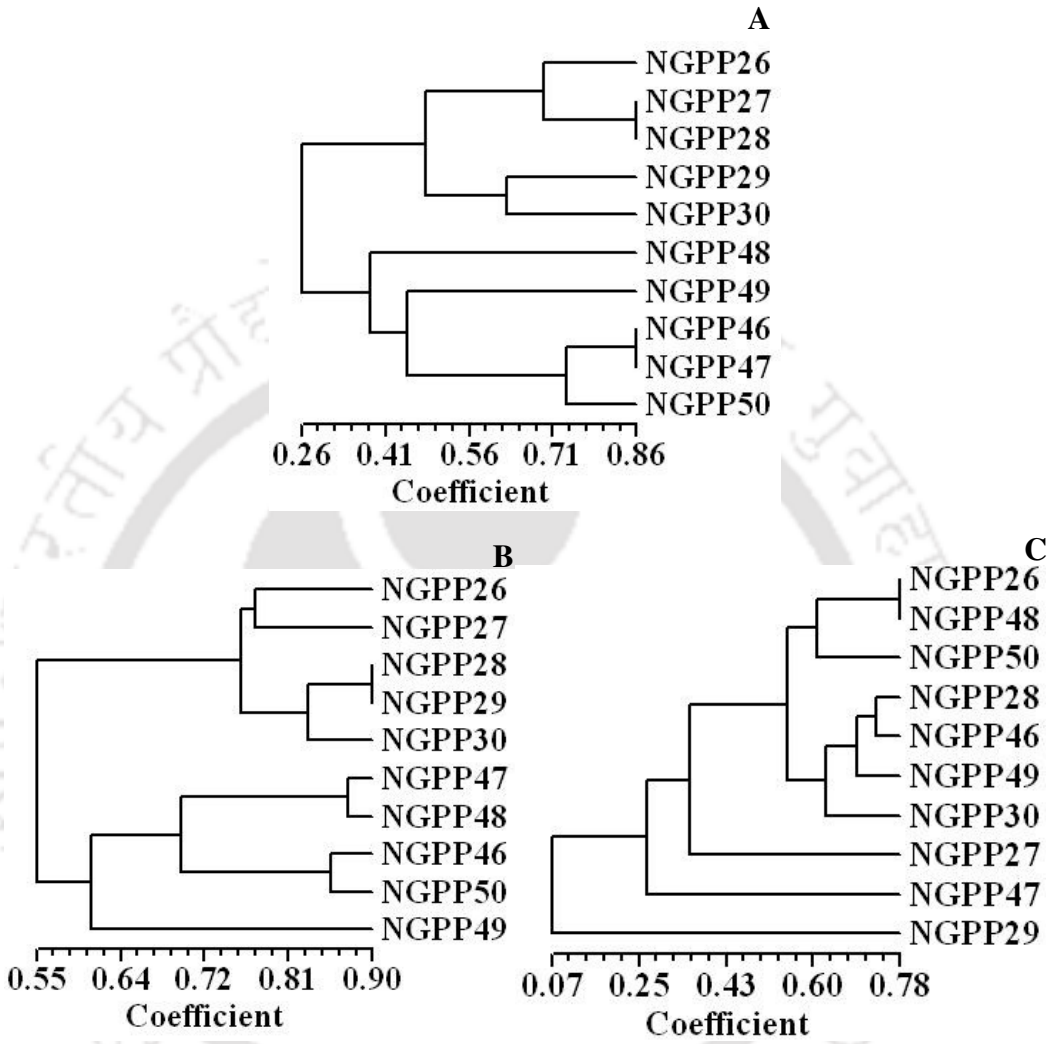


Figure 3.9. Phenograms representing the phylogenetic relationship among 10 CPTs of *P. pinnata* belonging to population 6 and 10 from study site as revealed by UPGMA cluster analysis using SHAN neighbor-joining tree based on polymorphic markers. The genetic distances were from Dice similarity coefficient.

A. RAPD; **B.** ISSR; **C.** AFLP

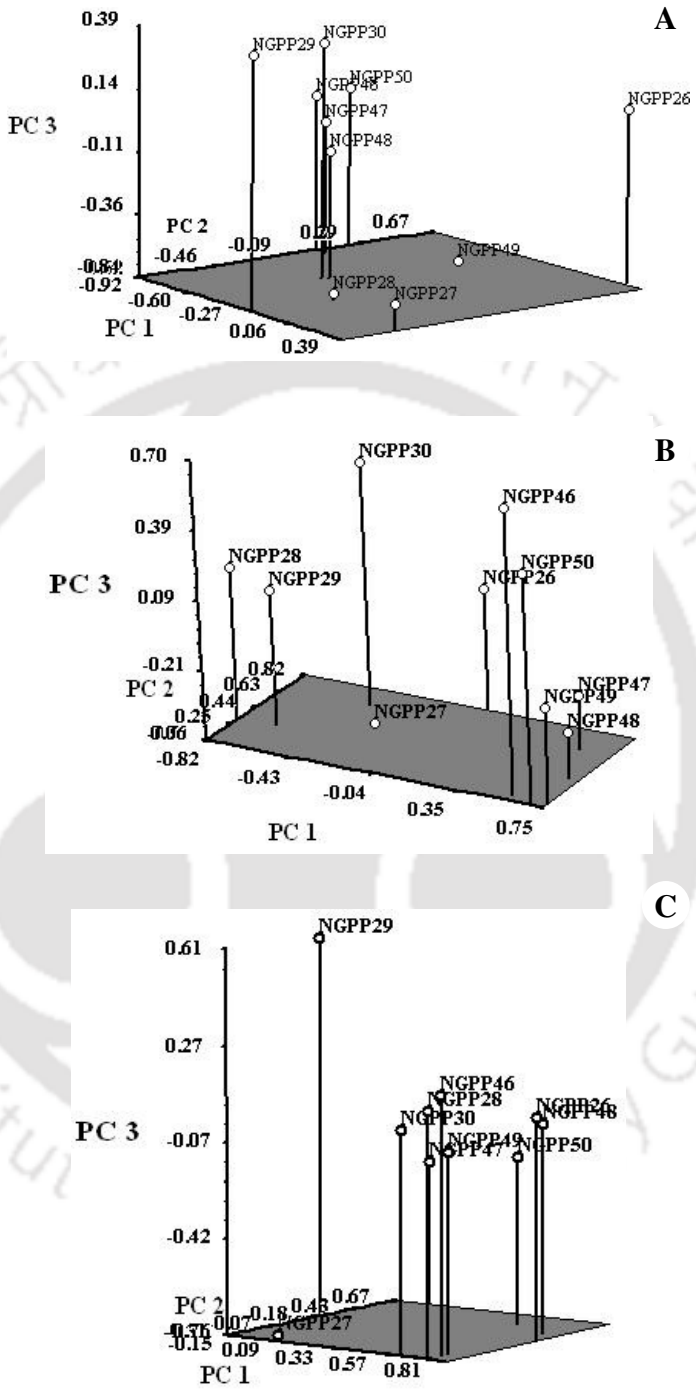


Figure 3.10. Principle co-ordinate map for the first, second and third principle- coordinate estimated for RAPD, ISSR, and AFLP markers for 10 CPTs of *P. pinnata*.

studies that smaller populations have a lower genetic diversity than larger populations due to genetic drift (Frankham, 1996; Fischer et al. 2000). Moderate low diversity and high population partitioning in tree species have previously been attributed to a number of factors, including the adaptation of genetic systems in small populations, recent fragmentation of continuous genetic systems (human activity), and limited gene flow due to the combination of wind pollination and a high inbreeding rate (Maguire & Sedgley, 1997). Genetic variation and it's partitioning among and within populations of a plant species are determined by a number of factors, of which reproductive biology system (outcrossing vs. selfing) is the most important (Hamrick & Loveless, 1989). Molecular markers are usually considered selectively neutral (Strauss et al. 1992) and thus do not necessarily reflect the diversity in functional characters (Karhu et al. 1996; Hintum van & Treuren van, 2002). The genetic variability of plants results from interaction of mutation, selection, random genetic drift and differential migration (Kumar et al. 2009). Mutation pressure and selection procedure play major role in changing the level of genetic equilibrium. Geographical, ecological and reproductive isolation have material effect on level of genetic diversity (Tripathi et al. 2007). RAPD markers proved to be useful by providing a bunch of simply inherited genetic markers for genome mapping (Kumar et al. 2009) or to discriminate among closely related taxa. RAPD markers could be used for estimation of genetic relationships, which ultimately help in characterization of *P. pinnata* germplasm as revealed in the present study. The disadvantage of RAPD, such as poor consistency and low multiplexing output should also be considered. Microsatellites have been found to vary in the polymorphism they detect depending sometimes on the length and sequence of the repeat motif they contain and their location in gene coding or non coding segments of the genome (Thouquet et al. 2002; Temnykh et al. 2000, 2001; Eujay et al. 2002). Polymorphic information content (PIC) analysis can be used to evaluate markers so that the most appropriate can be selected for genetic mapping, phylogenetic analysis or association genetics (Anderson et al. 1993). The advantages of using three different marker systems are that they exhibit no plasticity, unlikely to be similar because of convergent evolution and they can generate information at many different loci. ISSR markers owing to their co-dominant inheritance, detect multiple alleles at a given locus while RAPD and AFLP being dominant, detect multiple loci distributed throughout the genome. In addition, ISSR marker target multiple microsatellite

loci distributed across the genome while RAPD and AFLP markers scan the entire genome and hence, genome wide genetic variation could be detected with the use of two marker systems.

Different genotypes of each population clustered together in RAPD and ISSR based denrogram, hinting that inter-population differences were more significant compared to intra-population ones. Although the two marker systems, RAPD and ISSR analysis sampled different segments of genome, genetic information revealed was of almost comparable value and the dendrongrams structure based on RAPD/ISSR data was basically same with some minor changes. Genotypes belonging to the same populations were close to each other in genetic relation, than those from other populations. Due to different hierarchical positions in the dendrogram it is clear that the genome of 10 CPTs of *P. pinnata* is not exactly the same. In this study, AFLP analysis was found to provide a high resolution for the detection of genetic diversity and structures between and within populations of *P. pinnata*. According to Hamrick & Loveless (1989), the breeding system of a species is an important determinant of variability at both the species and population levels. The morphological, RAPD and ISSR data led to similar representations of the genotype relationships. AFLP marker demonstrated its usefulness in gaining information about genetic relationship by using less primer combinations. One advantage of AFLP-based DNA fingerprinting is its potential in revealing large genetic polymorphisms with a nearly complete coverage of the whole genome (Badr et al. 2000; Heun et al. 1997). In current study, four AFLP primer combinations were sufficient to disclose a proper number of polymorphic DNA fragments in support of all comparisons and of their statistical evaluation. AFLP detected polymorphism more efficiently due to greater number of loci detected/assay reaction. In fact, AFLP higher marker index (product of heterozygosity and number of polymorphic bands from a single amplification reaction), elucidate an overall measure of marker efficiency. In this sense, genotype identification should be possible even with only one or two AFLP primer combinations, a situation which is not so easily achieved with other molecular marker techniques (Ozkan et al. 2005; Karudapuram & Larson, 2005).

PCR based DNA markers technique has been successfully applied to investigate the genetic relationship in tree and crops. Since they are not subjected to environmental effects

and are independent of the development stage of the plant, these methods have been used to identify the germplasm of trees and crops (Ma et al. 2004). To perform genetic variability studies DNA extraction using seeds instead of leaves is time saving and less expensive (McDonald et al. 1994). The plants do not have to be individually grown and DNA can be extracted directly from seeds with the technical precautions of eliminating polysaccharides by supplementary DNA precipitation. The use of RAPD markers from seeds in a stable manner for varietals identification and genetic diversity evaluation has already been reported (McDonald et al. 1994; Fu et al. 2004), but, the current study might be of its kind where RAPD, ISSR and AFLP marker patterns from seeds have been characterized for genetic variability evaluation in biodiesel tree species. The three marker systems were used because polymorphisms obtained using RAPD, ISSR and AFLP have different underlying causes at the molecular level and thus may provide differing information in the analysis of genetic relationships. The amplification profiles of three marker systems resulted in differences in the detected banding patterns. In the current study the number of bands produced by RAPD and ISSR were comparable to that produced by SDS PAGE but from later no polymorphism detected among 10 CPTs. The results presented herein reveal that 10 CPTs of *P. pinnata*, growing in NG, Assam exhibited variations in their DNA fingerprinting profiles for each of the three different DNA markers type, in spite of being considered to have been derived from one geographical area or source.

3.5 Conclusion

In anticipation of new marker technology, the chapter deals with exploring basic approaches for extracting whole genomic DNA from dried seeds, as a part of a case study on a biodiesel plants (*Pongamia pinnata*). The ability to identify relevant gene(s) involved in fatty acid biosynthesis and the ability to reliably extract good quality DNA from seed samples is a fundamental step in the application of genetic techniques to the success of biofuel crops. The isolation procedure developed here confirms that for each plant species and tissues being studied, isolation protocols needs to be tailored and optimized (Bekesiova et al. 1999; Syamkumar et al. 2003) suited to the needs of molecular characterization. DNA isolated from seeds following SDS method of McCouch et al. (1988) after its modification gives

maximum yield of superior quality. The quality and yield of DNA is also appropriate for RFLP analysis. The PCR amplifiable high quality DNA extracted from the modified SDS protocol reported in current chapter have the potential to play a very important role in developing strategies for further improvement of biofuel crops through DNA polymorphism, genome mapping, identification of the QTLs and other plant breeding approaches such as marker assisted breeding. These approaches when complemented with ongoing conventional breeding programs will contribute to the application of sustainable management practices especially in the wastelands. The DNA extraction procedure described here for seeds is simple, rapid, effective and reproducible in different laboratories and can be scaled up as desired. This is the first of its study on standardization of protocol for isolation of DNA from seeds of potential biofuel crops viz., *P. pinnata*. The protocol described here can also be used for several other oilseed crops of commercial importance.

Furthermore, the present study also demonstrates the usefulness, limitations and resolution power of several types of biomolecular markers in comparison to classical morphological descriptors for analyzing the *P. pinnata* genome. Maintenance of high genetic diversity in *P. pinnata* is one of the most important issues as the species have immense multipurpose importance, yet genetically uncharacterized. The current study is again the first attempt at molecular characterization with multiple bio-molecular markers in CPTs of *P. pinnata* from study site that is NG, Assam, India. It will help to provide a genetic input into the *P. pinnata* for sustainable management and will also focus on a wider range of populations to widen the genetic base and for further genetic improvement of this crop. *Pongamia* is worldwide recognized as a versatile biodiesel tree species, with significant importance in maintaining soil fertility and medicine. However, there are problems in seeds availability, quality, yield and storage as they are highly infected with insects; another disadvantage is its long gestation period. Until recently, the genetic diversity at the molecular level is limited or not well investigated in *P. pinnata*. The accurate identification of CPTs is essential in breeding programs for successful production of elite genotypes. The effectiveness of the tree improvement program depends upon the nature and magnitude of existing genetic variability and also on the degree of traits transmission or heritability (Zobel & Talbert, 1984; Rout et al. 2009). The loss of genetic variation is thought to decrease both

the short term and long term adaptability of populations in variable and changing environments. Morphological markers for identifying the individuals are limited in number and they do not often reflect genetic relationships because of interaction with the environment, epistasis and the largely unknown genetic control of the traits (Smith & Smith, 1989). In contrast, seed storage proteins and DNA markers are found in abundance and are not influenced by the environment or developmental stage of a plant, making them ideal for genetic relationships studies (Reddy et al. 2002). In this study, the results demonstrated that RAPD, ISSR and AFLP markers are powerful tools for fingerprinting, analyzing variability and genome mappimg in CPTs of *P. pinnata*. From a tree improvement perspective, a large proportion of genetic variance of *P. pinnata* found among individuals within populations confirmed by AFLP is encouraging. The elucidation of the variability among the 10 CPTs and the identification of genotype specific markers are important resources for devising strategies for breeding and efficient management in *P. pinnata* germplasm. Introgression of desired traits like oil biosynthesis genes needs to be further conducted to enhance the germplasm for elite genotype development in *Pongamia*. However, it is worth stressing that this work needs to be further strengthened with more exhaustive sampling of populations and more advanced molecular techniques. The study of marker data described here is also of great value for other reasons such as by using these amplified products it is possible to construct SCAR markers in cases where little or no sequence data are available from public domain databases such as GeneBank. Furthermore, the selected amplified products from this study will be used for identification of elite genotypes of other potential biodiesel crops.


भारतीय प्रौद्योगिकी संस्थान गुवाहाटी
Indian Institute of Technology Guwahati

Chapter 4

Chapter 4

Bio-molecular characterization in CPT

4.1 Introduction

Oil yielding crop plants are very important for economic growth of the energy and agricultural sectors. The oil seeds containing polyunsaturated fatty acids are important source of biodiesel (Sarma et al. 2005; Sharmin et al. 2006). These organic seed oils are better than diesel fuels in terms of physico-chemical properties and biodegradability (Goering et al. 1982) as is the case in *P. pinnata*.

P. pinnata an oil yielding leguminous tree is an economically important worldwide due to the multifunctional characteristics of the plant (Scott et al. 2008; Kesari et al. 2009; Mukta et al. 2010). Of particular interest is the high oil content of the seeds which can be used for biodiesel production. The extraction of the oil from the seeds leaves a meal highly enriched in protein (crude protein ~ 40 %), providing a potential protein source for animal nutrition (NOVOD, 2009).

However, while utilizing these species as a source of biodiesel there is a further need for research into its various areas of production and utilization. As reported in chapter 1 systematic characterization and seed oil analysis in *P. pinnata* enabled to tag an elite genotype (CPT) from natural populations occurring in NG that can be included in programmes aimed at genetic improvement of the species (Kesari et al. 2008). Although chemical composition and bioassay of the seed oil of this plant has been previously reported separately (Wagh et al. 2007; Kumar et al. 2007), there has been no systematic study or concerted efforts to characterize the oil content from the elite genotype and its efficacy against range of microorganisms acting as pathogenic indicators.

Therefore in the first subsection of the chapter 4 best reporting CPT (henceforth referred with original tag number NGPP 46) was progressed further with the following

objectives: (i) extraction and quantification of the total oil yield from the seeds of the best reporting CPT of *P. pinnata* using different solvents and their azeotropic mixtures (ii) to analyze and compare the physico-chemical properties of the seed oil of n-Hexane and ethyl acetate fraction (iii) to determine the fatty acid profile of the fraction yielding maximum oil and, (iii) to investigate the antimicrobial activity of the seed oil against referential bacterial and fungal strains. All experiments were performed using the seed material of NGPP 46, earlier characterized, based on their phenology and reproductive characters from natural populations occurring in North Guwahati, Assam, India (Kesari et al. 2008).

To develop applications with a higher added value, knowledge of the seed proteins of *P. pinnata* apart from their other storage products (lipid), is of prime importance. In addition, characterization of seed proteins is important for improving the yield and quality of the seed through a traditional breeding and genetic engineering techniques. Despite the fact that the first report on *Pongamia* seed protein was reported by Scott et al. (2008) little information is available concerning the characteristics of the seed proteins that contribute a major component of sexual seed development. So far no report on the protein profiling in other vegetative tissues of the same plant is available. The same is true of its comparison with seed germination stages. In order to characterize *P. pinnata* currently cultivated mainly for its seeds, subsection 2 studies involved changes in phenotypic traits of the developing seeds, changes in seeds protein content and protein profile using SDS-PAGE during its maturation stages. Also comparisons were drawn between SDS-PAGE profiles of seed proteins with other tissues of *P. pinnata*. To understand protein mobilization during germination, qualitative (SDS-PAGE) and quantitative (protein content) changes in the germinating seed protein (cotyledon and embryonic axis) of *P. pinnata* were also investigated. The study might be first of its kind to elucidate the changes in the total soluble protein pattern associated with *Pongamia* sexual seed development and their comparison with seed germination stages.

Observing the differences in the biochemical localization of storage reserves in the cotyledon of developing seeds during its sexual maturation (stage I, 90 DAF-stageVII, 350 DAF) could provide insight in exploiting the improvement of *P. pinnata* seeds for the desired traits. Earlier reports have proposed that during germination, seed storage reserves are degraded by endo- and exo-proteases to monomeric units to support the growth of

germinating seedlings (Bhandari & Chitralekha, 1984). The process of degradation of storage reserves in the seeds of *P. pinnata* is not known till date. The present study was also undertaken to elucidate the changes in the pattern of storage reserves degradation in the germinating cotyledon of *P. pinnata* to bring comparisons with seed maturation. Thus, in the subsection 3 of the current chapter simultaneous analysis and comparison of *P. pinnata* seed maturation with germination in terms of ultrastructural changes and spatial localization of storage reserves of cotyledon especially proteins and lipids were presented through microscopic studies using scanning electron microscope (SEM) and transmission electron microscope (TEM). This study was done as a prelude to physiological studies on seed storage behaviour. The information obtained from the present study may be needed to develop a better understanding of how the deposition of storage reserves in seed maturation is related to the germination and seedling growth in *P. pinnata* seeds.

The unique expression of a specific suite of genes can provide insight into the molecular mechanisms involved in an organism's response to its tissues, developmental age and environment. Although progress has been made in the elucidation of the regulatory mechanism of desaturase and oleosin gene expressions in oil yielding plant species, no information is available on their expression profile in different vegetative tissues and developmental stages of seeds from *P. pinnata*. In *P. pinnata* approximately 29-40 % by seed weight consists of lipid and among them 45-75 % comprises oleic acid, depending on genotype (Sarma et al. 2005). To identify regulatory genes involved in fatty acid biosynthetic pathway and TAG (triacyl glycerol) assembly, expression profile of 3 desaturase genes and 1 Oleosin protein have been analyzed using semi-quantitative RT-PCR in subsection 4 of the current chapter.

The isolation of RNA can be quite difficult particularly in plant tissues especially storage organs such as seeds that are rich in polyphenolics, polysaccharides, proteins and other unidentified compounds (Bugos et al. 1992; Sharma et al. 2003; Almaraza et al. 2006). These compounds tend to co-precipitate with RNA and contaminate RNA extracts because they display physical and chemical properties similar to those of nucleic acids (Salzman et al. 1999; Azevedo et al. 2003; Sharma et al. 2003; Singh et al. 2003). Moreover phenolic compounds readily oxidized to form covalently linked quinones (Loomis, 1974; Salzman et al. 1999;

Mattheus et al. 2003), and avidly bind nucleic acids. In addition to this RNA degradation by ribonucleases (RNases) drastically reduces the recovery of intact RNA (Birtic & Kranner, 2006). Existing RNA isolation methods specifically alleviate one or two of these challenges, but are usually designed for a single species rather than a broad biodiversity (Ding et al. 2008). Even plant species belonging to the same genus or related genera can exhibit enormous variability in the complexity of pathways of dispensable functions (Sharma et al. 2003). Thus the biochemical compositions in plant tissues of different species are expected to vary considerable. This renders RNA unusable for molecular biology methods such as semi-quantitative RT-PCR, cDNA library construction and investigating gene expression profile using quantitative Real-Time PCR. The current objective has been to study the spatial and temporal expression of the fatty acid biosynthetic (FAD 2, FAD 3, SAD) and oleosin genes from vegetative (leaf and root) and cotyledon of different developing stages of the seed tissues in *P. pinnata*.

4.2 Review of Literature

The characteristic attributes of *Pongamia* make them an ideal biofuel crop as they support the production of large quantities of seed, a wide genetic base, ease of growth and shorter generation time as the plant flowers twice in a year (Kesari et al. 2008; Scott et al. 2008; Mukta et al. 2010). Oilseeds contain three major classes of storage products, i.e. storage lipids or oils, storage proteins, and oleosins.

The seeds of *Pongamia* are reported to contain on an average about 28-34 % oil with high percentage of polyunsaturated fatty acids (Sarma et al. 2005). Historically, *P. pinnata* has been used as folk medicinal plant, particularly in Ayurvedha and Siddha systems of Indian medicine (Meera et al. 2003). All parts of the plant have been used as a crude drug for the treatment of tumours, piles, skin diseases, itches, abscess, painful rheumatic joints wounds, ulcers, diarrhea etc (Shoba & Thomas, 2001; Meera et al. 2003). Besides, it is well known for its application as animal fodder, green manure, timber and fish poison. It has also been recognized to possess applications in agriculture and environmental management, with insecticidal and nematicidal activity. More recently, the effectiveness of *P. pinnata* as a

source of biomedicines has been reported (Brijesh et al. 2006), specifically as antimicrobial and therapeutic agents targeting host pathways and processes.

Oil seed proteins attracted the attention of scientists in view of their importance in the plant system. Seeds proteins are conservative and are widely used in plant genetic studies (Cooke, 1995; El-Naggar 2001; Jha & Ohri, 2002; Syros et al. 2003). The electrophoretic profiles of seed proteins are widely used as biochemical markers in breeding programs and in seed purity control (Cooke, 1999). Seed proteins include in addition to the storage proteins (40-70 % in legumes), housekeeping proteins (maintains normal cell metabolism) and biologically active proteins (protease inhibitors, lectins and allergens) which are minor proteins. Seed proteins based on their solubility were empirically classified into albumins (1.6S-2S), globulins (7S-13S), prolamins and glutelins (Osborne, 1924). Albumins and globulins comprise the seed proteins of dicots (e.g. pulses), whereas prolamins and glutelins are major proteins in monocots (e.g. cereals). Globulins that includes legumins (11S), vicilins (7S) are storage proteins, whereas albumins are mostly enzymatic and non storage proteins (Bhatty, 1982; Lqari et al. 2004; Kirmizi & Guleryuz, 2007). The seed storage proteins can be distinguished from other proteins by some of their characteristics such as: (1) these accumulate in high amounts in seed during mid-maturation stage of seed development and are used up during germination, (2) these are synthesized only in the seed (in cotyledon or in endosperm) and not in other tissues, (3) they lack any other functional activity besides storage, and (4) they are deposited mostly in special storage organelles called protein bodies. It is reported that substances such as trypsin inhibitors, hemagglutinins, polyphenols, glucosinolates, colour and bitter principles, which are inherent constituents of some of the oilseeds, are generally associated more with the 2S or 7S fraction (Prakash & Rao, 1988). During germination, storage proteins are degraded by a variety of proteases, which convert the insoluble storage proteins into soluble peptides, which are then further hydrolysed to free amino acids. These free amino acids are mobilized to the embryonic axis to support its growth and also to provide energy (Shutov & Vaintraub, 1987; Bewley & Black, 1994; Muntz et al. 2001). Two hypothesis (*source sink and plant growth substance stimulus*) proposed for regulation and mobilization of storage proteins during germination in dicots (Davis & Slack, 1981; Bewley & Black, 1994; Nandi et al. 1995;

Muntz et al. 2001; Wang et al. 2003; Gall et al. 2005). Albumin proteins of cotyledons were degraded during germination and thus behave like the globulin proteins or storage proteins (Murray, 1979).

Given the importance of *P. pinnata* seeds in earlier chapters of this thesis, there is an extensive literature on fatty acid composition of seed oil (Kesari et al. 2009; Sarma et al. 2005; Scott et al. 2008; Mukta et al. 2010). *P. pinnata* seeds contain 29-33 % fats (Sarma et al. 2005) and approximately 40 % protein (NOVOD, 2009) on a dry weight basis. These lipid and protein bodies are membrane-bound, and later one may be homogenous without any inclusions, or heterogenous containing only globoids or both crystalloids and globoids. The globoidal inclusions vary in shape, size and number, and contain principally phytin, a cation salt of myo-inositol hexaphosphoric acid (Lott, 1981). The protein matrix contains some inorganic elements (Buttrose & Lott, 1978 a, b) as well as hydrolytic enzymes (Yatsu & Jacks, 1968; Ory & Henningsen, 1969; Adams & Novellie, 1975; Konopska & Sakowski, 1978). No studies were available neither on the phenotypic features nor the localization of storage reserves and ultrastructural analysis during any stage of seed maturation or germination in *P. pinnata* seeds.

Recent technological advances have allowed the generation of plants characterized by increased oil content and/or a novel fatty acid composition (Jaworski & Cahoon, 2003; Hildebrand et al. 2005). However, a number of significant technical challenges still remain to be solved. Indeed, most of the fundamental research on oilseed metabolism has been performed in Arabidopsis. Although the majority of genes encoding lipid biosynthesis in plants have been identified and isolated (White et al. 2000; Ruuska et al. 2002; Beisson et al. 2003; Murphy, 2005), the specific pattern and fine regulation of gene expression in crop seeds are largely unknown. Seed desaturase genes and other related genes, which are developmentally regulated by various factors, play an important role during seed development in determining seed storage lipid composition (Seffens et al. 1990; Murphy et al. 1998; Holdsworth et al. 1999; Jin et al. 2001). Information from biochemical and molecular biological studies on fatty acid biosynthesis has rapidly accumulated recently (Thelen & Ohlrogge, 2002; Poghosyan et al. 2005; Tong et al. 2007; Chen et al. 2007). The first fatty acids desaturation step is catalyzed by a plastidial stearoyl-ACP desaturase (SAD)

(Thelen & Ohlrogge, 2002). After termination of the plastidial fatty acid elongation by acyl-ACP thioesterases which hydrolyze acyl chains from ACP, the free fatty acids are transported into the cytoplasm, onto the endoplasmic reticulum (ER), where they are subjected to further elongation and desaturation steps. The ω-6 and ω-3 FADs (fatty acid desaturases) introduced the second and third double bonds respectively, in the biosynthesis of 18:2 (linoleic) and 18:3 (linolenic) fatty acids which are important constituent of plant membranes and storage lipids (Voelker & Kinney, 2001; Han et al. 2001). In leaf tissues, there are two distinct pathways for polyunsaturated fatty acid biosynthesis, one located in the microsomes and the other located in the plastid membranes. In non green tissues and developing seeds, the microsomal pathway predominates (Ohlrogge & Browse, 1995; Jin et al. 2001). The microsomal ω-6 and ω-3 fatty acid desaturations are controlled by the *fad* 2 and *fad* 3 loci, respectively (Yadav et al. 1993). It has been postulated that these loci correspond to structural genes for the desaturase enzymes, which have been recalcitrant to purification and study. The free fatty acids are then esterified and transferred to glycerol to form triacylglycerol (TAG). The storage lipids of seeds consist usually of TAGs that accumulate during the maturation phase of the embryo and/or the endosperm. Oleosins are distinct from seed storage proteins, with respect to the timing of their synthesis and degradation, their sub cellular localization, their structure and their biological function (Murphy, 1990). In contrast to the storage proteins, oleosins are relatively hydrophobic proteins in nature, and are localized exclusively on the surface of storage oil bodies. It has been suggested that an important role of oleosins is to stabilize storage oil bodies during tissue dehydration (Murphy, 1990).

4.3 Methods

4.3.1 Oil extraction, chemical composition and bioassay of seed oil from CPT

4.3.1.1 Plant material

The plant material used in the current study were healthy and fully ripened matured seeds from characterized CPT individual of *P. pinnata* collected from Sila Forest Range, NG,

Assam during the month of April-May 2007. The favourable attributes of characterized elite genotype of *P. pinnata* are that they are high seed yielding tree that can be subsequently translated to maximum oil production possessing desirable quality (Kesari et al. 2008). The seeds of NGPP 46 (around 1 kg) were dried at 65 ^{o}C in an oven for approximately 5-7 days till the dry weight was constant, manually crushed and stored at 4 °C until further use (max. 3-6 months). All experiments were performed with the same dried material.

4.3.1.2 Oil extraction

The seed oil was extracted from 100 g powdered seeds following the Soxhlet extraction method as per the standard AOAC procedure (American Oil Chemical Society, 1995) using different solvents (n-Hexane, petroleum ether, ethyl acetate, isopropanol) at their boiling points. Azeotropic mixtures like ethyl acetate (91.53 %) + water (8.47 %) and isopropanol (87.8 %) + water (12.2 %) at their boiling point were also used for extraction of oil. Extracted oil was subjected to simple distillation and evaporation under reduced pressure to remove the solvent and finally lyophilized. The oil yield was expressed as % vol wt^{-1}. The crude oil was stored at 4 ˚C until further use (max. 1 month) for chemical analysis and bioactivity studies.

4.3.1.3 Determination of physical properties of seed oil

Basic physical, chemical and fuel related properties of *Pongamia* seed oil of n-Hexane fraction viz; colour, odour, refractive index, density, acid value, cloud point, pour point, kinematic viscosity, calorific value and flash point were determined in accordance in accordance with the ASTM (2000) and AOAC (1995) standard procedures. Cetane index was calculated according to Goering et al. (1982).

4.3.1.4 GC-MS analysis

Aliquots of the n-Hexane phase were analyzed by GC-MS. One microlitre of the n-Hexane extract was injected in split less mode onto a CBP-5 fused silica capillary column (30 m long × 0.25 mm i.d. × 0.25 mm film thickness composed of 5 % phenylmethylpolysiloxane), at an injector temperature of 250 ^{o}C oven initial temperature 60 ^{o}C and final temperature 260

^{o}C (5 ^{o}C min^{-1} increase every min), oven equilibration time 1.01 min, helium carrier gas pressure 100 kPa, total flow 50 ml min^{-1} and interface temperature of 300 ^{o}C, sampling time 3 min. Fatty acids were identified by standard MS Library Wiley 139.LIB programme.

4.3.1.5 Antimicrobial and antifungal assay

Bacterial cultures, viz., *Listeria monocytogenes, E. coli* (MTCC 108), *Yersinia enterocolitica* (MTCC 859) and *Salmonella paratyphi* grown in nutrient agar media (stored slants maintained at 4-5 ºC) were transferred to 10 ml of nutrient broth and incubated overnight at 37 ºC. A preculture was prepared by transferring, 1 ml of this culture to 9 ml nutrient broth (Hi-media) and incubated for 48 h. The cells were harvested by centrifugation (2795 *g* for 5 min), washed and suspended in saline. Inoculum (100 µl) was then spread onto the solidified agar plate. The assay was carried out using disc diffusion technique. Observations for growth inhibition zone were recorded after 24 h.

Fungal slant cultures viz., *Aspergillus niger, Aspergillus terreus, Candida albicans* were maintained in potato dextrose agar (PDA) medium. Inoculum (300 µl, 10^{6} spores ml^{-1}) from the spore suspension cultures of the different fungal strains was then spread onto the solidified Czapek Dox Agar (CDA) plates. The assay was done out in the same way as described for bacterial cultures and observations was recorded after 48 h. Ampicillin (USB Amersham 50 mg ml^{-1}) and Co-trimoxazole (HiMedia, 100 µg disc^{-1}) were used as positive controls. The results are represented as the diameter of the zone of inhibition (mm) ± SD, excluding the disc diameter (6 mm). Minimal inhibitory concentration (MIC) of the n-Hexane extracted seed oil of *P. pinnata* (NGPP 46) was determined according to Lennette et al. (1974). The different concentrations (35 % to 100 %) of seed oil were obtained by diluting it in pure DMSO. The minimum inhibitory concentration (MIC) was recorded as the lowest dilution of the tested sample inhibiting the visible growth of organism after 24 and 48 h for the bacteria and fungal cultures respectively on the agar plate.

4.3.1.6 Statistical analysis

Each assay was performed three times and the results were expressed as their mean ± SE.

The data were analyzed by one-way analysis of variance (ANOVA), using the SPSS 16.0 software package. Differences between means were tested by LSD tests and were considered significant at $p < 0.05$.

4.3.2 Total soluble protein pattern during sexual seed development and germination stages in CPT

Total soluble protein content and electrophoretic spectra (SDS-PAGE) in the course of development of seed maturation, germination as well as in vegetative and other reproductive tissues in CPT of *P. pinnata*, NGPP 46 were studied.

4.3.2.1 Plant material

Different tissues such as leafs, flowers, buds, seeds and roots were harvested from the CPT, NGPP 46 of *P. pinnata* (Kesari et al. 2008). During the course of development, seeds at specific stages/ages were harvested and transported to the laboratory in sealed polythene bags, where they were processed as described below. Seeds of different developmental stages were scored for various qualitative and quantitative variables. The qualitative seed characteristics scored were: shape and colour whereas quantitative seed characteristics were: fresh weight, dry weight, moisture content, length, breadth and thickness. Seeds used for protein extraction were collected during different developmental stages from 90 days after flowering (DAF) to 350 DAF periodically within an interval of 40-45 days in the year 2007 (August)-2008 (April). The matured seeds collected during end of April were used for germination. Seeds were rinsed in 25 % ethanol solution for 10 minutes and washed with distilled water two to three times before being placed in polythene bags containing sand and clay at the ratio of 1:4 in mist chamber at 28±2 ^{o}C and 70-80 % relative humidity respectively. Roots, germinated saplings (uprooted from the polybags), cotyledons, embryonic axes (cotyledonary notch portion) were harvested till 22 days of seed germination i.e. on 5th, 8th, 11th, 14th, 18th and 22nd day. The outer hard seed coat of the cotyledons of the germinated seedlings were removed, frozen in liquid nitrogen and stored in sealed containers at -20 ^{o}C until use for protein extraction and analysis respectively. All other plant parts were also stored in similar fashion. Five seeds of each

stages/ age were examined at different stages of seeds germination. Also *in vitro* callus cultures initiated from leafs and roots (described in chapter 2 section 2.2.3.2 and 2.2.3.3) stored at -20 ^{o}C were used as explants for protein extraction and SDS-PAGE analysis.

4.3.2.2 Total soluble protein extraction and quantification

Plant material (leafs, root, flowers, cotyledons) approximately 0.5 g were ground in a mortar and pestle using liquid nitrogen with an extraction buffer containing 100 mM Tris-HCl, pH 8.1, 10 mM EDTA, pH 8.0, 10 mM β-mercaptoethanol and 0.2 mg/ml phenylmethylsulfonyl fluoride (PMSF). All extractions were normalized on the basis of fresh weight to buffer ratio (1:1; 500 mg/500µl). The homogenate was then centrifuged at 13,000 *g*, 30 min twice and the supernatant (crude extract) was collected as protein sample. The extracts were dissolved in the sample buffer (250 mmol/L Tris-HCl, pH 6.8, 4 % SDS, 10 % β-mercaptoethanol, 20 % glycerol, and 0.03 % bromophenolblue) and boiled for 5 min, the collected supernatants were stored at -80 ºC till further use. Protein concentration was determined colourimetrically according to the method of Bradford (1976) using bovine serum albumin as standard. All the experiments were performed in triplicates.

4.3.2.3 SDS-PAGE analysis of total soluble seed storage proteins

Denatured proteins were separated by SDS-PAGE with the discontinuous buffer system (Laemmli, 1970) using a vertical slab gel (1.5 mm thick, Biorad Mini Protean II). The concentrations of stacking gel (pH 6.8) and resolving gel (pH 8.8) were 5 % and 12.5 %, respectively. Tris glycine buffer (pH 8.5) containing 0.1 % SDS (w/v) were used as the electrode buffer. Protein samples dissolved in the sample buffer and marker were denatured at 100 °C for 5 min before being loaded onto the gel. Briefly, 50-100 µg protein samples, as quantified by the Coomassie brilliant blue G250 assay (Bradford, 1976) were loaded onto each lane and electrophoresis was carried out at initial voltage of 120 V and raised to 180 V when the tracking dye reached the gel mold. After electrophoresis, proteins were visualized by staining the gels with 0.05 % (wt/vol) Coomassie Brilliant blue-R 250 in 40 % methanol and 10 % acetic acid and the band intensities were analyzed by gel documentation system. Molecular weight of polypeptide was determined by the mobility of the standard molecular

weight markers for SDS-PAGE (Bangalore Genei, India). The protein profiles of various plant samples were confirmed by three separate extractions. Each experiment was repeated thrice and only a representative gel is shown in results.

4.3.3 Localization and distribution of storage reserves in the cotyledon tissues during different stages of seed maturation and germination in CPT

4.3.3.1 Plant material

Localization of storage reserves in the cotyledons of CPT of *P. pinnata*, NGPP 46 was carried out for the same seed material as used for protein analysis mentioned in section 4.3.2.1. Sexual seed development (~10 months or 350 DAF; days after flowering) and seed germination (22 days) was followed by observations of serial transverse sections (TS) of cotyledons through whole seeds fixed in fixatives. Localization of storage reserves in the cotyledons of CPT of *P. pinnata*, NGPP 46 was carried out from the four different stages of sexual seed development viz.; early immature (90-135 DAF), late immature (180-225 DAF), early mature (225-270 DAF) and late matured (315-350 DAF) stages. Similarly, localization of storage reserves in the cotyledons of germinated seedlings of CPT of *P. pinnata* were performed from early germination (within 8 days) to late germination stages (within 45 days). All the experiments were performed in duplicates. Two seeds were examined at each developmental stage.

4.3.3.2 Electron microscopic studies

4.3.3.2.1 Sample Preparation for SEM

For SEM observation, cotyledonary tissues of different stages of sexual seed development and germination were fixed in 2.5 % glutarldehyde for 48 h and post fixed in 1 % osmium tertraoxide (OsO_4) at 4 ºC. Sections 10-12 mm thick were dehydrated at room temperature at 15-30 min intervals in a graded series of aqueous ethanol (10, 30, 50, 70, 90, 96 and 100 %), critical point dried using CO_2, mounted directly on aluminum stubs using double-side adhesive tape, and sputter-coated with gold. Observations were made in a LEO 1430 VP;

Leo Electron Microscopy Limited, Cambridge, UK at an accelerating voltage of 10 kV. For localization of lipids, cotyledonary tissues of matured ripend seeds were first defatted in acetone and n-Hexane for 48 h and then fixed in 2.5 % glutarldehyde.

4.3.3.2.2 Sample Preparation for TEM

Cotyledons were cut with a sterile razor blade and fixed in Karnovsky's fixative (Karnovsky, l965) for 48 h at 4 ºC. The material was post-fixed for 1 h in 1 % (w/v) OsO_4 in 0.1 M sodium phosphate buffer (4 ºC, pH 7.0), rinsed three times 10 min in buffer and dehydrated at room temperature at 15-30 min intervals in a graded series of aqueous ethanol (10, 30, 50, 70, 90, 96 and 100 %). Dehydrated tissues were infilterated and embedded in Spurr's resin for long pot-life (Spurr, 1969). Ultrathin sections cut with an ultramicrotome were stained with 40 g/l uranyl acetate for 45 min, followed by 4 g/l lead citrate for 4 min, mounted on grids and observed in a Philips CM 10, Philips (Eindhoven, The Netherlands).

4.3.4 Transcript analysis of fatty acid genes involved in triacylglycerol biosynthesis during developmental stages of sexual seed maturation by RT-PCR

4.3.4.1 Plant material

Seeds of 4 different developmental stages viz., stage II (90-135 DAF, early immature), stage IV (180-225 DAF, late immature), stage V (225-270 DAF, early mature) and stage VII (315-350 DAF, late mature) and leafs were harvested from CPT, NGPP 46 of *P. pinnata*. Roots of the same genotype mentioned above were harvested from the germinated saplings grown in the green house conditions. All the collected tissues were immediately frozen in liquid nitrogen and stored at -80 °C for later RNA extraction and single step RT-PCR amplification.

4.3.4.2 Reagents and solutions

4 M Guanidinium iso thiocyanate; 1 M Tris Base (pH 8.0); 0.5 M EDTA (pH 8.0); 10 % sodium dodecyl sulphate (SDS); 2 M NaCl; β-mecaptoethanol; 2 M sodium acetate (pH 5.2);

25 mM Sodium citrate; 10 % N-laurosylsarcosine (Sarkosyl); Tris saturated Phenol (pH 8.0); Chloroform; Isopropanol, -20 °C; Absolute ethanol, -20 °C; 70 & 75 % ethanol (v/v); TE: 10 mM Tris-HCl (pH 8.0); 1 mM EDTA (pH 8.0); DEPC (Sigma); Oligo dT (10 mM); 3M NaOH; 1X TAE. RNA extraction buffer (GITC-Denaturing Buffer): 4M GITC, 25mM Sodium citrate pH 7.0; 1 % N-laurosylsarcosine (Sarkosyl); and 0.1M β-mercaptoethanol add immediately before use.

4.3.4.3 RNA quantification, primer design and transcript analysis by RT-PCR

RNA quality and quantity was estimated by measuring the $A_{260/280}$ ratio and absorbance at 260 nm with a spectrophotometer respectively. The integrity of total RNA extracted was characterised by resolving 2 µl RNA on 1 % agarose gel under denaturing conditions with (0.4 M) formaldehyde followed by staining with EtBr (0.5 µg/mL). All the experiments were repeated twice.

Oligonucleotide primers were designed for the genes involved in fatty acid biosynthesis pathway based on the sequence information of the central cDNA fragments available from NCBI. Table 4.1 shows the sequence of the gene specific primers and there expected product size. The primer for glyceraldehyde-3-phospate dehydrogenase gene was used as an internal control. For RT-PCR reaction 1µg of total RNA isolated from roots, leaf and 4 different developmental stages of seeds (stage II, stage IV, VI and VII) were used as a template in first strand cDNA synthesis using an Oligo dT primer (0.5 µg/ reaction) and ImProm-II™ Reverse Transcription system (Promega), according to the manufacturers protocol. The products were quantified by running 2µl cDNA on 1 % agarose gel and the samples were stored at 4 °C for short term storage and -20 °C for long term storage. For second strand synthesis corresponding to four fatty acid genes namely microsomal FAD 3, oleosin 1 mRNA, SACPD A, FAD 2 microsomal FAD 3, oleosin 1 mRNA, SACPD A and FAD 2 gene specific primers mentioned in Table 4.1 was used. PCR amplification was performed in 25 µL reaction volume containing cDNA amount corresponding to 200 ng total RNA as a template, together with 200 µM of each dNTP, 2 mM $MgCl_2$, 15 µM of each

oligo, 5 U *Taq* DNA polymerase, using a DNA Thermal cycler (Applied Biosystems, USA). The PCR thermal-cycling parameters were as follows: an initial denaturizing step of 95 °C for 5 min, followed by 35 repeats of 95 °C for 1 min, annealing time was 1 min 30 sec with varied temperatures as per the melting temperature of the gene specific primer, extension at 72 °C for 2 min and final extension at 72 °C for 7 min. Amplified products were loaded in 1.5 % agarose gel containing 0.5 µg/mL of EtBr and documented by a gel documentation system (Bio Rad, USA). All the experiments were repeated twice.

Table 4.1. Details of gene specific primers and internal control used for single step RT-PCR amplification.

Gene name	Primer Designed (5-3)	Length of the primer	Expected Product size
Microsomal FAD 3	F1-CCAGGGAAAGAAGGTTCA R1-TACTTTCCCAGCACTGGC	18	424 bp
Oleosin 1 mRNA	F2-CGAGTATGAGCGACGACC R2-CCTTCTGGCCCAATTGAC	-do-	394 bp
SACPD A	F4-GAAGCCATTCACTCCTCC R4-TCAACTCGACCACTCAAG	-do-	437 bp
FAD2	F7-CACTCCAACACCGGTTCG R7- CGAGGAACGCGTTGACTA	-do-	369 bp
GAPDH	F8-GCAGGAACCCTGAGGAGATC R8-TTCCCCCTCCAGTCCTTGCT	20	360 bp

F-Forward; R-Reverse;FAD- fatty acid desaturase;
SACPD A-steroyl ACP desaturase; GAPDH-glyceraldehyde 3-phosphate dehydrogenase

4.4 Results and discussion

4.4.1 Chemical composition and bioassay of seed oil from CPT

4.4.1.1 Plant material

For *Pongamia* to be an effective, economical and promising biodiesel plant it should possess traits of significance such as high seed yield with high oil content of desirable quality. The CPT is an individual tree of *P. pinnata* possessing superior morphological characters than other individuals of the same species as shown in chapter 1. Ten CPT belonging to two different populations (6 and 10) were identified from a total of 50 tagged trees (each of the

50 trees is a different genotype) based on several vegetative and reproductive markers from natural populations growing in North Guwahati, Assam. Ten CPT were analyzed for pod and seed traits, the two most important aspects with regard to oil and in the second stage the best performing genotype was identified (Kesari et al. 2008). This best reporting CPT (henceforth referred with original tag number NGPP 46; Fig. 4.1) was progressed further for determination of physical and chemical composition related to fuel properties and bioassay of seed oil against reference pathogenic indicators.

4.4.1.2 Oil extraction and yield

The favourable attributes of characterized elite genotype of *P. pinnata* are that they are high seed yielding trees with maximum oil production. Thus the next important step is to exploit the right oil extraction technique for the recovery of maximum oil from the seeds or kernels for biodiesel production. Seed oil yield extracted using Soxhlet method was found to be in the range of 26.0 to 33.0 % (vol wt^{-1}) by using different solvents as shown in Table 4.2. The oil is dark in colour with a disagreeable odour. Soxhlet extraction yielded best results with maximum yield being closer to 33 % using n-Hexane. Advantage of using organic solvents in extraction technique is that they can be recovered during the process and is easy for implementation and has been employed in many potential biodiesel crops (Akintayo, 2004).

For all the extractions, seed to solvent ratio taken was 1:6 (wt/ vol) as this has been found to be the optimal ratio for getting higher yield in less time with good quality oil (Kesari et al. 2008). However, it was found that minimum time was required for extraction of oil using non-polar solvents (n-Hexane and petroleum ether) whereas for polar solvents (ethyl acetate and isopropanol) and their azeotropic mixtures, the time taken for extraction was more than double for the same amount of the seed material. Minimum oil yield of 23.5 % was obtained when water was used as a solvent for extraction using hot water bath method (Kesari et al. 2008). From the Table 4.2 it is evident that the oil yield in azeotropic solvents is less in comparison with pure solvents, possibly because of decrease of solubility of oil in presence of water. The oil yield in isopropyl alcohol was found to be better but as a safety measure one can use ethyl acetate since isopropyl alcohol at higher temperature forms ketonic compound which is harmful to human health (Lusas et al. 1991).

Figure 4.1. *Pongamia pinnata* CPT (NGPP 46) from study site.

A. Growing in natural habitat; **B.** Matured seeds.

4.4.1.3 Physical and fuel related properties of seed oil from NGPP 46

The oil obtained using ethyl acetate (polar) and n-Hexane (non polar) organic solvents from the seeds of NGPP 46 i.e. CPT of *P. pinnata* were used for the determination of physico-chemical properties, important from biodiesel aspect. Table 4.3 gives the comparative study of fuel related physical properties for the seed oil from two different solvents viz., n-Hexane and ethyl acetate.

The density of n-Hexane and ethyl acetate extracted seed oil from NGPP 46 was found to be 0.927 and 0.932 kg L^{-1} respectively. The density of the *P. pinnata* seed oil is although similar to those of the other vegetable oil, it is higher than the acceptable ranges of petroleum diesel (0.820–0.845 kg L^{-1}) as per the current ASTM requirement and European specifications (prEN:590:2003) for automotive diesel fuel. Therefore, volumetric metering of the vegetable seed oil would result in the delivery of greater mass of fuel as is the case in unit injectors used in modern diesel engines. The calorific value for petro-diesel is 45.4 MJ kg^{-1} whereas those for the seed oil of NGPP 46 extracted from the two solvents are 44.34 MJ kg^{-1} and 42.88 MJ kg^{-1} respectively. Thus it has lower energy content on both the volumetric and a mass basis. This low energy content is due to the presence of chemically bound oxygen in the fatty acid chains as has been reported by Srivastava & Prasad (2000). However, based on its higher density and volume, the energy content of *P. pinnata* seed oil is closer to diesel fuel. The viscosity of a liquid fuel is important for it to flow through pipelines, injector nozzles, orifices and for atomization of fuel in the cylinder (Goering et al. 1982). The kinematic viscosity values for the oil recorded for NGPP 46 for the two solvents used in the current study were 24.9 and 26.3 cSt respectively. The kinematic viscosity of *P. pinnata* seed oil is lower than those reported for other vegetable oils (in the range of 30-40 cSt at 40 °C) used for biodiesel production (Akintayo, 2004). To reduce viscosity of *Pongamia* seed oil and improve its performance in a diesel engine steps like transesterification; pyrolysis or emulsification is recommended (Altin et al. 2001). The flash point recorded for elite genotype NGPP 46 was 205 °C for non polar solvent and 215°C for polar solvent which were less as compared to the other vegetable oils, but far greater than that of the conventional diesel fuel (55 °C). All vegetable oils have relatively high flash points,

Table 4.2. Oil yield from seeds of CPT of *P. pinnata* [a]

Solvents	Oil yield (%, vol wt^{-1})	Solvent recovery (%, vol wt^{-1})
n-Hexane	33.00± 0.15	82.50 ± 0.20
Petroleum ether	31.00 ± 0.21	77.50 ± 0.36
Ethyl acetate	31.50 ± 0.27	79.00 ± 0.23
Isopropanol	32.00 ± 0.29	80.00 ± 0.21
Ethyl acetate + H_2O (91.53 + 8.47) %	26.00 ± 0.32	74.50 ± 0.32
Isopropanol + H_2O (87.80 + 12.20) %	28.00 ± 0.31	76.00 ± 0.25

[a] seed to solvent ratio was 1:6 in all cases; Each value= mean ± SE of three replicates

Table 4.3. Physical properties from seed oil of CPT of *P. pinnata.*

Characteristics	Organic solvent	
	n-Hexane	Ethyl acetate
Colour and Odour	Dark brown and Repulsive	Dark brown and Repulsive
Oil content (%)	33.00 ± 0.26	31.50 ± 0.46
Refractive index (at 28.6 ºC)	1.4782 ± 1.73×10^{-4}	1.4750 ± 1.65×10^{-4}
Density (kg L^{-1} at 15 ºC)	0.927 ± 2.65×10^{-3}	0.932 ± 2.00×10^{-3}
Calorific value (gross) (MJ kg^{-1})	44.34 ± 0.12	42.88 ± 0.15
Kinematic viscosity (cst at 40 ºC)	24.91 ± 0.03	26.28 ± 0.03
Flash point (ºC)	205.00 ± 0.50	215.00 ± 0.60
Cetane index	35.00 ± 1.00	--
Cloud point (ºC)	4.00 ± 0.10	4.90 ± 0.13
Pour point (ºC)	3.00 ± 0.36	3.70 ± 0.42

Each value= mean ± SE of three replicates

so they are considered to be safe fuels under normal conditions. It was also earlier reported that *Pongamia* oil has a high viscosity and flash point but once chemically modified can fulfill the requirement of being a potential biodiesel alternative (Karmee & Chadha, 2005).

The calculated cetane index of *Pongamia* seed oil was 35 for n-Hexane fraction. This was within the typical cetane index range for all vegetable oils. The cetane numbers of vegetable oils increase with trans-esterification. High flash point and high cetane index, however, are good indications from the view point of auto-ignition and improvement of knocking properties. In current study, the cloud point was found to be 4 °C and 4.9 °C for n-Hexane and ethyl acetate extracted oil respectively. NGPP 46 seed oil had the lower cloud point when compared to the other vegetable oils. Moreover, its pour point was maximum of 3.7 °C in case of ethyl acetate extracted oil, which is within the range of 12 to -31 °C reported for vegetable oils (Goering et al. 1982). One of the disadvantages of the commonly used seed oils is the inadequate cold flow performance during winter (Adhvaryu et al. 2003). This problem can be overcome by the use of anti-gelling additives or by recirculation the exhaust gas to warm the fuel tank (Lang et al. 2001). From the results it is clear that not much difference lies in the physical properties of seed oil of NGPP 46, extracted using solvents of two different polarities and that n-Hexane fraction showed only a marginal improvement in physical parameters with regard to fuel properties.

4.4.1.4 Fatty acid profile

Table 4.4 presents the fatty acid profile for n- Hexane fraction of seed oil from CPT of *P. pinnata*. There was a predominance of compounds containing an even number of carbon atoms, especially C_{16} and C_{18} in *Pongamia* seed oil. The total saturated and unsaturated fatty acid composition was 20.5 and 79.4 %, respectively. Fatty acid profile shows that low molecular weight fatty acids such as lauric and capric acids occur in very small amount of about 0.1 % each. The concentration of arachidic acid was also found to be less (0.8 %) but higher than the above two acids. The major saturated acid present in this oil was palmitic acid (10.8 %) followed by stearic acid (8.7 %). Thus total of saturated fat present in *Pongamia* seed oil is higher than what has been reported earlier (Ahmad et al. 2003). The major mono unsaturated fatty acid was oleic acid (46 %) whereas linoleic (27.1 %) acid and linolenic acid (6.3 %) constitutes the total polyunsaturated fatty acid. The composition of unsaturated fatty acid was again different from what has been reported earlier (Sarma et al. 2005; Sharmin et al. 2006). The

differences in fatty acid composition may be due to the reason that the seed material is from different genotypes and from different ecological conditions. It is well known fact that local edaphic and environmental factor plays a crucial role in growth and characteristic of a particular plant making germplasm rich and unique and imparting characteristic traits specific for a region. So it is not surprising that the fatty acid profile from CPT in present study was different and possibly better from earlier studies. It should be kept in mind that any analyses should be site specific since plant characters; both quantitative and qualitative varies from place to place.

Table 4.4. Fatty acid composition from seed oil of CPT of *P. pinnata*.

Fatty acids	Composition (% by wt)			References
	Structure[a]	Current study	Previous study	
Capric acid	10:0	0.1		-
Unidentified	-	0.1	1.05	Sarma et al. 2005.
Lauric acid	12:0	0.1		-
Palmitc acid	16:0	10.8	9.23, 11.30	Ahmad et al. 2003, Sarma et al. 2005.
Stearic acid	18:0	8.7	4.48, 9.80	Ahmad et al. 2003, Sarma et al. 2005,
Oleic acid	18:1	46.0	71.0, 45.25	Ahmad et al. 2003, Sarma et al. 2005.
Linoleic acid	18:2	27.1	14.5, 24.75	Ahmad et al. 2003, Sarma et al. 2005.
Arachidic acid	20:0	0.8	1.75	Sarma et al. 2005.
Linolenic acid	18:3	6.3	2.90	Sarma et al. 2005.
Behenic acid	22:0	-	3.20	Sarma et al. 2005.
Myristic acid	14:0	-	0.23	Ahmad et al. 2003.
Saturated fat	-	20.5		-
Monounsaturated fatty acid	-	46		-
Polyunsaturated fatty acid	-	33.4		-

[a]xx:y indicates the number of carbons (xx) in the fatty acid chain and the number of double bonds (y) in the carbon chain.

4.4.1.5 Antibacterial and antifungal assay

Although different parts of *P. pinnata* have been used in the traditional systems of medicine for treating various skin problems and have been widely tested for insecticidal, nematicidal, antifungal, antibacterial and antiviral activities (Elanchezhiyan et al. 1993; Simin et al. 2002)

there is however no report as regards to the antimicrobial activity from seed oil of an elite genotype of *P. pinnata* against the pathogenic indicators. The results indicate that seed oil from elite genotype of *P. pinnata* (NGPP 46) inhibited the growth of all the tested bacteria at a minimal concentration limit of 50 % (except *S. paratyphi*) and maximum inhibition observed at 90 % (Table 4.5). For antibacterial assay 90 % oil with DMSO gave more inhibition rather 100 % seed oil. The seed oil of *P. pinnata* showed maximum antibacterial activity against *Y. enterococcai* followed by *L. monocytogens, E. coli, S. paratyphi* respectively (Table 4.5). Various authors have reported that Lauric, palmitic, linolenic, linoleic, oleic, stearic and myristic acids which are important constituents of plants are known to have potential antibacterial and antifungal agents (McGaw et al. 2002; Agoramoorthy et al. 2007). These antibacterial actions of fatty acids are usually attributed to long-chain unsaturated fatty acids including oleic acid, linoleic acid, and linolenic acid which are bactericidal to important pathogenic microorganisms including S*taphylococcus aureus, Helicobacter pylori, Klebsiella pneumoniae, Escherichia coli* and *Mycobacteria* (Agoramoorthy et al. 2007). Zheng et al. (2005) also reported that long-chain saturated fatty acids, including palmitic acid and stearic acid, tested were less active or not active on either Gram positive or Gram negative bacteria. This differential activity of saturated and unsaturated fatty acids was well correlated with the inhibition of *S. aureus* FabI (trans-2-Enoyl-ACP reductase I) *in vitro* (Zheng et al. 2005). However, the precise mechanism for this antimicrobial activity remains unclear. Zheng et al. (2005) found that linoleic acid inhibited bacterial enoyl-acyl carrier protein reductase (FabI), an essential component of bacterial fatty acid synthesis, which has served as a promising target for antibacterial drugs. Additional unsaturated fatty acids including palmitoleic acid, oleic acid, linolenic acid, and arachidonic acid also exhibited the inhibition of Fab I.

In the present study, the antimicrobial screening of the n-Hexane fractionated seed oil from CPT of *P. pinnata* revealed that the seed oil were more effective against fungal than bacterial cultures. Zone of inhibition by the action of seed oil against tested microorganism (*L. monocytogens* and *A. terreus)* is depicted in Fig. 4.2. Antifungal activities exhibited by linolenic, linoleic and oleic acids against plant pathogenic indicators have been reported earlier (Raynor et al. 2004). The possibility of the therapeutic use of various unsaturated fatty acids from *Pongamia* as antimicrobial agents is thus worthy of note. The membrane disruption could be one

of the likely mechanisms of action of the seed oil, inactivating the microbial adhesions, enzymes, and transport proteins. The findings in current study agrees with the earlier reports (Kumar et al. 2007; Wagh et al. 2007) on strong activity of *P. pinnata* (seed oil or extract) against various fungal and bacterial pathogens. There are also some reports on the antimicrobial and antifungal activity of the main components (Alam, 2004). The oil of *P. pinnata* inhibited efficiently all the micro organisms tested with MIC value mostly 50 %. The results are interesting and confirm the importance of the correlation between the chemical content of the oils and the antimicrobial activities. This investigation therefore justifies its ethnomedical use and can thus be used to discover bioactive natural products that will lead to the development of new pharmaceuticals. The antimicrobial activities can be enhanced if the active components are purified and adequate dosage determined for proper administration. Such screening of various natural organic compounds and identification of active agents must be considered as a fruitful approach in the search of newer antimicrobial chemotherapeutic agents. This is also imperative because there is increasing treatment failure rates of microbial infections due to drug-resistant antibiotics (Selwyn et al. 1980). In the present study, the n-Hexane fraction of the *P. pinnata* seed oil has a very high potential to be used as a source for drug discovery for antimicrobial agents.

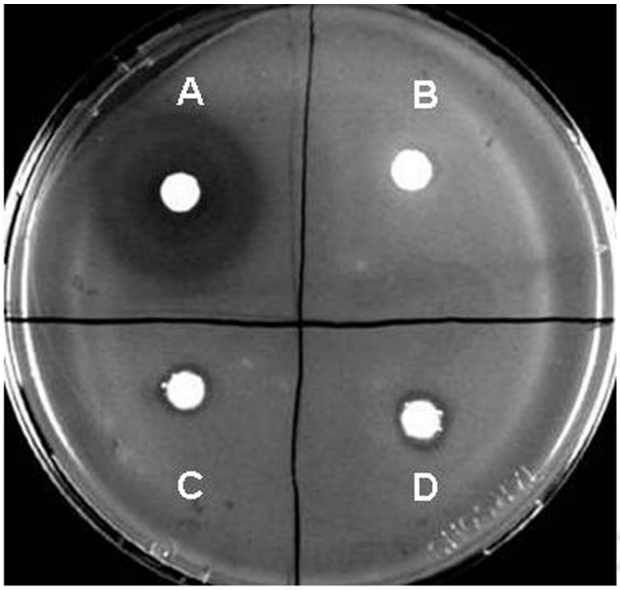
Table 4.5. Antibacterial and antifungal activities of the seed oil from CPT of *P. pinnata*

		Microbial cultures						
		Diameter of Zone of inhibition[a] (mm)[#] ± S.E.						
		Listeria monocytogens	*Escherichia coli* MTCC 108	*Yersinia enterococcai* MTCC 859	*Salmonella paratyphi*	*Candida albicans*	*Aspergillus niger*	*Aspergillus terreus*
Oil concentration %	**100**	2.63 ± 0.15b	2.10 ± 0.12b	3.48 ± 0.09c	1.32 ± 0.15a	10.97 ± 0.18d	20.50 ± 0.45e	11.08 ± 0.41d
	90	6.07 ± 0.13c	5.13 ± 0.39b	6.43 ± 0.18c	2.68 ± 0.15a	7.98 ± 0.20d	10.10 ± 0.12e	9.07 ± 0.22f
	80	2.13 ± 0.22bc	1.72 ± 0.13b	2.45 ± 0.09c	1.02 ± 0.04a	5.02 ± 0.16d	6.03 ± 0.23e	7.05 ± 0.18f
	50	1.17 ± 0.15b	0.62 ± 0.12a	1.03 ± 0.09ab	0.00	3.03 ± 0.22d	3.50 ± 0.12e	1.97 ± 0.18c
RA*		20.50 ± 0.47d	7.77 ± 0.18a	16.63 ± 0.13c	12.05 ± 0.08b	22.15 ± 0.25e	25.22 ± 0.49f	45.43 ± 0.47g
MIC (% of oil)		50	50	50	60	40	40	45

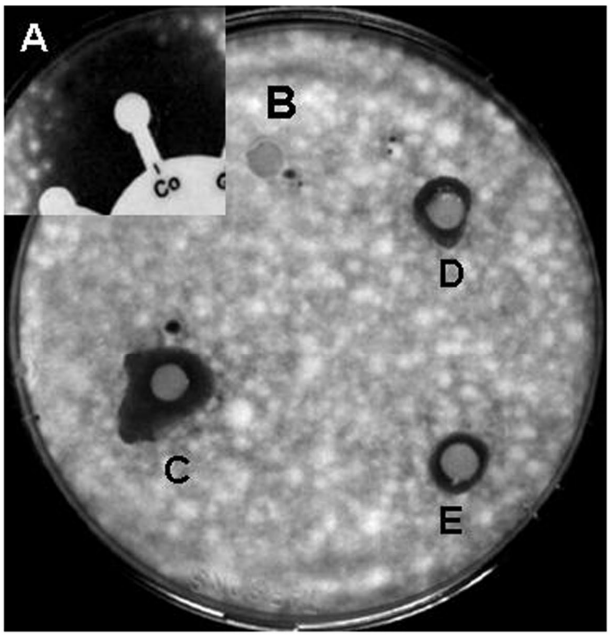
[a]Zone of inhibition from oil and reference antibiotics are given as mean ± SE of three replicates.

[#]Excluding the disc diameter, 6mm

RA*: reference antibiotics (Ampicillin (50 mg ml^{-1}) for bacteria and Co-trimoxazole (100 µg $disc^{-1}$) for fungus. Values followed by the same letter within the same row, are not significantly different ($p>0.05$) according to LSD test.



I



II

Figure 4.2. Microbial assay of the seed oil against **I.** *L. monocytogens*; **II.** *A. terreus*. **A.** Reference antibiotics (Ampicillin 50 mg ml^{-1} for I and Co-trimoxazole 100 µg $disc^{-1}$ for II); **B.** Negative control DMSO; **C**. Crude oil; D- 90 % oil; **E.** 80 % oil.

4.4.2 Total soluble protein pattern during sexual seed development and germination stages in CPT

4.4.2.1 Morphological parameters of developing seeds

Seeds collected from CPT, NGPP 46 of *P. pinnata* from NG, Assam at various stages of its maturation (starting from mid August to end April) were categorized into seven developmental stages viz; stage I (highly immature; 90 DAF, days after floweing) to stage VII (highly mature; 350 DAF). The whole of the seed maturation process in *P. pinnata* takes approximately one year from the month of flowering (May). Moisture content of the seeds ranged from 10.50 to 88.32 %; lowest observed in mature seeds in stage VII and highest at stage I (Table 4.6). In general, seed development and acquisition of the ability to germinate are associated with an overall loss of moisture (Adams & Rinne, 1980), and this was absolutely true for *P. pinnata* seeds. Germinability of the embryo was coupled with a decrease in moisture content of the seed plus endocarp unit (Li et al. 1999). Seeds vary w.r.t. shape, size and colour at each of the seven stages of development. Seeds of *P. pinnata* were ovate or oblong in shape. Seed colour from stage I (90 DAF) to stage IV (225 DAF) was dark-green, yellowish-green in stage V (270 DAF), pale yellow in stage VI (315 DAF) and turned dark brown in stage VII (350 DAF) i.e. fully ripened seeds (Fig. 4.3A). Increase in fresh weight from stage I to V was nearly 57 times and subsequently decreased at stages VI and VII when seeds ripened and turn yellow or brown in colour. Similarly, seeds phenotypic traits (length, breadth & thickness) that contributes to the increase in seed mass proportionately increased from stage I to stage V, however, it shrunk considerably at stage VI & VII (Fig. 4.3B). The data thus point that the greatest change in mass occurred in *P. pinnata* seed cotyledons, in which most of the reserve accumulation occurred. Early seed development in *P. pinnata* (morphogenesis phase) is characterized mainly by cell divisions followed by seed filling phase during which cell divisions cease or slow down. During seed filling and subsequent maturation, most of the dry weight increase occurs (Table 4.6). Evaluation of 7 phenotypic or morphological traits (both qualitative and quantitative variables) of seeds (fresh weight, dry weight, moisture content, colour, length, breadth and

thickness) enabled differentiation of all of the seven stages of sexual seed maturation process, with a clear separation between immatured and matured stage.

Table 4.6. Fresh weight, dry weight and moisture content (%) in different developmental stages of sexual seeds maturation in CPT, NGPP 46 of *P. pinnata*.

Seed developmental stages	Fresh weight	Dry weight	Moisture %
I (Mid August; 90 DAF)	0.086 ± 0.008	0.010 ± 0.001	88.32
II (End September; 135 DAF)	0.331 ± 0.019	0.083 ± 0.000	74.79
III (Early November; 180 DAF)	1.135 ± 0.083	0.337 ± 0.035	70.35
IV (Mid December; 225 DAF)	2.771 ± 0.181	1.295 ± 0.107	53.27
V (Early Feburary; 270 DAF)	4.915 ± 0.083	2.771 ± 0.435	43.62
VI (Mid March; 315 DAF)	4.388 ± 0.227	2.836 ± 0.541	35.38
VII (End April; 350 DAF)	3.377 ± 0.031	3.022 ± 0.122	10.50

Values are mean ± SE of triplicates.
DAF; days after flowering

4.4.2.2 Changes in total protein content during sexual seed development and germination stages

Significant changes in total soluble protein content were observed amongst different stages of seed maturation, vegetative and other reproductive tissues as well as in cotyledons and embryonic axis of germinating seedlings of *P. pinnata*. Time-course of protein synthesis in *Pongamia* seeds were investigated from 90 days after flowering (DAF) till seed ripening (350 DAF). Total soluble protein content varied from 5.59±0.45 mg/ml in immatured seeds of stage I to 18.92±0.90 mg/ml in matured ripened seeds of stage VII (Fig 4.3B). The increase in protein content obtained from stage I to stage VII of seed maturation is 73 %. *Pongamia* seed development could be roughly divided into 3 phases according to the presence of differential rates for protein synthesis: initial, early synthesis period (before 135 DAF, stage I), middle, rapid and massive synthesis period (135-270 DAF, stage II-V) and last, slow synthesis period (270-350 DAF, stage V-VII). Stage II to V was the period showing the highest rate of protein synthesis. On a dry weight basis, most of the total protein

synthesis in *P. pinnata* seeds took place within this period (Fig. 4.3B). The protein contents reported in this study also corroborates the fact that *Pongamia* seeds contain protein levels comparable with that of other legume species. While comparing the total soluble protein content amongst the different tissues, highest protein content were observed in mature ripened seeds (approx. 19.78±0.47 mg/ml) and lowest concentration in the root calli (3.03±0.21 mg/ml) (Fig. 4.4). Data indicates large variation in the protein contents amongst different stages of seed development and different tissues of *P. pinnata*.

The quantitative changes in total soluble protein content in the cotyledons of germinating seedling of *P. pinnata* varied between 18.92±0.47 (stage I) to 14.68±0.21 (stage VII) whereas for the germinated embryonic axis (cotyledonary notch) it varied between 18.92±0.47 mg/ml (stage I) to 10.27±0.32 mg/ml (stage VII) (Fig. 4.5). It is inferred from the present investigation that there is an almost linear decrease in protein concentration till 11^{th} day of germination, followed by slight increase on 14^{th} day (in both cotyledons as well as embryonic axis of germinating seedlings), again declines and becomes stationary. Thus the maximal rate of protein depletion were observed during the first (0-11 day) and last stages (18-22 day) from both cotyledon and embryonic axis of the germinating seedling of *P. pinnata*. The data demonstrated higher rate of protein degradation in embryonic axis than cotyledon for the same time interval i.e. 22 days of seed germination. The overall losses of total soluble protein content were 22.41 % and 41.75 % from cotyledon and embryonic axis respectively within 22 days of seed germination. The results elucidated that the protein bodies of the seeds are degraded and utilized to support the growth of germinating seedlings. *Pongamia* seed storage proteins has not been previously evaluated for its development and germination but it would be of interest to compare the results presented here with those obtained by other workers on the use of the seed storage proteins for seedling growth and development. Many authors reported decrease in the level of protein content in germinating cotyledons in *Macrotyloma uniflorum* (Karunagaran & Rao, 1990; Rajeswari & Ramakrishna, 2002), *Lupinus albus* L. (Nandi et al. 1995) and *Lathyrus sativus* (Chandna et al. 1995). Storage protein mobilization was observed in *Pisum sativum* L., *Glycine max* L., *Vicia sativa* L. and *Phaseolus vulgaris* L. (Schlereth et al. 2000; Tiedemann et al. 2001).

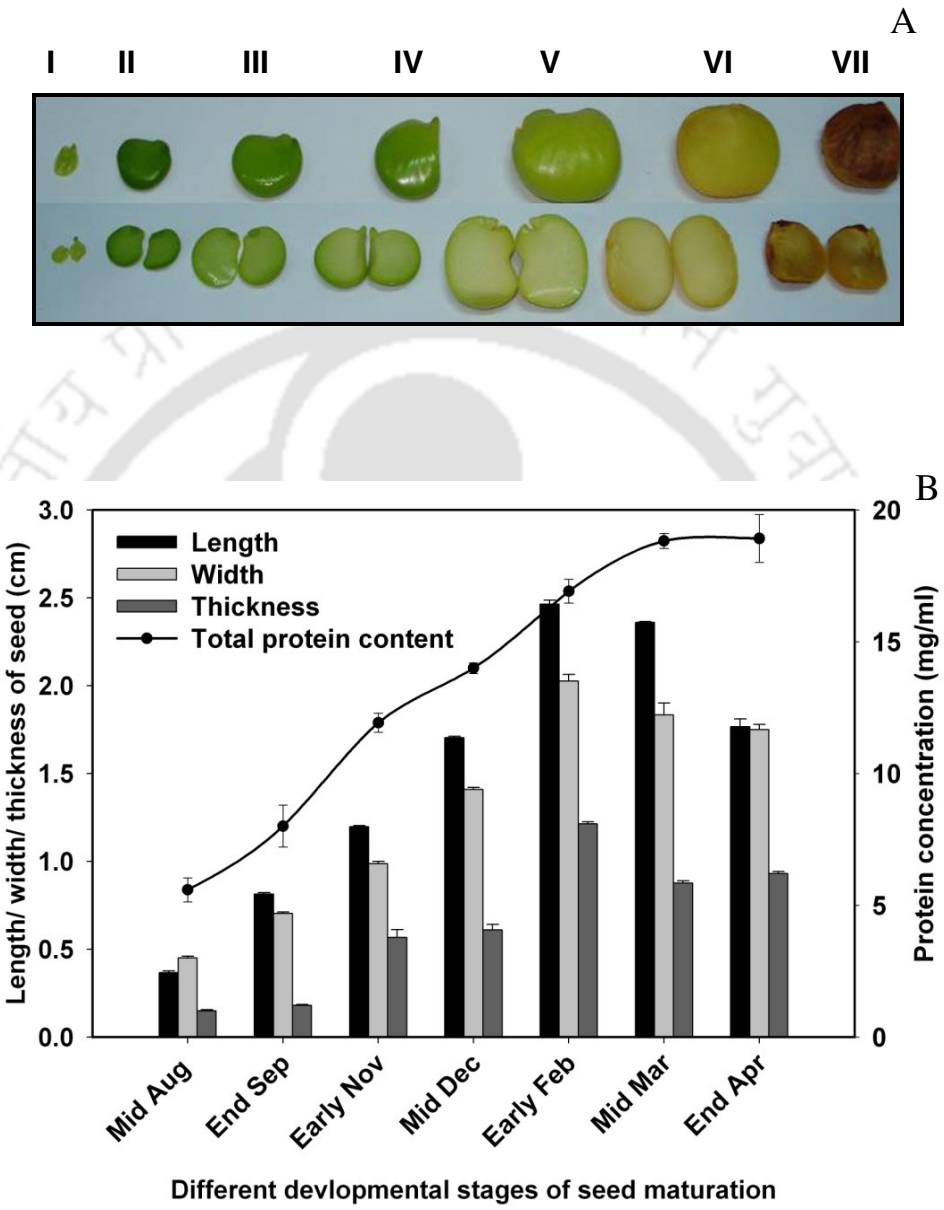


Figure 4.3. Developmental stages of seeds maturation in CPT, NGPP 46 of *P. pinnata.*

A. Stage I-VII;

B. Length, breadth, thickness and comparison of total soluble protein content

Values are mean ± SE of triplicates.

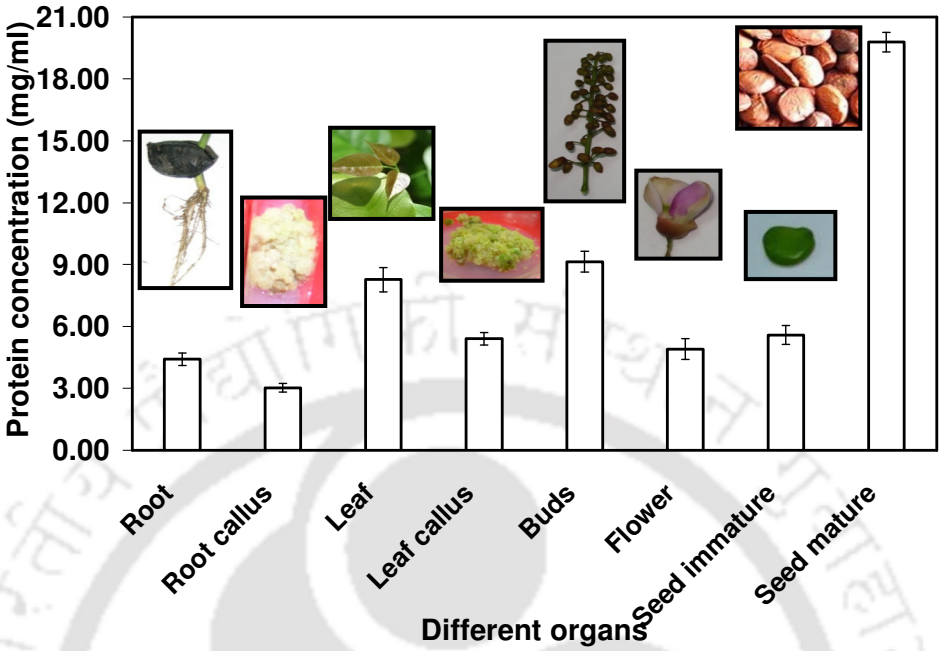


Figure 4.4. Total soluble protein content in vegetative and reproductive tissues of CPT, NGPP 46 of *P. pinnata.*

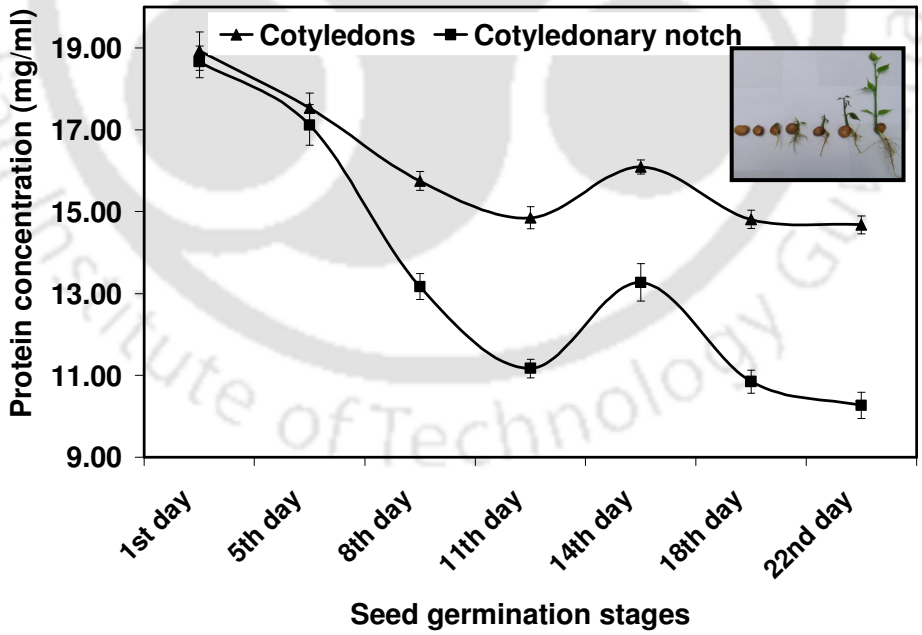


Figure 4.5. Changes in total soluble protein content during seven stages (I to VII) of seed germination in CPT, NGPP 46 of *P. pinnata*.

4.4.2.3 SDS-PAGE analysis of total soluble seed storage proteins

In order to characterize more precisely *Pongamia* seed storage proteins during different developmental stages of maturation (stage I to stage VII) SDS-PAGE was used. The electrophoretic spectra of total soluble seed proteins (crude extracts) under reduced conditions at different growing stages of seed maturation showed 15-20 number of polypeptide bands (Fig. 4.6). This number of polypeptides coincides with the number, reported by Ghafoor & Arshad (2008) for *Pisum sativum* (25) and Ghafoor & Ahmad (2005) for *Vigna mungo* seeds. The results from comparison with standard molecular weight marker revealed that the highest molecular weight protein observed in SDS-PAGE was approximately 150 kDa (stage I to stage III) and the lowest 14 kDa (stage III to stage VII). This is similar to the observations made in groundnut (Prakash & Rao, 1986), sesame seed (Prakash & Nandi, 1978), sunflower seed (Rodriguez et al. 2002), mustard seed (Aluko & Mclntosh, 2004). During the developmental stages (I to VII) of *P. pinnata* seeds, polypeptides having the MW of 14, 18, 21, 23, 25, 29, 35, 39, 50, and 95 kDa were observed (Fig. 4.6). SDS-PAGE analysis of reduced seed protein extracts of *P. pinnata* showed the following results: at stage I, highly immature seeds (90 DAF) contained only trace amounts of detectable proteins. At stage II (135 DAF), some polypeptides of low intensity were major constituents of seed extracts. Only from 135 DAF onwards, seed proteins began to be synthesized. As development of seed proceeded (stage II and V), three main polypeptides of MW 50 kDa, 18 kDa and 14 kDa became the major protein and there was a clearly marked increase in the expression level with maturation (Fig. 4.6). The similar findings were reported earlier in *Brassic kaber* and *Cleome gynandra* where reduced seed proteins showed high intensity bands of MW 27-28 kDa, 18-20 kDa and 13-15 kDa respectively (Ochuodho et al. 2006). Of all the investigated seed maturation stages in *P. pinnata* the difference among polypeptides are in the relative intensity of the stained bands as well as in number. In the present study, *P. pinnata* seeds of 270 DAF (stage V) and more (stage VI) exhibited no significant difference in SDS-PAGE profiles with mature seeds (stage VII) and share polypeptides of similar molecular weights. This may suggest that most of the seed storage proteins were synthesized within 270 DAF in *P. pinnata*. There are reports on *Pongamia* seed proteins showing bands of MW 90-10 kDa (Scott et al. 2008). Low MW proteins are highly expressed in *P. pinnata* seeds. Globulins are the main seed storage proteins in *Pongamia* that

includes legumins (11S) and vicilins (7S), both of which are multisubunit proteins. No high molecular components are observed in the electrophoretic spectra. This is because of the presence of ß-mercaptoethanol that breaks disulphide-bonded monomers of 11S globulins to acidic and basic polypeptides. This behaviour is typical of the disulfide bonded acidic and basic chains of 11S storage proteins (globulins) found in other legume such as *Pisum* (Matta et al. 1981), *Olea europaea* (Wang et al. 2001). The endoproteolytic cleavage of 11S proteins into two disulfide-bonded chains is evolutionarily conserved in seeds of conifers, monocots and dicots too (Nielsen et al. 1995). There are a large number of reports in legumes which demonstrate that globulins represented by two main fractions: legumin like, legumins (MW 350-450 kDa) and vicilin-like, vicilins (MW 150-250 kDa) are the predominant polypeptides. Legumins exist as a mixture of trimers and hexamers comprising subunits of MW 50-60 kDa held together by non covalent interactions (Hayashi et al. 1988, Shotwell et al. 1988; Wang et al. 2001). In the present study due to the presence of reducing agent ß-mercaptoethanol, these polypeptides were cleaved to give rise to an acidic (50 kDa) and a basic (20 kDa) polypeptide joined via a disulfide bond. The vicilin-like 7S globulins are typically trimeric proteins of MW150-190 kDa, with subunit molecular mass in the range MW 40-80 kDa, joined together via weak interactions: hydrogen and hydrophobic bonds. There are reports on legume and oil yielding seed proteins showing bands at 21-97 kDa (Wang et al. 2001). Three main groups of polypeptides were identified: one basic (approximately 20 kDa), and two acidic groups (30 and 40 KDa). Polypeptide with molecular masses of 40-80 kDa, 37 kDa, 29 kDa, 20 kDa, and 17 kDa has been reported in legumes (Ghafoor & Arshad , 2008; Wang et al. 2001). The most representative bands of soluble proteins are 43.3 and 14.4 KDa (Navari et al. 1992). These results concur with our study in the banding pattern observed. There were 3 high intensity bands 1 of approximately 50 kDa, and 2 of approximately 18 and 14 kDa respectively (Fig. 4.6). Oleosin proteins of about 16-20 kDa can be resolved by SDS-PAGE from oil seeds, each of which has been purified to homogeneity (Li et al. 1992; Cummins et al. 1993). The authors elucidated that total soluble protein fraction prepared from young seed tissues contained very little oleosin protein whereas identically prepared fraction from matured /dry seeds contained distinct band of oleosin protein as examined by SDS-PAGE. Oleosins are distinct from seed storage proteins, with respect to the timing of their synthesis and degradation, their subcellular localisation, their structure and their biological function (Murphy, 1990). In contrast to the

storage proteins, oleosins are relatively hydrophobic proteins, and are localised exclusively on the surface of storage oil bodies. To date a number of seed oleosins have been described in rapeseed, maize (Huang, 1992; Keddie et al. 1992; Cummins et al. 1993). In the present study also in developing seeds of *P. pinnata* from stage III (180 DAF) polypeptide of MW 14-18 kDa appeared and gets darkened at stage IV (225 DAF), V (270 DAF) and remains the same till stage VII (350 DAF). Virtually all the minor polypeptides are present at very low levels in protein fractions prepared from young seed tissues. Altogether, the data reflect that the content of the seed proteins increased as the seeds of *P. pinnata* matured as shown by sequential increase in the number and intensity of polypeptide bands. In some dicotyledonous plants, e.g. pumpkin (Hara-Nishimura et al. 1993), sunflower (Anisimova et al. 1995) and pulses a third type of seed storage proteins 2S albumins, are available in abundance. SDS-PAGE of the seed proteins did not show any definite correlation between any polypeptide and seed characteristics (Odeigah & Osanyinpeju, 1998). *P. pinnata* seeds accumulate large quantities of protein at maturity (39 % of dry weight; NOVOD, 2009). *Pongamia* seed protein may have similarities with the other well-known oilseed protein such as soy, Canola, sunflower *Jatropa* protein. The accumulation of storage proteins represents a major metabolic event during development of legume seeds (*P. pinnata*) and is accompanied by a multitude of changes in gene expression that adapt the seed tissue to assume this role.

Further investigation in the current section of this chapter involves SDS-PAGE banding profile in vegetative (leaf and root) and reproductive tissues (flower, bud and seeds) to evaluate the effect of tissues on their protein profiling pattern and storage proteins respectively. In comparison, the protein profiling pattern of *in vitro* raised callus from leafs and roots were also studied. Polypeptides showing considerable heterogeneity were found in the different vegetative and reproductive tissues in CPT, NGPP 46 of *P. pinnata* analyzed by SDS-PAGE. Differences in terms of number and relative staining intensities of bands were observed (Fig. 4.7). The results elucidated that there was an absence of practically all key seed storage proteins/ polypeptides in vegetative tissues and reproductive tissues (other than seeds) except in leafs. In leaf total soluble protein extract, two prominent polypeptide band of MW 55 and 16 kDa were visible that represents Rubisco larger and smaller subunit polypeptides respectively (Fig. 4.7).

The data obtained suggests clearly expressed differences in electrophoretic profiles of total soluble reduced proteins amongst different vegetative and reproductive tissues.

Another experiment was set up to examine the effect of germination on protein using SDS-PAGE to follow the changes in protein profile within 22 days of germination in cotyledon and embryonic axis of the investigated *P. pinnata* seeds. SDS-PAGE is widely used for monitoring protein mobilization in early stages of seed germination (Hussain et al. 1988; Ahmed et al. 1995; Krochko & Bewley, 2000). During germination the results showed that *P. pinnata* seeds (both cotyledon and embryonal axis) contained 15-20 bands ranging from 100-14 kDa as shown in Fig. 4.8A & B, lane 2-8, these results are in line with those reported in other legume crops (Bhatty 1982; Ahmed et al. 1995; Ramakrishna, 2007; Kirmzi & Guuleryuz, 2007). SDS-PAGE profile of cotyledon and embryonal axis (cotyledonary notch) under reducing and denaturing conditions showed changes in the number of protein bands as well in the intensity of some polypeptides with germination. High MW polypeptide of 50 kDa starts disappearing rapidly with germination from both cotyledon and embryonic axis and almost undetectable on 22^{nd} day whereas another polypeptide of MW 29 kDa disappeared completely within 14^{th} day of germination Fig. 4.8A & B. Studies with seeds of *Lathyrus sativus*, *Dolichos lablab*, *Cicer arietinum*, *Vicia faba*, and *Gossypium hirsutum* revealed various protein fractions ranging in MW from 92-12 kDa with faster degradation of high MW proteins (Chandna et al. 1995; Vigil & Fang, 1995; Muntz et al. 2001; Ramakrishna, 2007). There are reports that showed that protein fractions exhibit different degradation patters, globulins being degraded in the early stages after the start of imbibition and albumins in the later stages of seed germination (Shutov & Vaintraub, 1987). Savelkoul et al. (1992) suggested the high molecular weight proteins that disappeared during germination were legumins and vicilins. Thus the present results revealed faster degradation of high MW polypeptides of globulins in the early stages of germination from both cotyledon and embryonal axis of *P. pinnata* seeds. The new low molecular weight polypeptides of size from 25 kDa to 14 kDa started appearing on day 5^{th}, and further intensified with the progress of germination Fig. 4.8A & B. The results of present study are supported by other investigators who have found that though some protein bands were disappeared, some other bands were appeared for the first time as reported in *Zea mays, Vicia faba* and other legumes (Mitsuhashi & Oaks, 1994; Kirmzi & Guuleryuz, 2007; Ahmed et al. 1995). Ahmed et

al. (1995) suggested that degradation of some protein indicates that these proteins are reserve proteins, and appearance of some other proteins on specific days during germination indicates that these proteins might be enzymes or subunits of enzymes required at this stage of seed germination. On the other hand, the constant proteins might also be enzymes or proteins which build the structure of cells, while the gradually decreasing proteins indicate that these proteins are sources for amino acids required to build up other proteins or enzymes during the germination time. Focusing on the metabolic changes, previous studies reported that during germination period, storage proteins are degraded by a variety of proteases (acidic, neutral and alkaline), which convert the insoluble storage proteins into soluble peptides, which are then further hydrolysed to free amino acids. These free amino acids are mobilized to the embryonic axis to support its growth and also to provide energy (Shutov & Vaintraub, 1987; Bewley & Black, 1994; Muntz et al. 2001).

The developmental expression of polypeptide MW 40, 18 and 14 kDa during seven stages of seed maturation exhibited inverse relationship with seed germination stages. The electrophoretic spectra of reduced seed storage proteins of all the seven stages of seed maturation as well as seed germination contain 11S subunits and components of 7S globulins. The mobilization or degradation of polypeptide of MW 50 kDa during germination confirmed its storage role. During seed development and seed germination, certain specialized regulatory and structural genes are turned on. The SDS-PAGE gel pictures revealed that the seed storage proteins starts accumulating from 180 DAF and continue till 270 DAF i.e. mid maturation stage of seed development and after that it becomes constant and are used up during germination. The SDS-PAGE gel pictures also revealed that they are synthesized only in the seed (in cotyledons) and not in other tissues. These proteins lack any other functional activity besides storage. The proteins are synthesized during seed development and are packaged in special storage organelles called protein bodies (PBs) via one or more steps of the secretory pathway. The seed storage proteins of plants are a group of evolutionary conserved proteins that serve as a source of carbon, nitrogen, and sulfur for the germinating seedling (Shotwell & Larkins, 1989). The comparison of protein electrophoretic patterns of the investigated seeds allowed in identifying the effects of maturity on protein content and the complex effect of both the factors on the composition of seed proteins in current chapter.

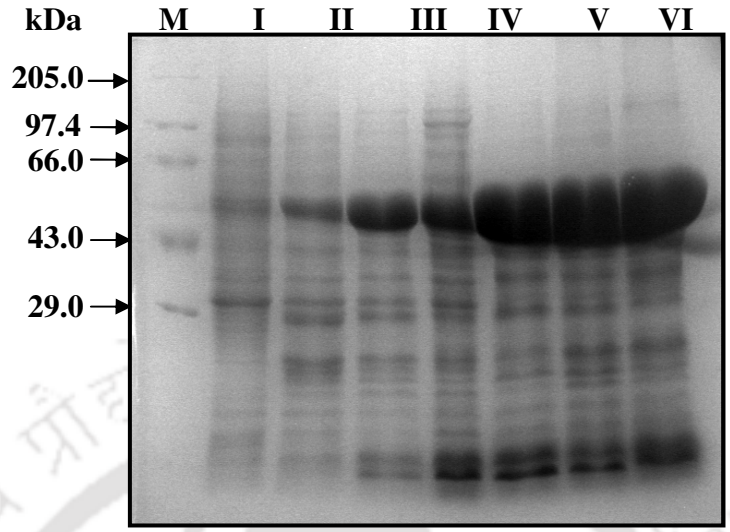


Figure 4.6. SDS-PAGE of total soluble seed protein at various developmental stages of seeds maturation in *P. pinnata.* Lane 1, Marker; Lane 2-8, Stage I to VII.

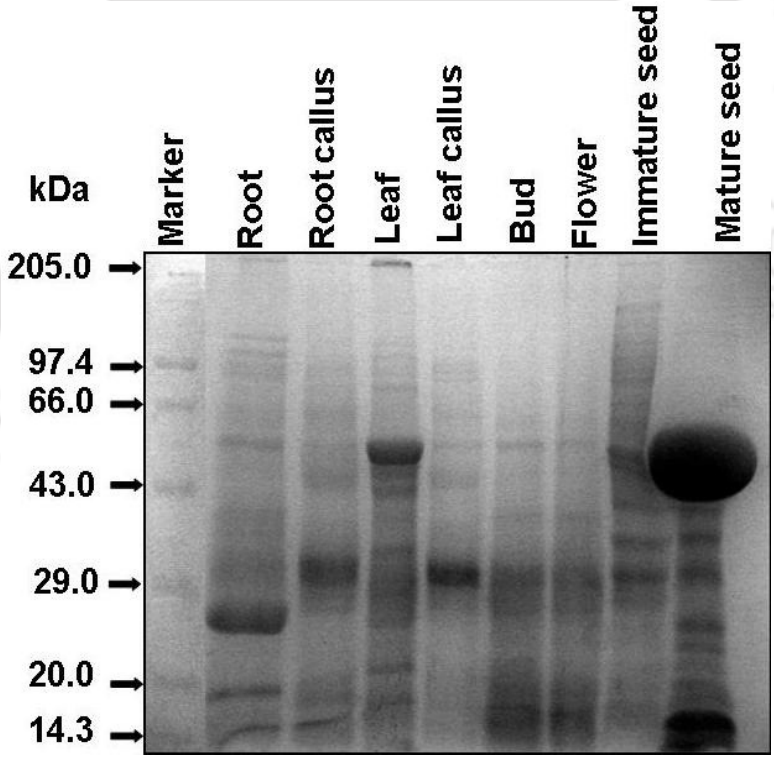
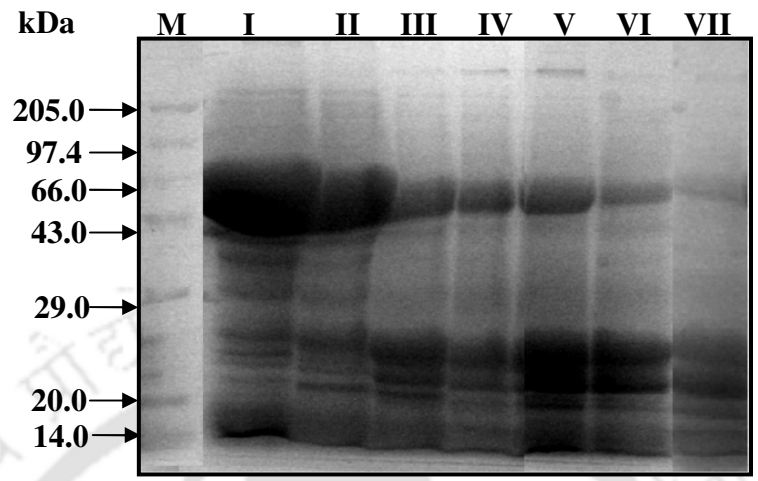


Figure 4.7. Electrophorogram showing banding pattern of total soluble proteins in vegetative and reproductive tissues of *Pongamia.*

A



B

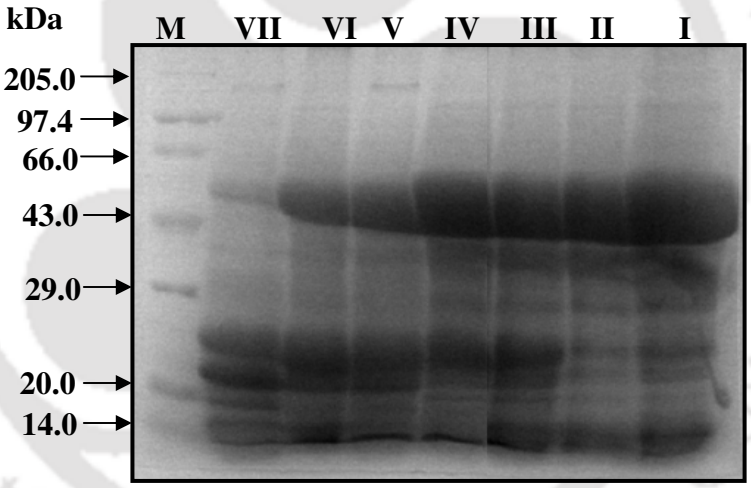


Figure 4.8. SDS-PAGE of total soluble protein at various developmental stages of seed germination in *P. pinnata*.

A. Cotyledon: Lane 1, Marker; Lane 2-8, Stage I to VII;

B. Cotyledonary notch: Lane 1, Marker; Lane 2-8, Stage VII to I.

4.4.3 Localization and distribution of the cotyledon reserves in CPT during sexual seed development and germination stages

4.4.3.1 SEM

The seed genetics of *P. pinnata* have been studied with respect to total lipid profile, total seed protein profile and RAPD (Scott et al. 2009; Kesari et al. 2009; Kesari et al. unpublished data). Developing seeds of *P. pinnata* displayed variation in phenotypic traits (size and shape), as described in the earlier section of this chapter. During early immature stage of seed development (90-135 DAF), the cotyledon parenchyma showed formation of large intercellular air spaces and cells were not well differentiated as shown in Fig. 4.9A SEM TS at a magnification of 100 µm. However the cells displayed large and great number of intercellular air spaces during late immatured seeds (180-225 DAF) (Fig. 4.9B). As the seeds become more matured (225-270 DAF), well defined vascular bundles distinctly appeared (Fig. 4.9C). By increasing the resolution at higher magnification well differentiated vascular bundles were seen at this stage of seed maturation (inset 4.9C). In the cotyledons, elongated palisade parenchyma cells just beneath the epidermal cells may be distinguished from the more rounded spongy parenchyma present in the central regions (Fig. 4.9C). The cotyledon parenchyma cells of highly matured seeds of *P. pinnata* (315-350 DAF) showed more compact, highly differentiated cells with reduced size and number of intercellular air spaces respectively (Fig. 4.9D).

Fine structural observations during the 4 different stages of sexual seed maturation revealed a considerable increase in cell wall thickness that varied from approximately 60 µm to 100 µm in size for early immatured seeds to late matured seeds respectively (Fig. 4.10 A-D). The epidermal cells of the flat inner cotyledon surface of a highly immature seeds were observed to be characterized by an irregular shape. During this stage, to distinguish stomata is impossible. However, the epidermal cell of matured seed is very closely packed and possesses angular shaped distinct stomata. SEM images demonstrated apparent cell wall thickening and appearance of stomatal grooves as the seed matures. Dodd et al. (1989) showed that seed development in *Podocarpus henkelii* occurred with increase in wall thickness. The authors reported that wall thickenings were irregular and frequently

associated with numerous microtubules, ribosomes, polysomes and dictyosomes of active appearance.

Young cotyledons (early immatured stage, 90-135 DAF) of *P. pinnata* showed homogeneous parenchyma containing very few reserve substances. Young mesophyll or spongy parenchyma cells started accumulating protein reserves in their central area (Fig. 4.11A) and showed lot of intercellular spaces in the seed cotyledon. When single cell of the cotyledon were focused at higher magnification it was observed that protein bodies were deposited centrally inside the cytoplasm whereas lipid droplets accumulated adjacent to the cell walls (Fig. 4.11B). In addition, thin cytoplasmic strands or networks are found to be interspersed with lipid and protein droplets. In further stages of seed development the microscopic studies (SEM) demonstrated the accumulation of great pools of storage reserves even during early matured stages (225-270 DAF) of seed development in *P. pinnata* (Fig. 4.11C). It has been previously shown that *P. pinnata* seeds display large and a great amount of protein expression from 270 DAF in SDS-PAGE. Intense reserve materials synthesis occurred in all the parenchyma cells of cotyledons in early and late matured seeds (Fig. 4.11C & D). The individual parenchyma cells were full of proteins, lipids and other storage reserves. During seed development deposition of storage reserves (proteins lipids and carbohydrates) begins at late immature stage and increases till late maturation stage. The SEM images of the cotyledon parenchyma cells of the developing *P. pinnata* seeds elucidated that the syntheses of storage components mainly lipids and proteins followed a maturation gradient inside the fruit. To confirm the localization of lipid bodies within a cotyledon cells, another experiment was done in which the matured ripened seeds (within 350 DAF) were given an acetone and hexane treatment for 48 h prior to fixation. Intracellular spaces was formed within parenchyma cells of treated seeds (Fig 4.12 A-D) which were initially absent in the untreated matured seed.

To examine the utilization pattern of seed storage reserves during germination of *P. pinnata* seeds, SEM analysis of cotyledonary tissues of germinated *P. pinnata* seeds (5^{th} to 45^{th} day after germination) were performed. Cotyledons were fixed after 5^{th} day of germination to assay cells in which mobilization of reserve substances had just commenced to 45^{th} day after germination. Within 8^{th} day of seed germination, lipids, proteins and other

cytoplasmic networks were detectable (Fig. 4.13A) but their appearance were different from the one present in matured seeds in *P. pinnata*. It is interesting to note that the SEM images of the sections of initially germinated cotyledons showing the localization of storage reserves were similar in appearance to the acetone treated cotyledons of the study material. Within 22 days of seed germination the protein bodies of a cell increase in size and become rounded (Fig. 4.13B & C) and the smaller protein bodies fuse to form two or four larger ones. Cells completely devoid of storage reserves were observed at 45th day of seed germination (Fig. 4.13D). In addition to this it was observed that cotyledonary tissues of 22 days old germinated seedlings showed a highly differentiated vascular bundles with distinct xylem and phloem cells (Fig. 4.14A & B). While observing the changes in the cell wall appearance, shrinkage and distortion of cell walls observed within 45 days of seed germination in *P. pinnata* (Fig. 4.15A-C).

4.4.3.2 TEM

The high moisture content of the immature seeds (90-135 DAF) of *P. pinnata* was supported by the highly vacuolated nature of cells when viewed by electron microscopy (Fig. 4.16A). Cells were 13 to 16 µm in diameter with well defined wall and had large central vacuoles. Cells large vacuoles were dissected by sheets of cytoplasm that extended to the periphery. These confined regions contained well differentiated mitochondria and abundant granular material possibly of a proteinaceous nature (Fig. 4.16B & C). These observations suggested that reserve deposition had began at the immatured stage of seed development in *P. pinnata*.

Rudimentary plastids were seen in the cytoplasm of cotyledonary cells of green matured seeds of *P. pinnata* (Fig. 4.16D). All cells of matured seed contained abundant protein and lipids in the form of protein and lipid bodies (Fig. 4.16D & E). When close to maturity, the lipid and protein reserves prevailed over the cell structure components, as, for instance, the starch granules. Being oil yielding leguminous tree, lipids and proteins were definitely the main cell reserves in the developing seeds of *P. pinnata* (Fig. 4.16F). The lipid globules, with diameters ranging from 1 to 3 µm, were found in a spatial association with the protein reserves (Fig. 4.16F). In fact, due to the interaction of the lipid globules with the protein reserves, it was not possible to define the oleosin membrane. Guilloteau et al. (2003)

reported the existence of two classes of oleosins in *T. cacao*. Authors stated that the rapid coalescence amongst the lipid bodies occurs when exposed to temperatures above 28 °C (fresh histological sections) that did not help to confirm the existence of the oleosin membrane enclosure. In the earlier section of this chapter, determination of the fatty acids composition of the same genotype CPT, NGPP 46 from the same geographical location and demonstrated the predominance of polyunsaturated fatty acid viz., oleic acid.

Further lipid and protein accumulation was always associated with dense cytoplasm containing nucleus, mitochondria, numerous ribosome, polyribosome and endoplasmic reticulum (smooth and rough) (Fig. 4.16G). In TEM images it was revealed that both the smaller and larger protein bodies contain globoid inclusions. Protein bodies contained one or more globoid crystals in the proteinaceous matrix (Fig. 4.16H & I) and varied in diameter from 0.5 to 3 µm. Seed storage proteins are synthesized from the expression of large gene families that are highly conserved among diverse species (Kinney et al. 2001). The increase in dry weight and total soluble protein content as reported in the previous sections of this chapter was reflected especially by the accumulation of lipids and protein, which was detected by ultrastructural changes. The origin of lipid bodies was not clear but it was reported for other lipid rich plants such as *Brassica napus* L. and *Cucumis sativus* L. to arise from the swelling of ER (Wanner et al. 1981). The cotyledon of *Pongamia* showed a great amount of phenolic cells interspersed with the other cell constituents in TEM section which were not visible in SEM images.

Examination of maturing seed by electron microscopy with osmium post fixation demonstrates that the cytoplasm contains numerous electron-dense vesicles. The proportion of normal constituents, i.e. cytoplasm, endoplasmic reticulum, plastids and mitochondria was visible. Endoplasmic reticulum occurred as closely packed sheets of cisternae. The functional significance of this plastid type is unclear. Proplastid with phytoferritin in seed tissues has been reported in cotyledons of some members of Fabaceae: *Pisum sativum* L. (Lobreaux & Briat, 1991), *Vicia faba* L. (Johansson & Wales, 1994) and *Cercis siliquastrum* L. (Baldan et al. 1995). Few starch grains were evident in the cotyledons of matured seeds of *P. pinnata* in TEM sections which were indistinguishable from protein bodies in SEM images. According to Greenwood (1976) the grain shape depends on the amylose content: the less angular, rounded

grains having relatively higher amylose levels. The grain shape of *P. pinnata* starch granules, like that of Quinoa and *Amaranthus hypocondriacus* seems to indicate that they contain mainly amylopectin (Prego et al. 1998).

To examine the ultrastructural changes in cotyledonary tissues of germinating *P. pinnata* seedlings, electron microscopic analysis was conducted (Fig. 4.17A-D). The PBs (protein bodies) structure in germinated seed was different from the PBs observed in maturing seed. Although the PBs retained a limiting membrane, they expanded to create a much larger space between the included storage protein aggregation and the membrane (Fig. 4.17E). During germination protein bodies of the seed breakdown to provide free amino acids to the growing seedling. The digestion of PBs may be of the more common internal type, the entire protein matrix breaking down uniformly. The digestion of protein bodies may also be of the external type, digestion beginning at the surface of the matrix in the form of numerous peripheral vacuoles which increase in size and fuse with each other, resulting in a large vacuole (Bhandari & Chitralekha, 1984). Similarly, in the present study TEM images showed that protein bodies blebs as germination proceeds and fuses with germination, fused with each other to form a single large vacuole. Subsequent fusions result in protein masses occupying the centre of the cell, completely pushing the cell contents to the periphery (Fig. 4.17E & F). At this stage they are devoid of all their globoidal inclusions. Subsequently, networks of proteinaceous particles replace the masses, which ultimately disappear leaving large vacuoles in the centres of the cells (Fig. 4.17G & I). The breakdown of protein bodies during germination has been ascribed on one hand simply to the amount of water in the cells (Bain & Mercer, 1966) or on the other hand to the influence of the embryonic axis (Varner et al. 1963; Opik, 1966; Penner & Ashton, 1967; Guardiola & Sutcliffe, 1971; Yomo & Srinivasan, 1973; Halmer et al. 1978; Kern & Chrispeels, 1978). Davies & Chapman (1979) have suggested that the seed coat might also have some role to play in controlling protein hydrolysis during seed germination in Cucumis. Thus, views concerning the source of the factor initiating protein body degradation are controversial. The ultrastructural study in *P. pinnata* seeds during germination and subsequent seedling growth reflects that mature seed constituents such as protein, oil and sugar are degraded and utilized to support growth of the embryonic axis.

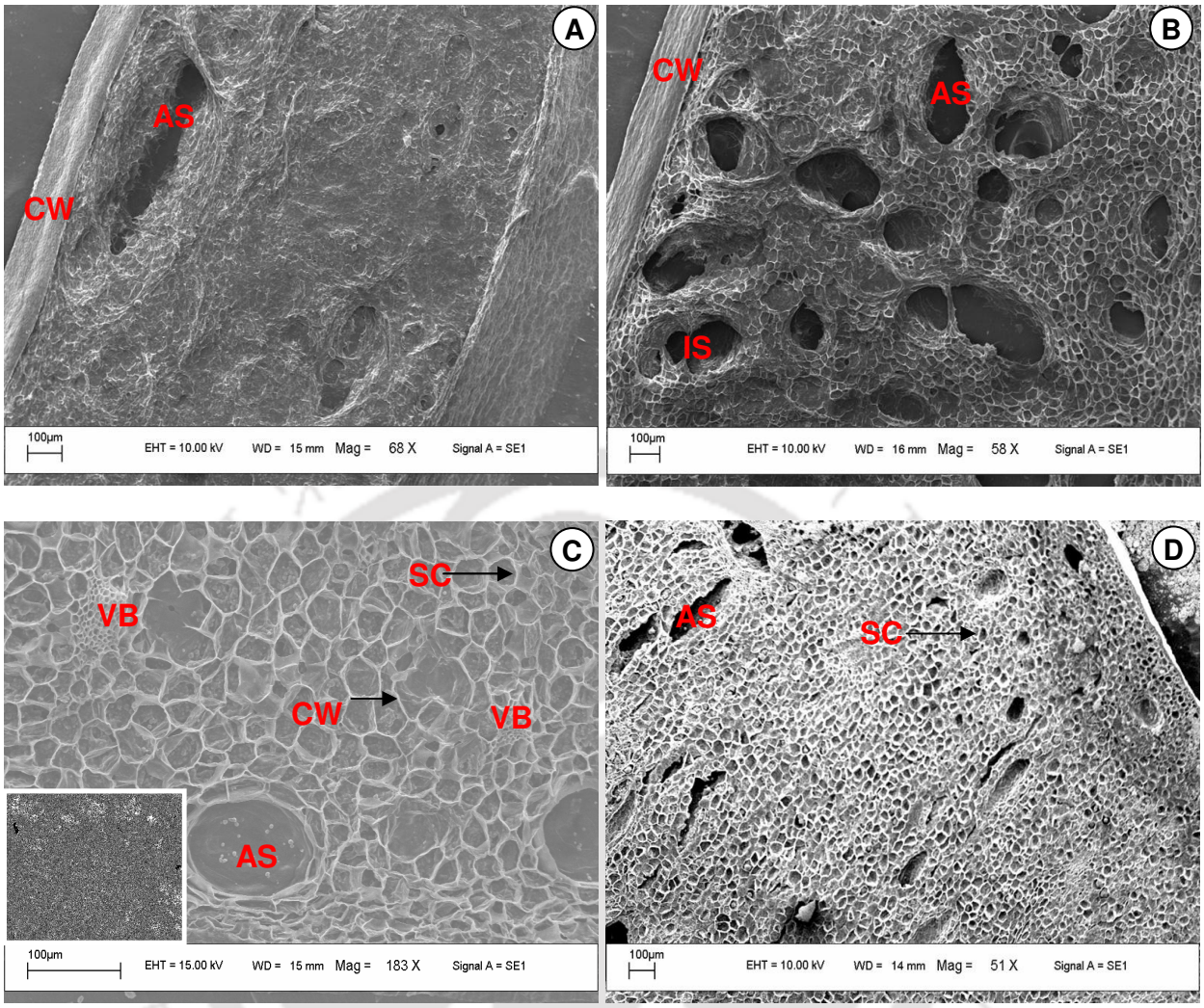


Figure 4.9. Scanning electron micrographs of transverse section of cotyledonary tissues of developing seed of CPT, NGPP 46 of *P. pinnata* at lower resolution (50-70 X) covering cotyledon parenchyma cells as a whole.

A. Early immature stage (90-135 DAF); **B.** Late immature stage (180-225 DAF);

C. Early mature stage (225-270 DAF); *inset* vascular bundle, bar 20 µm;

D. Late mature stage (315-350 DAF).

AS, air spaces; CW, cell wall; VB, vascular bundle; SC, single cell

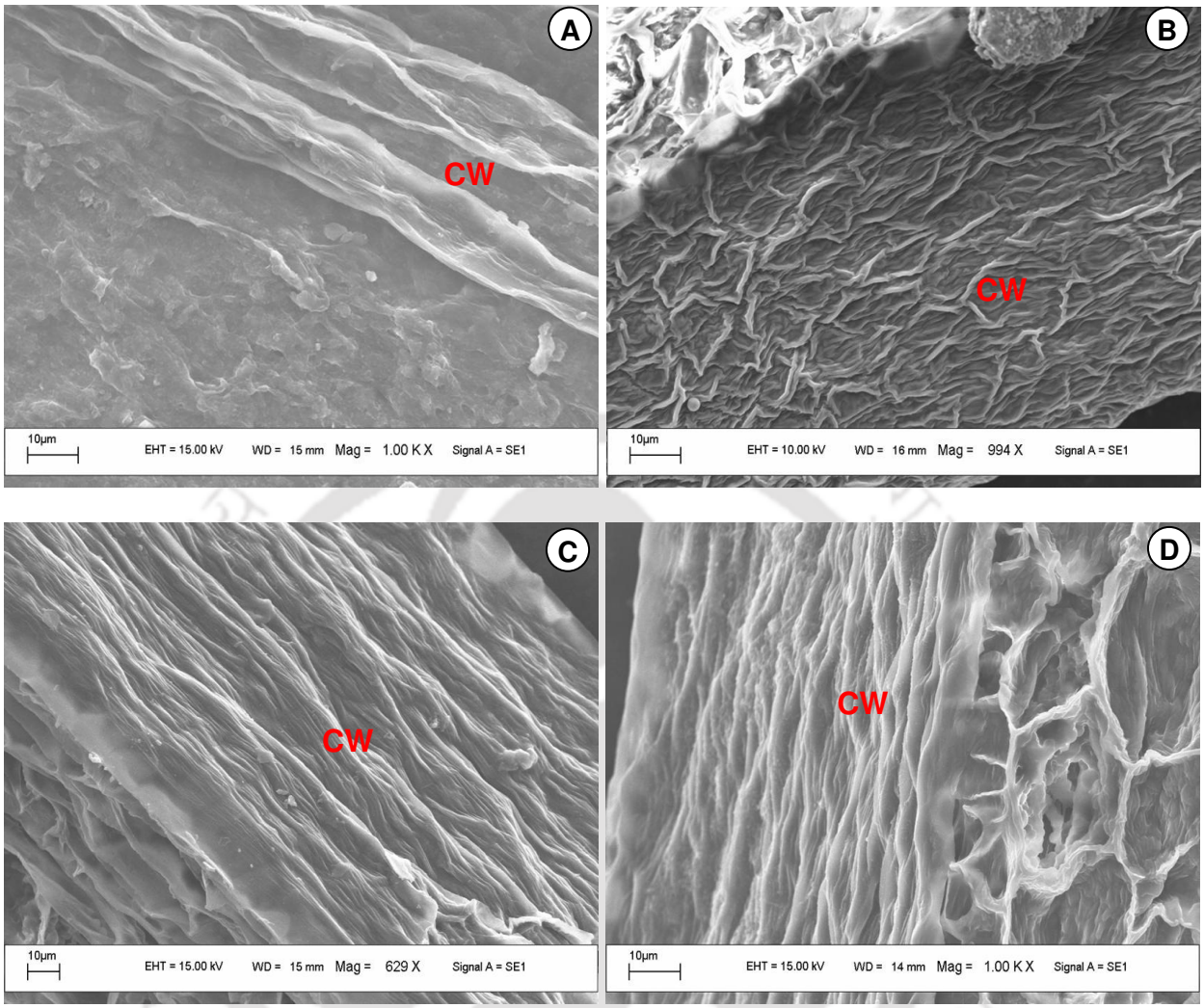


Figure 4. 10. Scanning electron micrographs transverse section of outer wall of the cotyledons of developing seed of CPT, NGPP 46 of *P. pinnata*.

A. Early immature stage (90-135 DAF); **B.** Late immature stage (180-225 DAF);

C. Early mature stage (225-270 DAF); **D.** Late mature stage (315-350 DAF).

CW, cell wall

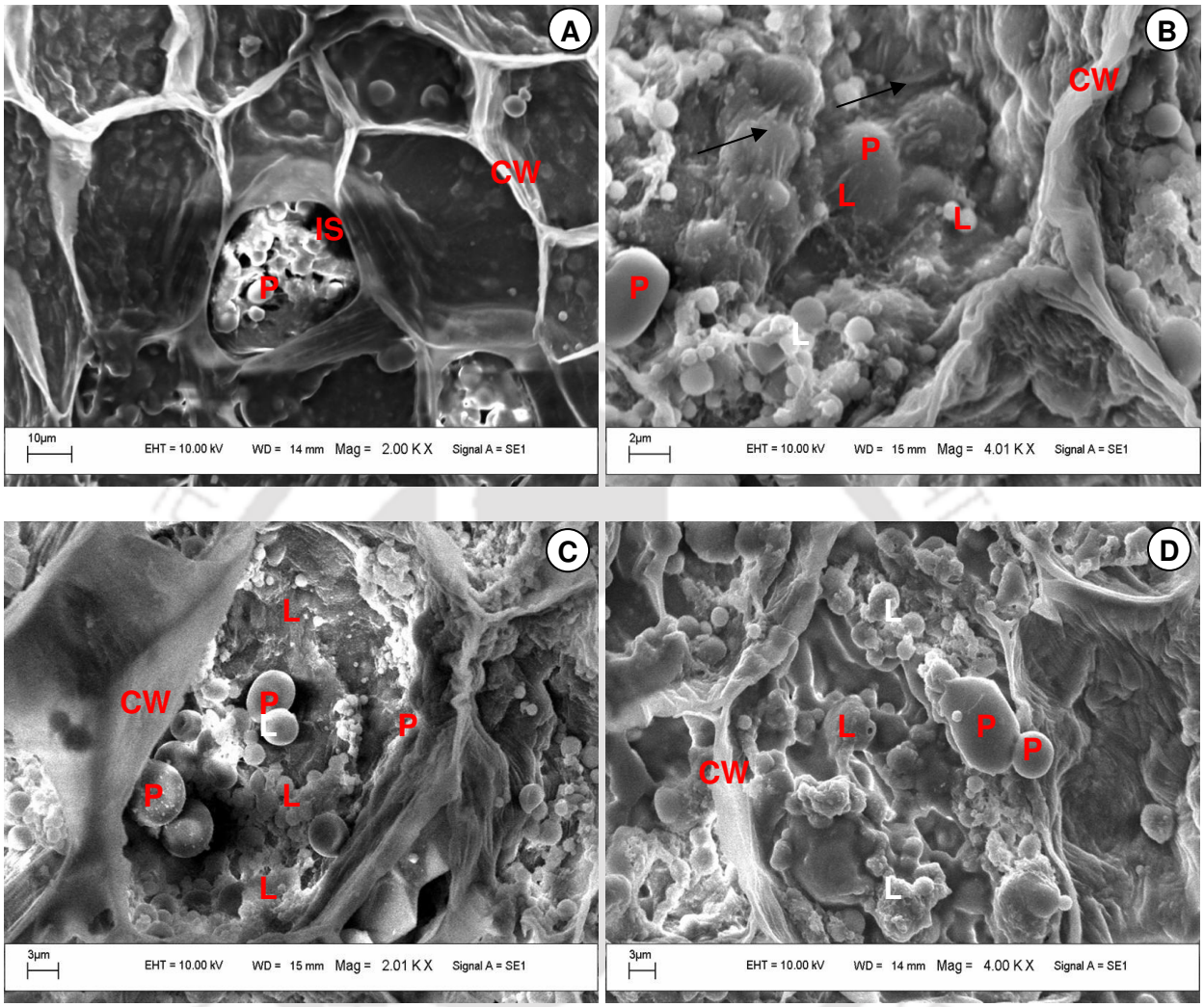


Figure 4.11. Scanning electron micrographs focusing the single cells of cotyledon parenchyma at 4 different developmental stages of seed maturation in CPT, NGPP 46 of *P. pinnata.*

A. Early immature stage (90-135 DAF); **B.** Late immature stage (180-225 DAF);

C. Early mature stage (225-270 DAF); **D.** Late mature stage (315-350 DAF).

P, protein; L, lipid; CW, cell wall; IS, intracellular space; arrowheads, cytoplasmic network

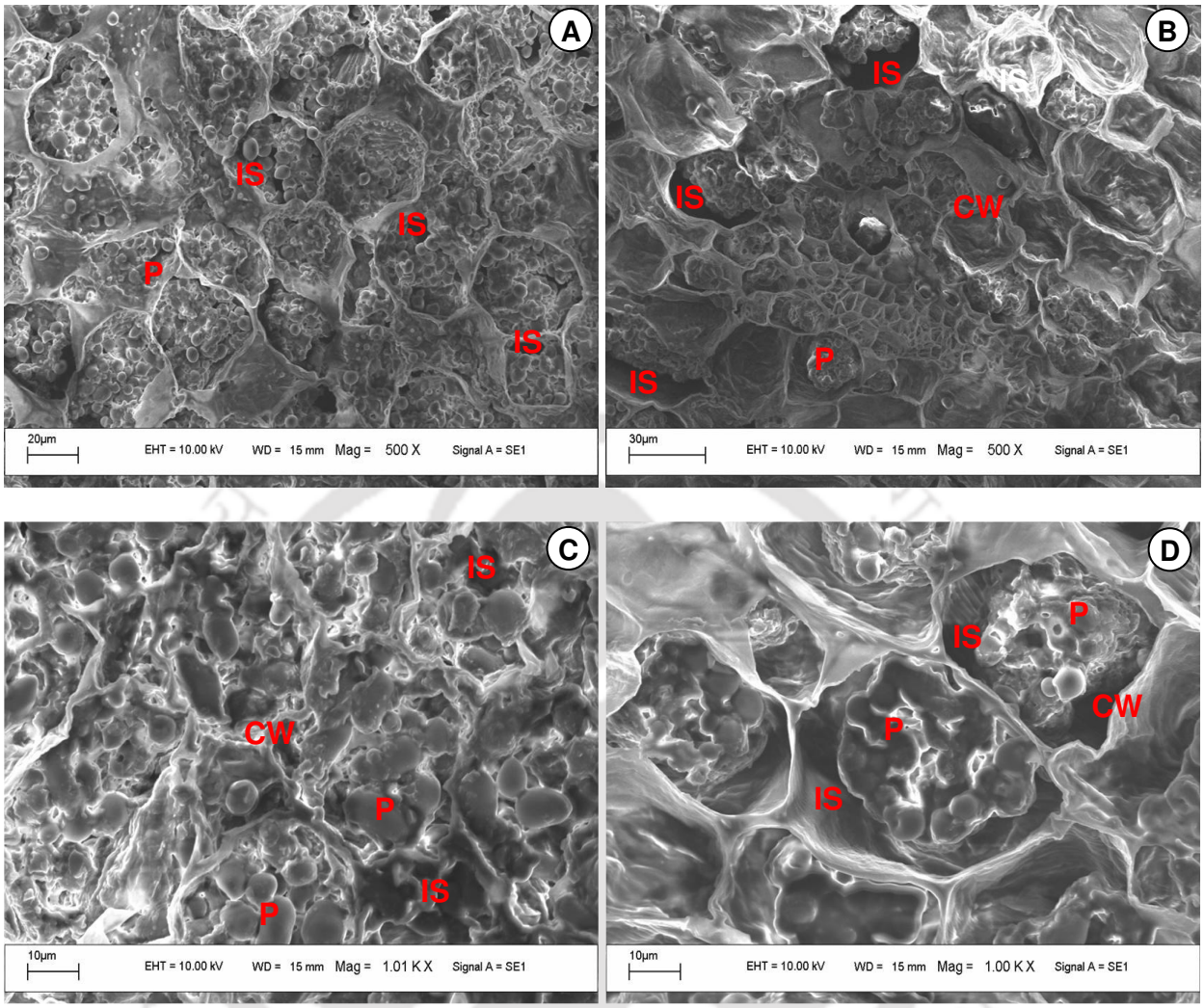


Figure 4.12. Scanning electron micrographs of the cross section of cotyledon tissues of late matured stage (315-350 DAF) of CPT, NGPP 46 of *P. pinnata* seed.

A. & B. Hexane treated; **C. & D.** Acetone treated.

P, protein; CW, cell wall; IS, intracellular space

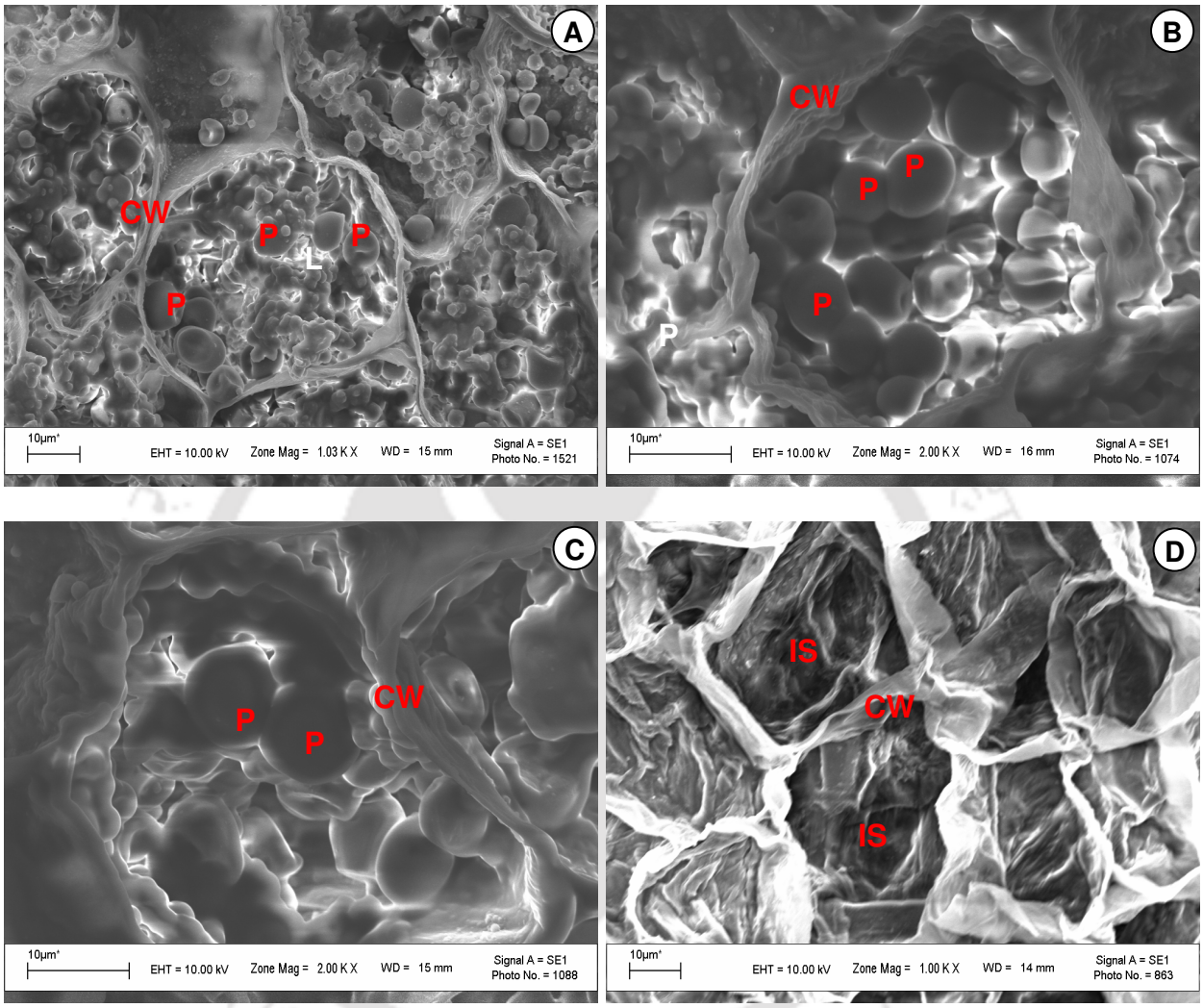


Figure 4.13. Scanning electron micrographs focusing the single cells of cotyledon parenchyma at 3 different developmental stages of seed germination in CPT, NGPP 46 of *P. pinnata.*

A. Early germination stage (within 8 days);

B. & C. Middle germination stage (within 22 days);

D. Late germination stage (45 days).

P, protein; L, lipid; CW, cell wall; IS, intracellular space

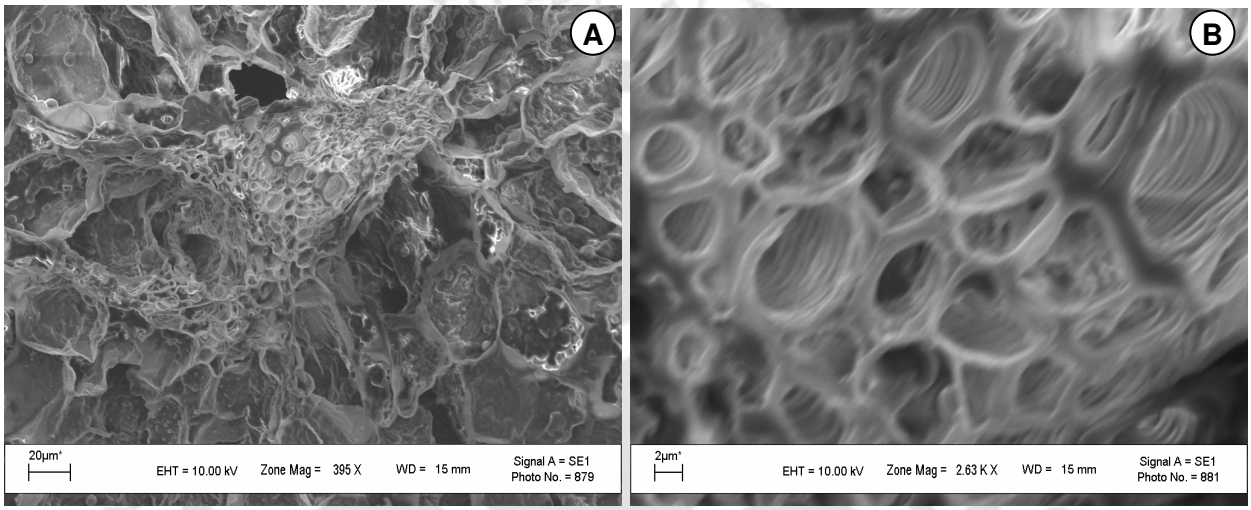


Figure 4.14. Scanning electron micrographs of the cross section of cotyledon of 22 day old seedlings showing well developed vascular bundles in CPT, NGPP 46 of *P. pinnata*.

A. Vascular bundle connecting a number of neighboring cells;

B. Highly differentiated conducting elements (xylem and phloem cells) at a advanced stage of seed germination.

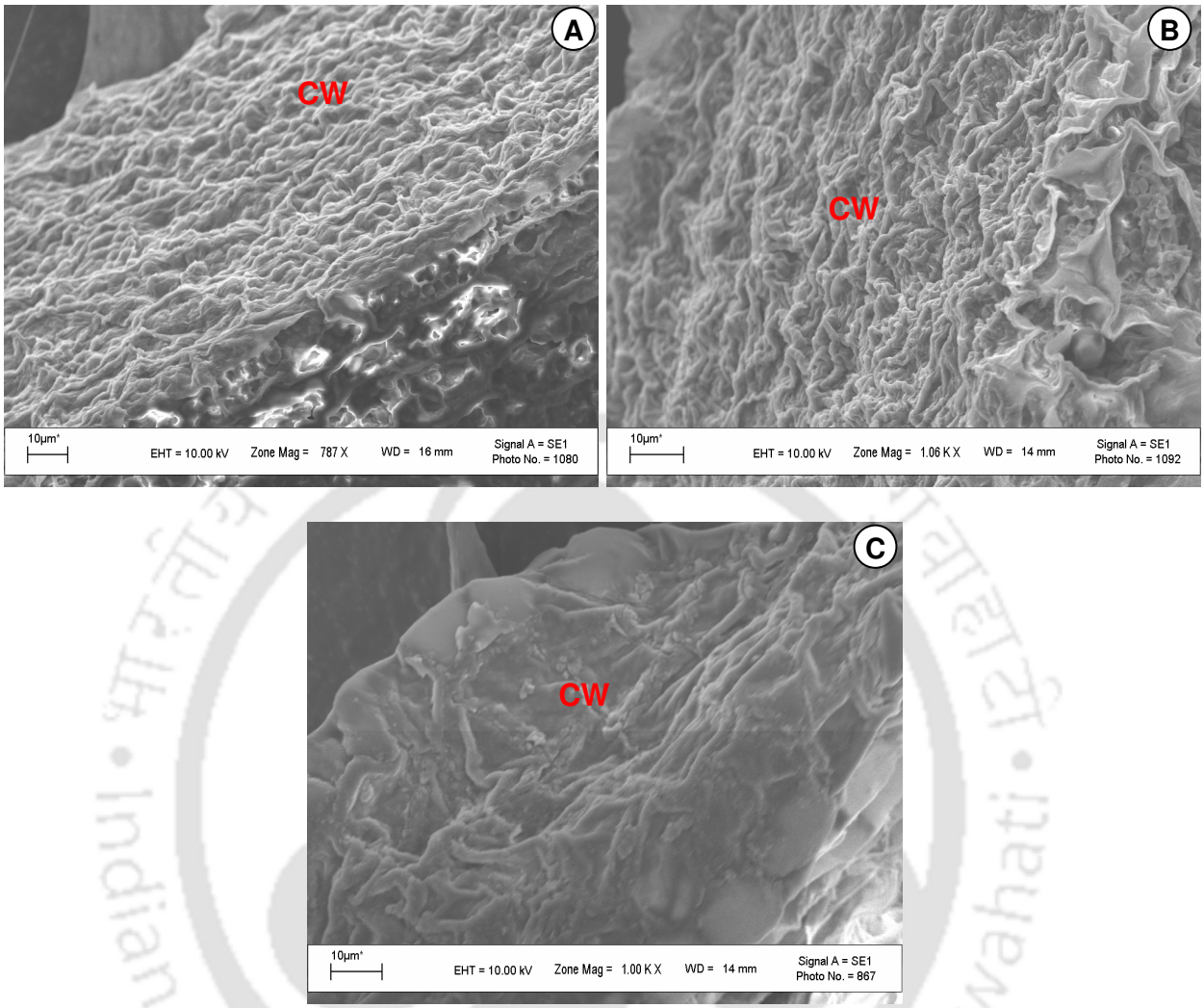


Figure 4.15. Scanning electron micrographs TS of outer wall of the cotyledon of germinating seeds of CPT, NGPP 46 of *P. pinnata*.

A. Initial germination (within 8 days);

B. Middle germination (within 22 days);

C. Late germination (45 days).

CW, cell wall

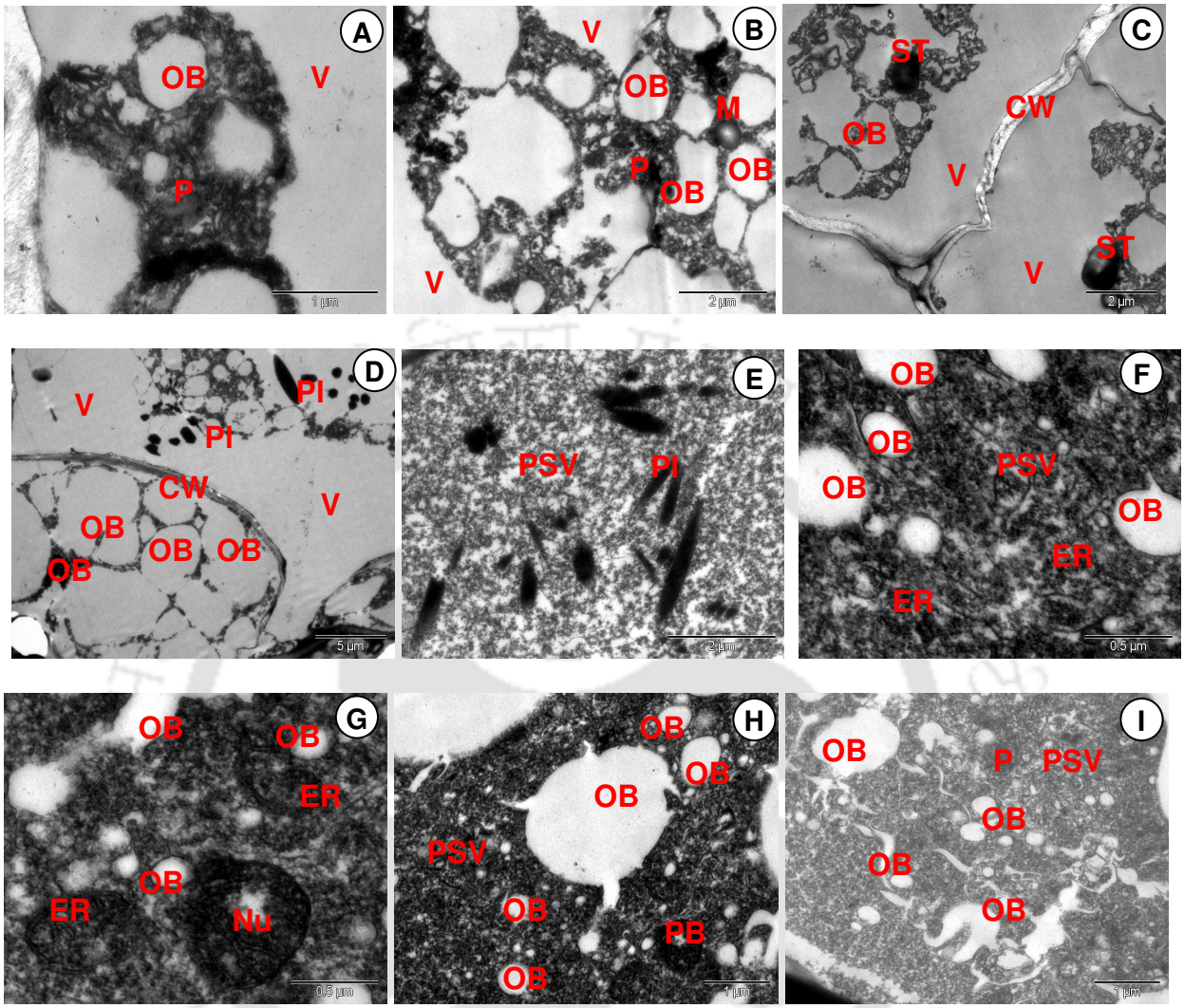


Figure 4.16. The ultrastructure of the cotyledons of developing seed of CPT, NGPP 46 of *P. pinnata*.

A-C. Late immature stage, large central vacuole (V) with few oil bodies (OB) and non membrane bound protein granules (P) were found associated with regions of active cytoplasm; mitochondria, oil bodies, starch (St), cell wall (CW) and vacuoles were indicated. The starch grains were often found in amyloplasts with a vacuole like structure. Cell walls displayed an irregular thickening thought to be the deposition of reserve material.

D-I. Late matured stage, illustrate dense cytoplasm with rudimentary plastids (Pl), number of oil bodies, vacuoles, protein storage vacuoles (PSV) containing globoid crystals, nucleus (Nu) and endoplasmic reticulum (ER) forming sheets of cisternae, numerous ribosomes and amylopasts.

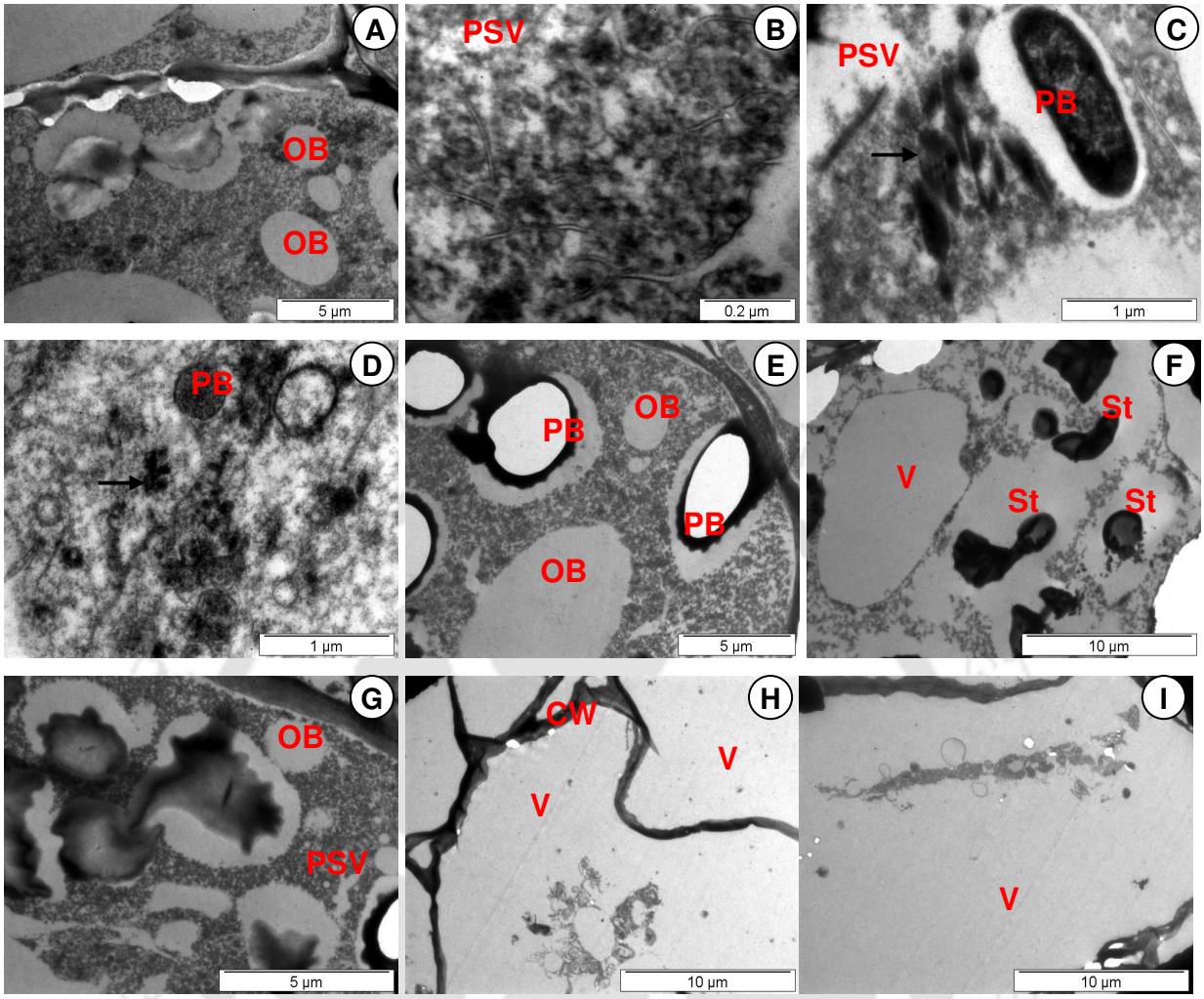


Figure 4.17. Transmission electron micrographs of the cross section of cotyledon of germinating seed of CPT, NGPP 46 of *P. pinnata*.

A-D. Initial germination (within 8 days); **E-G.** Middle germination (within 22 days); **H & I.** Late germination (45 days).

PSV, protein storage vacuole; PB, protein bodies; OB, oil bodies; CW, cell wall; V, vacuole; St, starch

4.4.4 Transcript analysis of fatty acid genes involved in triacylglycerol biosynthesis during developmental stages of seed maturation by RT-PCR

4.4.4.1 RNA extraction and quantification

An attempt was made to check the expression (presence or absence) of fatty acid biosynthetic genes from leaf, root and developing seeds of CPT, NGPP 46 of *P. pinnata* in current study. Initial few attempts failed to yield high quality intact RNA by using standard GITC method of Chomczynski & Sacchi (1987) and TRIZOL reagent. This was possibly due to either no RNA recovery or an inability to reverse-transcribe the RNA produced. To circumvent this problem, total RNA was isolated from seeds (135-350 DAF), roots and leaf using guanidinium isothiocyanate (Sigma) method of Chomczynski & Sacchi (1987) with slight modifications. The freeze-dried *P. pinnata* tissue ~0.1 g ground to a fine powder in a pre-chilled mortar and pestle containing 1ml of GITC buffer (4 M GITC; 25 mM Sodium citrate, pH 7.0; 1 % N-laurosylsarcosine and 0.1 M β-mercaptoethanol added immediately before use). The homogenate was properly mixed by gently inverting the tubes (5-6 times), followed by vortexing for 30 sec. Sample containing tubes were kept at room temperature for 10-15 min then kept on ice. About 100 µl of 2 M Sodium acetate (pH 4.0) was added by placing the tubes on ice, vortexed for 30-60 sec followed by chloroform-phenol extraction. After centrifugation at 10,000 rpm for 20 min at 4 °C, the upper aqueous layer containing RNA was carefully transferred to a fresh sterile tube. Pre-chilled isopropanol (equal volume) and 5M NaCl (half volume) was added, and allowed for precipitation on ice for 30-45 min. Pellet was collected by centrifugation at 12,000 rpm for 10 min at 4 °C, washed with 75 % ethanol twice, and resuspended in DEPC treated water for further analysis. Total RNA was treated with 200 units of RNase-free DNase I (Invitrogen) at 37 °C for 15 min. DNase was removed by phenol/chloroform extraction. Total RNA pellet was recovered by sodium acetate precipitation, re-suspended in 50-70 µl DEPC-treated water, vortexed and incubated at 65 °C for 10 min. Tubes containing RNA were briefly centrifuged and was quantified by using spectrophotometer by monitoring the absorbance at 260 nm. RNA quality is a concern in extraction process. Spectrophotometer analysis revealed that $A_{260/A280}$ ratios of RNA

samples ranged from 1.71 (seeds, 350 DAF) to 1.97 (roots), elucidating that there was no contamination of proteins, DNA, polyphenolics, and polysaccharides in isolated RNA by using modified GITC method described (Fig 4.18). The A_{260}/A_{280} ratios of the RNA samples isolated by the guanidine thiocyanate method of Chomczynski & Sacchi (1987) and TRIZOL were very low that varied from 1.22-1.45 (matured ripened seeds of stage VII) 1.65-1.73 (leaf and root). By using the modified protocol of Chomczynski & Sacchi (1987) high-quality RNAs from leaf (lane 1), root (lane 2) and developing seeds (lane 3-6) of CPT, NGPP 46 of *P. pinnata* was successfully isolated (Fig. 4.19A). RNA samples showed intact 28S and 18S bands on 1 % agarose gels and intensity of 28S rRNA band equal to or more than 18S rRNA band suggesting that little or no RNA degradation had occurred during the extraction. The RNA yields ranged from 350-500 μg g^{-1} and 600-700 μg g^{-1} from seeds (stage V, 270 DAF and stage VII, 350 DAF) and leaf of *P. pinnata* respectively, sufficient enough to carry 350-700 RT-PCR amplifications. RNA yield obtained were 70-400 μg g^{-1} from GITC method of Chomczynski & Sacchi (1987) and 55-200 μg g^{-1} from TRIZOL method. Using the protocol described in the current study, the yields of RNA were higher or within the range of previously published data. For example, RNA yields of 200-800 μg/g (Ruuska & Ohlrogge, 2001) and 150-200 μg/g (Vicient & Delseny, 1999) have been reported from seeds of *Arabidopsis thaliana*, and 55-195 μg/g for polyphenol-rich pine tissues (Chang et al. 1993). The original method of Chomczynski & Sacchi (1987) and TRIZOL reagent was ineffective for the isolation of good quality RNA from *Pongamia* samples especially seeds visualised from the gel electrophoresis (Fig. 4.19B & C). The main problem was that the homogenate of *Pongamia* seeds of stage V and VII formed clumps like structure that could not be properly mixed in GITC extraction buffer as well as in TRIZOL reagent. This interferes in initial phase separation (aqueous and organic) by centrifugation. In addition another problem was faced in the last step i.e. RNA precipitation using standard protocols of Chomczynski & Sacchi (1987) and TRIZOL reagent. Pellet was found to contain lot of mucilage which co-precipitated with RNA and formed a glue-like gel structure that cannot be dissolved or suspended in DEPC-treated water. The reason for this is that tree species often contain large amounts of proteins, DNA, polyphenolics, polysaccharides and phenolic compounds that are difficult to separate from RNA but are easily identified, because they make the RNA pellet sticky and gelatinous (polysaccharides) (Gao et al. 2001).

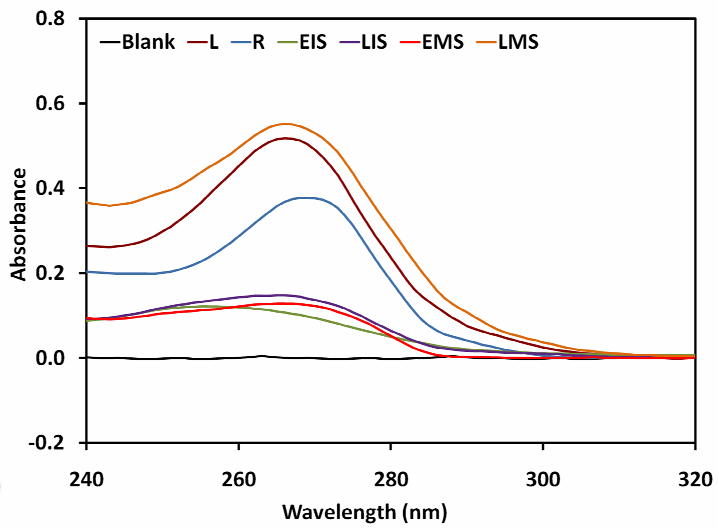


Figure 4.18. Purity index of total RNA from vegetative tissues and developing seeds of *P. pinnata* as shown by UV absorption spectra. (L, leaf; R, root; EIS, early matured seed; LIS, late immatured seed; EMS, early matured seed; LMS, late matured seed).

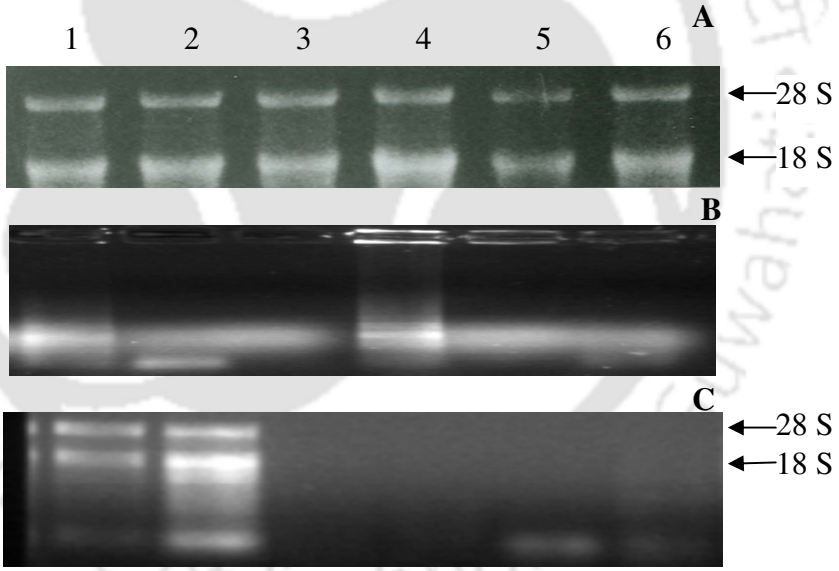


Figure 4.19. Visualization of total RNA isolated by three different methods.

A. modified guanidine thiocyanate method of Chomczynski & Sacchi (1987) (5 µg/lane); **B.** guanidine thiocyanate method of Chomczynski & Sacchi (1987); **C.** TRIZOL reagent. Total RNA were separated on 1 % non denaturing agarose gels containing EtBr and photographed under UV light.

Lane 1, root; lane 2, leaf; lane 3, early immatured seed (stage II); lane 4, late immatured seed (stage IV); lane 5, early matured seed (stage VI); lane 6, late matured seed (stge VII).

In the current modified protocol, combined use of high concentration of 5M NaCl and pre-chilled isopropanol at the precipitation step significantly increased the efficiency of proteins, secondary metabolites and polysaccharide removal. DNase I was applied to eliminate genomic DNA. When DNase is added at the end of the RNA isolation procedure, RNA migration on the agarose gel is abarrent. Therefore, DNaseI was applied immediately after precipitation with isopropanol.

4.4.4.2 Transcript analysis of the fatty acid genes

Based on NCBI sequence search for fatty acid genes from different oil yielding tree species, primers were designed corresponding to each desaturase and oleosin gene from the coding regions. Further to investigate the temporal gene expression of three desaturae (Stearoyl-acyl carrier protein desaturase, SAD; fatty acid desaturase, FAD2 and FAD3) and oleosin 1 transcripts in different tissues of *P. pinnata* tree, RT-PCR analysis was carried out with total RNA extracted from root, leaf and developing seeds (stage II, 135 DAF; stage IV, 225 DAF; stage V, 270 DAF and stage VII, 350 DAF) using modified method of Chomczynski & Sacchi (1987) described earlier. To scrutinize the tissue specific expression patterns of SAD gene at the transcript level, RT PCR was done for total RNA isolated from roots, leaf and developing seed tissues (135-350 DAF). A ~440 bp amplified fragment was strictly restricted in seed tissues of four different developmental stages (Table 4.7), revealing that *Pongamia* SAD gene has a seed specific expression pattern. SAD is a key enzyme in fatty acid biosynthetic metabolism of higher plants. The enzyme is present in plastid stroma, and the main function of the enzyme is to catalyze desaturation of stearoyl-ACP, which introduces a double bond into the fatty acid chain between C9 and C10 to form oleoyl-ACP (Tong et al. 2007). The desaturation of stearoyl-ACP is the last step of de novo synthesis of fatty acids in plants, but its desaturated product (Oleoyl-ACP) is the substrate forming many poly-unsaturated fatty acids, such as linoleic acid and linolenic acid, because double bonds are further introduced into the monounsaturated acyl-lipids. Saturated acyl groups do not generally serve as substrates for desaturation at the 6, 12, or 15 positions in the carbon chain. Therefore, SAD plays a key role in determining the ratio of saturated to unsaturated fatty acids in plants (Thompson et al. 1991). SAD is closely related with many life activities of plants based on catalyzing desaturation of stearoyl-ACP.

Table 4.7. Desaturase and oleosin gene expression studies in vegetative tissues and developing seed of *P. pinnata*.

Genes	Root	Leaf	Seeds			
			Stage II (135 DAF)	Stage III (225 DAF)	Stage V (270 DAF)	Stage VII (350 DAF)
SAD	-	-	+	+	+	+
FAD3	+	+	+	+	+	+
Oleosin	-	-	-	+	+	+
FAD2	+	+	+	+	+	+
GAPDH	+	+	+	+	+	+

The housekeeping gene GAPDH is constitutively expressed in all the tissues of *P. pinnata* used in the current study and the size of the amplified product is approximately 350 bp (Fig. 4.20A). Amplified fragment of 650 bp were generated for FAD2 (Fig. 4.20B) and ~440 bp for FAD3 gene respectively from all the tissues of *P. pinnata* used. Based on semi-quantitative RT-PCR analysis a "house keeping" expression pattern of FAD2 and FAD3 gene found in all tissues examined, reflecting its major role in desaturation for the biosynthesis of membrane lipids. Based on these observations, it could be said that FAD2 and FAD3 are constitutively expressed in all the tissues whereas SAD gene had a seed-specific expression pattern. Heppard et al. (1996) have isolated two different cDNA sequences, designated FAD2 - 1 and FAD2 -2 cDNAs, encoding microsoaml ω-6 desaturase in soybean and presented the molecular evidence that although both cDNAs are expressed in developing soybean seeds, their expression specificity and function in lipid biosynthesis are not identical. It was suggested that the FAD2 -1 gene, which has a seed-specific expression pattern, may be involved in desaturation of oleic acid of seed storage lipids. In contrast, the FAD2 -2 gene, which is constitutively expressed in both vegetative tissues and developing seeds, may be responsible for conversion of oleic acid to linoleic acid within membrane lipids. Current and earlier observations indicate that most of the desaturase genes are present in seeds of *P. pinnata* throughout its development. There is evidence that fatty acid biosynthesis is a constitutive function and it undergoes coordinated regulation through gene

expression to provide constant demands of fatty acids for membrane lipid biosynthesis and in seed storage TAG assembly (Chen et al. 2007).

The oleosin transcript expression was found in seed tissues of *Pongamia* and was observed at 225 DAF whereas untill 135 DAF was almost undetectable (Fig. 4.20C). The result demonstrated expression of oleosin gene only in seed tissues and was found to be temporally regulated during seed maturation. Oleosins are oil body-associated proteins that cover and stabilize oil bodies during TAG accumulation. The expression of oleosin gene is probably driven by the demand for newly synthesized oil bodies resulting from the growing ER membranes. It has been suggested that an important role of oleosins is to stabilise storage oil bodies during tissue dehydration. The semi-quantitative RT-PCR analysis showed that at 135 DAF of seed development, all the desaturase gene was expressed but the expression of oleosin gene was detectable 225 DAF. This reveals the differential timing of storage oil and oleosin gene expression in the cotyledon tissues of developing seeds of *P. pinnata.* Earlier results demonstrated the difference in timing of storage lipid, storage protein and oleosin accumulation and expression of their related genes in a number of oil yielding crops (Cummins & Murphy, 1990; Kater et al. 1991; Keddie, 1993; Mansfield & Briarty, 1992; Rest & Vaughan, 1972; Slocombe et al. 1992). SAD gene was relatively abundant during early stages of seed development, while oleosin mRNA transcripts were relatively rare at this stage (Murphy, 1993). Oleosin mRNA abundance reached a peak during the desiccation phase of seed development, while Δ_9-stearoyl-ACP desaturase mRNA levels had declined appreciably by this stage (Keddie et al. 1992; Slocombe et al. 1992). Tzen et al. (1993) reported the concomitant accumulation of oleosins and storage lipid during the early-mid stages of development in embryos of maize and rapeseed (Huang, 1992). Many previous studies have shown that during later stage of embryo development bulk of Oleosin accumulation takes place (Cummins & Murphy, 1990; Cummins et al. 1993).

Although the current study reports a clear difference in the temporal pattern of Oleosin and oil-related gene expression in *P. pinnata* seeds, these findings cannot necessarily be extrapolated to other plant species. In other species or under environmental conditions where seed development is relatively rapid, the onset of dehydration and the

accumulation of Oleosins may overlap considerably with oil accumulation. Indeed, in developing soybean seeds, this appears to be the case (E. Herman, personal communication). In contrast, however, Oleosin gene (D129) expression in cottonseeds persists rather later in embryo development and overlaps both storage protein and LEA gene expression (Hughes & Gaalau, 1989; Hughes & Gaalau, 1993), which is more similar to the pattern reported for rapeseed in the present study. It is possible that, although Oleosin genes are expressed at a low level relatively early in seed development, their expression is increased dramatically by the regulatory factors such as ABA that are associated with seed dehydration. The provision of the immature oil bodies with an annulus of Oleosin protein will then render them stable to desiccation. This will allow the oil bodies to maintain their small size even in tissues with very low water contents which would otherwise lead to their rapid coalescence. The synthesis of sufficient Oleosin protein to stabilize oil bodies in drying seeds may be an important factor in determining the viability of oil-rich seeds (Cummins et al. 1993).

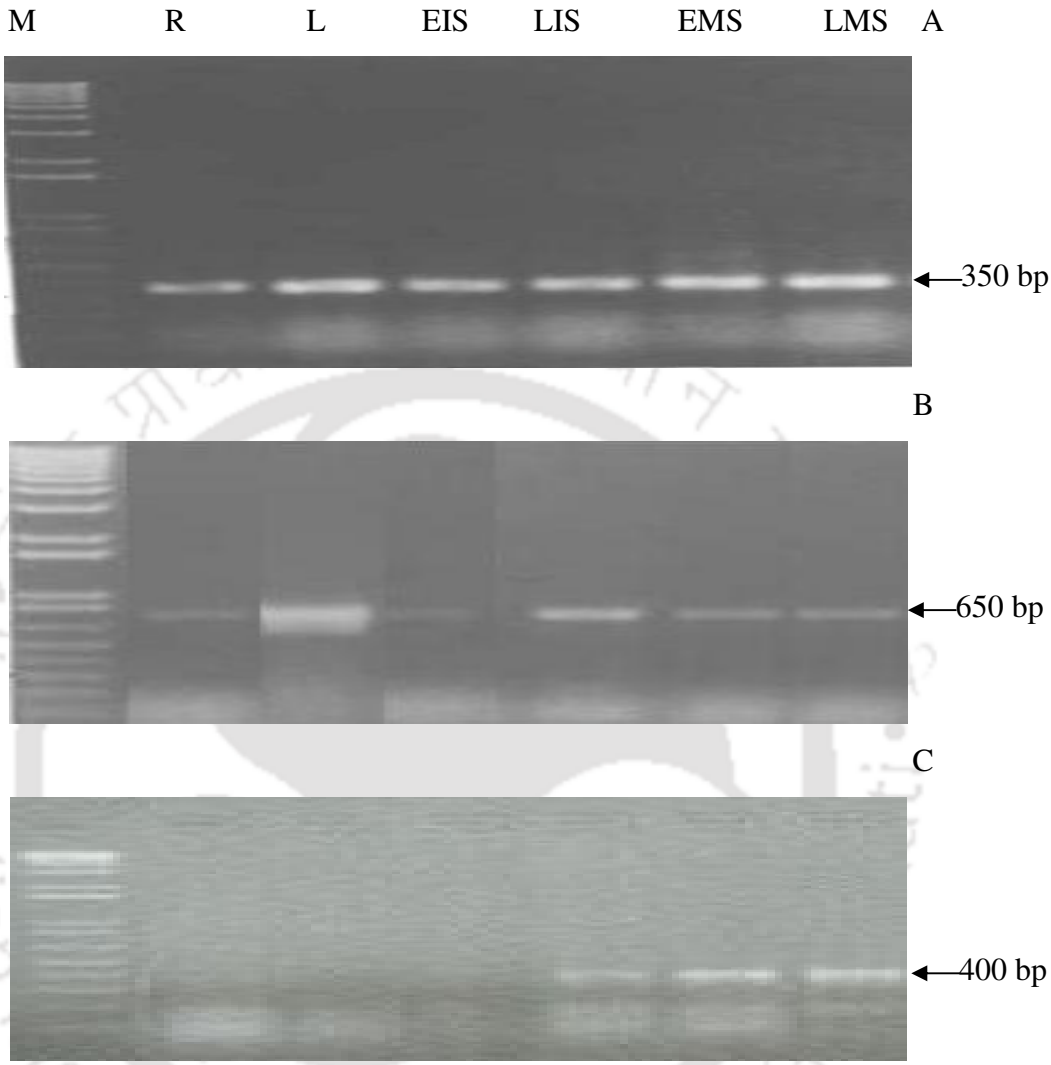


Figure 4.20. Fatty acid gene expression studies in *P. pinnata* tissues.

A. Control RT-PCR of the GADPH gene using total RNA;

B. RT-PCR amplification of FAD gene using total RNA;

C. RT-PCR amplification of oleosin gene using total RNA.

R, root; L, leaf; EIS, Early immature seed, LIS, late immature seed; EMS, early mature seed; LMS, late mature seed

4.5 Conclusion

The whole chapter is divided into 4 distinct objectives. In the first section characterization of crude oil for various physico-chemical properties demonstrated that, almost all the important properties of seed oil from the NGPP 46 (CPT) are in very close agreement with the biodiesel making it a potential candidate for the application in CI engines. Seed oil isolated from NGPP 46 also expressed significant bactericidal and fungicidal activity against selected strains of pathogenic microorganisms. Characterization of a CPT for its added attributes also has important implications for germplasm utilization and may establish a case study of genetic enhancement of biodiesel species (Rangan, personal communication). However, further studies will be needed to evaluate the cost, efficacy and safety of the oil and their products. The results of current study on chemical composition and bioassay of seed oil from NGPP 46 nevertheless would help in addressing key issues in the genetic enhancement of biodiesel species.

The second section deals with in depth study on various stages of seed development with respect to phenotypic traits and protein profile during maturation and germination stages (I to VII) respectively. The results indicate that seed storage proteins in *P. pinnata* consist of three major polypeptides of MW 50 kDa, 18 kDa and 14 kDa respectively expressed significantly in high amount as compared to other polypeptides. In addition, a significant change in protein synthesis during seed development was also evident. During the early period of seed development (before 135 DAA), seed proteins were synthesized only in low quantities. In contrast, most seed proteins, and particularly 48-62 KDa and 14-20 KDa were rapidly synthesized and accumulated in high quantities over a period of time 135-270 DAF and 180-270 DAF respectively exhibiting a strict time-dependence. Therefore a tight gene control of the developmental programme is expected. Biochemical characterization of seed proteins is very important for improving the quality of the seed proteins through a traditional breeding and gene manipulation by using genetic engineering techniques. The mobilization of storage proteins during germination which is also one of the aims of this chapter is very important, since it will help in understanding how the seed proteins are digested and which enzymes play the key role in this process. In future, a

thorough comprehension of the regulatory mechanisms controlling seed storage protein gene expression may some day lead to the genetic engineering of seed crops containing higher seed yields for the desirable traits. Efforts are being made to clone the genes coding for the two major polypeptides mentioned above and to characterize the expression and regulation of seed storage proteins during *Pongamia* seed development.

The third section covered the tissue specific localization, gradients in storage reserve deposition and its subsequent utilization during seed development and germination in CPT of *P. pinnata*. The main purpose of the present study was to focus on the synthesis of the reserves from the first signals of cell deposition up to fruit maturity. Besides lipids, the cotyledonary parenchymatous cells were shown to synthesize a wide variety of reserves, such as proteins and starch. The pattern and the type of reserve accumulation in seeds of *P. pinnata* suggest a strategy of ongoing continuous development without the intervention of drying and developmental arrest. The present study of microscopic images (SEM and TEM) of seed tissues did not provide evidence of oleosin membrane in *P. pinnata*. The study also showed the structural changes of storage reserves deposition during seed germination in study material. It would be interesting to elucidate the identity and function of those storage reserves involved in the metabolic and biochemical pathways of plant development for future studies in this important legume plant.

In the fourth and final section, the RNA extraction protocol was defined that is efficient, inexpensive and highly reproducible to isolate total RNA from *P. pinnata* tissues. To further test the quality of RNA, total RNA from all the tissues of *Pongamia* were amplified using gene specific primers of interest. RT-PCR showed that the extracted RNA from vegetative (leaf and root) and developing seed tissues of *P. pinnata* were successfully amplified and were suitable template for gene expression, thus validating its high quality. The expression of desaturase and oleosin genes has been examined in different vegetative (leaf and root) and developing seed tissues of CPT, NGPP 46 of *P. pinnata* by RT-PCR. The temporal pattern of gene expression varied dramatically between the two classes of gene. Desaturase gene expression was relatively expressed in all stages of seed tissues whereas oleosin gene expression was detectable from stage IV (225 DAF). Of the three desaturase genes, FAD2 and FAD3 showed similar, relatively uniform patterns of spatial expression in

all the tissues of *P. pinnata*. It is suggested that, in developing *P. pinnata* seeds, the accumulation of storage oil and oleosin is not concomitant but that the eventual deposition of oleosin onto the surfaces of storage oil bodies is essential for their stability during seed desiccation. The findings of the current study have built a solid foundation for identification, characterization, gene cloning and standardization of seed storage proteins in *P. pinnata* for further studies. Knowledge gained from these studies facilitated efforts to improve yield and quality of seeds of *Pongamia* using genetic engineering techniques.


भारतीय प्रौद्योगिकी संस्थान गुवाहाटी
Indian Institute of Technology Guwahati

Chapter 5

Chapter 5

Rhizobium pongamiae from root nodules of CPT

5.1 Introduction

Legumes advantages over other tree species are its symbiotic association with *Rhizobium* that plays a critical role in sustainable nitrogen management in agriculture, afforestation and soil erosion control programs. The genus *Rhizobium* encompasses a genetically diverse group of gram negative, chemoorganotrophic aerobic soil bacteria that induce hypertrophisms in plants as root nodules with or without symbiotic nitrogen fixation (Young et al. 2001). This symbiotic process results in the de novo formation of a unique organ, the N_2-fixing root nodule. Root nodules arise from the coordinated expression of both rhizobial and plant genes in response to signals exchanged between the partners. Release of flavonoids/isoflavonoids from developing roots induces the expression of rhizobial nodulation genes and the synthesis of lipochitooligosaccharides (Schultze & Kondorosi, 1996). Lipochitooligosaccharides then activate the expression of a cascade of plant genes required for root nodule formation (Geurts & Bisseling, 2002). Signaling between rhizobia and legumes initiates development of a unique plant organ, the root nodule, where bacteria undergo endocytosis and become surrounded by a plant membrane to form a symbiosome (Liu et al. 2006).

Evaluation of rhizobial host range is one of the cultural characteristics used for the description of new species of rhizobia (Graham et al. 1991). The ability of indigenous rhizobia to nodulate a legume crop effectively, or to act as barrier to the successful establishment of inoculant strains, is critical to successful establishment and growth of legumes. *Pongamia pinnata* multipurpose benefits as a provider of green manure and medicine and its role in agroforestry makes it a potential candidate for large-scale plantation on marginal lands (Kesari et al. 2009b; Scott et al. 2009) as described in earlier chapters. *Pongamia* are able to grow in nutrient-poor soils by virtue of their ability to establish

symbiosome with nitrogen fixing rhizobia bacteria. Symbiotic relationships between leguminous plant *Pongamia* (Fabaceae) and nodule-forming bacteria *Rhizobium* remain poorly characterized despite their importance (Dayam, 1985; Siddiqui, 1989). Most studies have been focused on biodiesel aspect and medicinal value (Scott et al. 2008; Meera et al. 2003). There is a clear need to characterize in more detail the spectrum of rhizobia that can form an effective symbiotic relationship with *P. pinnata*, as well as the ontogeny of nodule formation. The characterization of indigenous populations of rhizobia inhabiting this important tree plant towards their resistance to stress environmental factors will be important before their selection for nursery inoculation. A detailed study of the bacteria may further support more adequate management practices. In present work, isolation and polyphasic taxonomic characterization of novel strain belong to genus *Rhizobium* from the root nodules of candidate plus tree (CPT) *Pongamia pinnata* occurring in North Guwahati, Assam is described. The objectives of this study were to search for nodules, to isolate bacteria from these nodules, to test the strain phenotypic richness, and to group the bacteria using phenotypic characteristics and multivariate approaches.

5.2 Review of Literature

Early classification of rhizobia was on the basis of the cross inoculation group concept, which groups rhizobia on the basis of their ability specifically to infect and fix N_2 with a discrete group of legumes (Fred et al. 1932). Although it quickly became obvious that nodulation ability overlaps across inoculation groups, this concept has been retained in a modified form because of its practical significance in inoculant use (Giller, 2001). Legumes includes approximately 19,000 species distributed world wide, of this 3000 species identified as potential nitrogen fixers (Dommergues, 1995). These nitrogen fixing trees (NFTs) are also the major source of nitrogen in tropical ecosystems: sequential cropping systems, agroforestry, and silvopastoril systems, providing timber, fuel, pulp, fodder and even human food. Excellent reviews and books have been published discussing the establishment and functioning of NFTs symbiosis (Brewbaker, 1990; Dommergues, 1995; Dommergues et al. 1999; Subba Rao & Rodriguez-Barrueco, 1993; Faria et al. 1989). Many papers have been written in relation to rhizobia taxonomy and phylogeny (Jordan, 1984; Martinez et al. 1990; Martinez-Romero & Caballero-Mellado, 1996);

phenotypic characteristics (Sprent, 1997) evolution (Young, 1996), the effects of environmental factors such as salinity (Singleton et al. 1982; Cordovilla et al. 1999) and acidity (Keyser & Munns, 1979; Wood & Cooper, 1985; Vance & Grham, 1995). Our knowledge of the symbiotic affinities among tropical tree rhizobia is still limited. Early reports, which were based on studies of a narrow range of species, indicated that tree legumes were exclusively nodulated by slow-growing rhizobia of the 'cowpea miscellany' (Allen & Allen, 1936, 1939, 1981). However, Trinick (1965, 1968, 1980) and subsequent reports (Dreyfus & Dommergues, 1981; Zhang et al. 1991; Turk & Keyser, 1992; Moreira et al. 1998) showed that trees were as often nodulated by fast-growing as by slow-growing rhizobia. Nitrogen fixing trees are often introduced into new parts of the tropics, but without knowledge of the efficacy of indigenous rhizobia with the new legume.

Despite being a legume, relatively little is known regarding the nodulation of *P. pinnata*. The identity and community structure of *Pongamia* root-nodule bacteria in India and Australia has been addressed by few studies that assessed bacterial growth rate and nodulation ability (Scott et al. 2009; Mukta et al. 2010). Dayam (1985) noted the nodulation of *Pongamia* grown in sandy loam soil and the stimulatory effect of foliar applied sucrose on nodule number and plant growth. There were some preliminary reports that demonstrated the effective nodulation of *P. pinnata* with three strains of rhizobia; *Bradyrhizobium japonicum* strain CB1809, *Bradyrhizobium sp.* strain CB564, a strain previously isolated in Australia from *P. pinnata*; and *Rhizobia sp.* strain NGR234, the same strain previously reported unable to form nodules on *P. pinnata* (Scott et al. 2008). The nodulation and associated nitrate reductase activity of *Pongamia* seedlings was reported by Siddiqui (1989). In both these studies the nodulation of plants was reliant on the presence of endogenous rhizobia and their ability to nodulate the host plant. The present result extend this work to assess the identity of root-nodulating strain isolated from *P. pinnata* occurring naturally in North Guwahati, Assam.

5.3 Methods

5.3.1 Plant material

Root nodules of CPT, NGPP 46 of *P. pinnata* were collected from the study site, NG, Assam.

Young seedlings were preferred as the nodules were easier to locate than on mature plants. Plant roots containing several nodules from each plant were sealed in plastic bags for transport (each plant in a separate bag). Nodules shapes and size were recorded. Individual nodules were used to perform scanning electron microscope (SEM) and *Rhizobium* isolation. *Rhizobium* bacteria were generally isolated the same day, if not; bags were stored at 4 °C.

5.3.2 Rhizobium strain isolation

Root material was washed in running tap water to remove adherent soil. Individual nodules were dissected from the roots, using a flame sterilized scalpel and tweezers. If nodules were very small, a little root tissue was left attached either side of the nodule. Nodules were washed thoroughly in running tap water and the non-ionic surfactant 'Tween 20' (100 $\mu L \cdot L^{-1}$) to remove all traces of soil. The nodules were then transferred to a sterile conical flask and were surface sterilized with 70 % ethanol and then given three rinses with sterile distilled water. Subsequently nodules were treated with 5 % sodium hypochlorite for 10–30 minutes depending on the nodule size and again rinsed five times with sterile distilled water. Individual nodule of size (approx. 2 mm) was cut into two half and central part was scooped out and aseptically streaked across the surface of a petridish containing Tryptone Yeast Extract (TYE) and Yeast Mannitol Agar (YEM; Vincent, 1970) (Table 5.1). Agar plates were incubated at 28 °C for three days. The length of time required for the first visible rhizobial colony to appear was recorded. Individual colonies appearing were re-streaked onto YEM or TYE plates. The purity of the cultures was verified by repeated streaking of single-colony isolate onto YEM agar with 25 mg Kg^{-1} (w/v) congo red. Strain was maintained or stored at 4 °C for short-term storage whereas for long term storage cultures were maintained in YEM broth containing 20 % (v/v) glycerol at -80 °C.

Table 5.1. *Rhizobium* bacteria medium composition

Name	Composition per liter	Ref
YEM	10 g yeast extract, 10 g mannitol, 2.5 g peptone, 15 g agar	Vincent (1970)
TYE	5 g Tryptone, 3 g yeast extract, 0.87 g $CaCl_2.2H_2O$, 15 g agar	-do-

5.3.3 Cross nodulation assessment of VKLR01^T in host plant

Pongamia seeds were soaked 4 - 6 hrs in running tap water to leach out possible inhibitory compounds. Seeds were surface sterilized in a Tween 20 detergent (10 µL L^{-1}) for 10-30 min. All treated seeds were thoroughly rinsed with sterile distilled water until all traces of detergent were removed. Seeds were germinated in plastic pots (diameter, 7 cm; height, 14 cm) containing vermiculite. Strain tested was grown on YEM broth for 12-24 h. Seedlings were inoculated by applying 1 ml of undiluted inoculant directly on the roots of seedlings on 10-15th day of transplanting and were reinoculated one week later. The plants were watered with a 1:4 dilution of Jensen nitrogen-free plant growth medium (composition in g/L: $CaHPO_4$ 1.0, $K_2HPO_4.3H_2O$ 0.262, $MgSO_4.7H_2O$, 0.2, NaCl 0.2, $FeCl_3$ 0.1) (Zhang et al. 1991). The strain VKLR01^T grown on YEM broth for 12 h was applied (1 ml of undiluted inoculants) directly to the newly emerged roots of 10-15 day old seedlings and was reinoculated one week later. Plants were grown in growth chamber under long day condition 16 h day 8 h night cycle at 28 ± 2 ºC with a relative humidity of 80 %. The experiment was performed in triplicate and nodulation was checked after 8-12 weeks. Uninoculated plants were used as controls. Results were recorded as positive (nodules formed) or negative (no nodules were found).

5.3.4 Characterization of purified VKLR01^T

The nodule forming *Rhizobium* isolate was classified according to their phenotypic features, numerical analysis and multivariate approaches. The biochemical and physiological tests were performed in triplicate.

5.3.4.1 Morphological and cultural characterization

5.3.4.1.1 Colony and cell morphology

Rhizobium colony was characterized according to color, colony size (the colony diameter measured with a ruler) after 24-48 h of incubation. Cell size and morphology of the root nodule isolate VKLR01^T were determined using scanning electron microscopy (LEO 1430

VP; Leo Electron Microscopy Limited, Cambridge, UK) at 10 kV. For the micromorphology study, cells from the exponential growth phase (grown in YEM broth at 28 °C, 180 rpm) were harvested by centrifugation and fixed in 2.5 % glutardehyde for 45 min. The cells were washed with phosphate buffer saline (PBS) and applied to ethanol dehydration series (50 %, 70 %, 90 % and at 100 % for 10 min each) followed by critical-point drying with CO_2 and sputter-coating with gold as described by Boyde & Wood (1969).

5.3.4.1.2 Effect of temperature and pH on growth

Growth rate of strain VKLR01^T was recorded at different temperatures (4, 10, 20, 30, 37 and 45 °C) in YEM broth and agar till 72 hrs of incubation. The ability to grow in acid and alkaline media was tested by inoculating strain VKLR01^T onto YEM broth and YEM agar plates that was adjusted to various pH values (pH 4.0–11 at intervals of 1 pH units). The pH was adjusted prior to sterilization to various levels by the addition of 1N HCl or 1N NaOH.

5.3.4.1.3 Determination of NaCl tolerance level and intrinsic antibiotic resistance

The NaCl tolerance of the strain VKLR01^T was tested by growing them in YEM broth and YEM agar plates containing 0, 1, 2, 3, 4 and 5 % (w/v) NaCl. The inoculation method used was the same as that described above for the pH tolerance test. The intrinsic antibiotic resistance tests for the strain VKLR01^T were performed by disc diffusion assay in YEM agar against 26 different antibiotics (ocatadsics, HiMedia): Gentamycin (10 µg), Streptomycin (10 µg), Tetracyclin (30 µg), Cephotaxime (30 µg), Cephalexin (30 µg), Carbenicillin (100 µg), Amikain (10 µg), Kanamycin (30 µg), Tobromycin (10 µg), Co-Trimoxazole (25 µg), Nitrofurantoin (300 µg), Fuezolidone (50 µg), Lincomycin (2 µg), Methicillin (5 µg), Oleandomycin (15 µg), Penicillin G (10 µg), Ampicillin (10 µg), Ciprofloxacin (10 µg), Colistin (10 µg), Chloromphenicol (30 µg), Nalidixe (30 µg), Norfloxacin (10 µg), Oxytetracyclin (30 µg), Co-trimazine (25 µg), Oxacillin (5 µg) and Cephaloridine (30 µg).

5.3.4.2 Biochemical characterization and FAME analysis

5.3.4.2.1 Biochemical tests

Gram staining was performed by using samples fixed with acetic acid, as described by Dussault (1955). Nitrate reduction was tested using the sulfanilic acid and α-naphthylamine method (Smibert & Krieg, 1981). Catalase production was tested using 10 % (v/v) H_2O_2. The oxidase reaction was tested on filter paper moistened with a 1 % (w/v) aqueous solution of N,N,N9,N9-tetramethyl-p-phenylenediamine. Gelatin hydrolysis and methyl red test were also performed. Analysis of respiratory quinones was carried out by the Identification Service and Dr. Brian Tindall, DSMZ, Braunschweig, Germany according to the method of Komagata & Suzuki (1987).

5.3.4.2.2 Utilization of carbon source

Utilization of 5 carbon sources by type strain VKLR01^T was tested for raffinose, sucrose, arabinose, mannitol, lactose, and glucose respectively (Lindstrom & Lehtomaki, 1988). Def 9 medium containing mannitol was used as a positive control, while Def 9 medium without any carbon source was used as a negative control (Zhang et al. 1991). Acid production from different carbohydrates was determined by employing the API 50 CH system (bioMerieux) according to the manufacturer's instructions.

5.3.4.2.3 Fatty acid methyl ester (FAME) profiles

The purified strain VKLR01^T was sent to Royal Life Sciences (Hyderabad, India) for the identification and quantification of the fatty acid methyl esters analysis using the Sherlock Microbial Identification System (MIDI, Inc.). Strain VKLR01^T was grown on TYE medium for 48 h at 28 °C. Bacterial suspension was saponified in NaOH/methanol (45 g NaOH, 150 mL methanol, 150 mL mQ water), then methylated at 80 °C in hydrochloric acid (6 M) and methanol. The organic phase was extracted in n-Hexane and methyl tert-butyl ether, and analysed by Gas Chromatography (GC) MODEL 6850 (Agilent Gas chromatography) equipped with an agilent ultra 2 capillary column. The fatty acid methyl esters extracts were analyzed as described by Sasser (1990). The Sherlock software automates all analytical operations and uses a sophisticated pattern recognition algorithm to match the unknown

FAME profile to the stored library entries for identification. Fatty acids were identified and quantified, and the relative amount of each fatty acid in a strain was expressed as a percentage of the total fatty acids in the profile of that strain. The technique used by the Sherlock System to present results is based on a Similarity Index (SI). The SI is a numerical value, which expresses how closely the fatty acid composition of an unknown compares with the mean fatty acid composition of the strains used to create the library entry listed as its match.

5.3.4.3 Molecular characterization

5.3.4.3.1 Identification of strain VKLR01^T based on 16S rRNA gene sequence

Strain VKLR01^T isolated from root nodules of *P. pinnata* (VKLR01^T) were grown on yeast extract mannitol broth (YEM) (12 h, 28 °C, 180 rpm) for genomic DNA isolaton using Sigma′s GenElute Bacterial Genomic Kit. Strain VKLR01^T was identified based on almost-complete 1428 bp of 16S rRNA fragment amplification using consensus primer fD1 and rD1 (Laguerre et al. 1994). 16S rRNA primers used was taken from previous studies, eliminating the need to design and optimize new primer. PCR composition and conditions for amplification of 16S rRNA fragment is shown in Table 5.2. PCR products were sequenced using the same forward and reverse primers from Bangalore Genei, India. The almost complete 16S rRNA sequence of strain VKLR01^T was compared to other gene sequences in the NCBI GenBank database using a BLAST search (Altschul et al. 1990) and the ribosomal database project II (Cole et al. 2007) using the sequence match tool. Analysis of the sequence data was performed using the software package MEGA version 4.0 (Tamura et al. 2007) after multiple alignment by using the ClustalX package (Thompson et al. 1997). Evolutionary distances were computed by the Neighbour-Joining method (Saitou & Nei, 1987). Distances according to Kimura-2 model (Kimura, 1980) were determined and clustering with the neighbor joining and maximum-parsimony methods was performed by using Boostrap values based on 1000 replications. A phylogenetic tree was constructed based on 16s rRNA gene sequences of strain VKLR01^T and other related organisms of the family Rhizobiaceae.

Table 5.2. 16S rRNA amplification. **A.** PCR Components; **B.** PCR conditions.

A		B		
Component	**Amount (μL)**	**Temperature**	**Time**	
10× PCR Buffer	2.5	95 °C	5 mins	1 Hold
2.5 mM dNTPs	2.5	95 °C	45 s	20 Cycles
25 mM $MgCl_2$	2.0	63→53 °C	45 s	
10 μM Forward primer (fD1)	0.5	72 °C	90 s	
10 μM Reverse primer (rD1)	0.5	95 °C	45 s	15 Cycles
50 ng genomic DNA	1.0	53 °C	60 s	
5U Taq DNA polymerse	0.3	72 °C	45 s	
Sterile miliQ H_2O	15.7	72 °C	7 mins	1 Hold
Total volume	25	4 °C	99.9 mins	1 Hold

[a]Buffer, $MgCl_2$, and Taq DNA polymerase are from the Bioline kit (Germany).

5.3.4.3.2 G+C content

The DNA G+C content was determined as described by Tamaoka & Komagata (1984) from DSMZ, Germany. Cells of strain VKLR01^T were disrupted using a French pressure cell (ThermoSpectronic) and the DNA in the crude lysate was purified by chromatography on hydroxyapetite as described by Cashion et al. (1977). DNA was hydrolysed, dephosphorylized (Mesbah et al. 1989) and was analyzed for their G+C content by HPLC. Non-methylated Lambda-DNA (Sigma) was used as a reference.

5.3.4.3.3 DNA-DNA hybridization

DNA-DNA hybridization was assessed for type strain VKLR01^T against reference strain *Agrobacterium radiobacter* (*Rhizobium radiobacter*) DSM 30147^T (=AB247615). DNA-DNA hybridization was carried out as described by DeLey et al. (1970) under consideration of the modifications described by Huss et al. (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with Peltier-thermostatted 6x6 multicell changer and a temperature controller with *in-situ* temperature probe (Varian). The highest and lowest

values obtained were excluded, and the means of the duplicate were quoted as DNA-DNA relatedness values.

5.4 Results and discussion

5.4.1 Morphological and cultural characterization

Root nodules were seen on almost all the *Pongamia* saplings uprooted in the sampling site, indicating that native root-nodules occur widely in *P. pinnata* in North Guwahati. However, plants varied in the extent to which they were nodulated. The nodules varied in shapes and sizes formed on primary as well as secondary roots (Fig. 5.1). Phenotypic traits of *Pongamia* saplings viz., mean shoot length, mean root length and the number of nodules observed in *P. pinnata* were 28.25 ± 3.24, 12.17 ± 2.19 and 9.90 ± 2.31 respectively. Different shapes may be related to different developmental phases of the nodule ontogeny and it is therefore not surprising that the nodule morphology has been used as a taxonomic marker (Corby, 1971; Felker & Clark, 1980). Sectioned of single nodule, approx. 20 µm thick, in SEM image revealed that in the outer wall portion of the nodule no bacteria were visible. But when the middle portion was focused, each cells were fully filled with rod shaped bacteria (Fig. 5.2A & B). This was the reason that middle portion of the root nodule were used further for isolation of *Rhizobium* bacteria specific to *P. pinnata*.

The strain VKLR01^T growth is visible as a small white shiny dot like structure which increased in size from 1.5-3.5 mm (24 h) to 4.0-5.5 mm (48 h) in both YEM and TYE plates at 28 ºC (Fig. 5.3A-D). The generation time noted was 0.67 h in YEM medium. The strain VKLR01^T produced was creamy or white opaque, round or convex and gummy colonies with little or moderate extracellular polysaccharide production (EPS). The SEM image of the purified strain VKLR01^T from an exponential phase showed that the bacterium isolated was rod shaped and the cell dimension varied between 0.4-0.5 µm in width & 1.4-1.6 µm in length respectively (Fig. 5.3E). Early reports of rhizobia associated with woody legumes described them as species that belong to the slow-growing, the cowpea miscellany (Jordan, 1984), but more recent reports (Zhang et al. 1991) have shown that this population includes a very diverse type of rhizobia including fast, intermediate and of slow-growing bacteria.

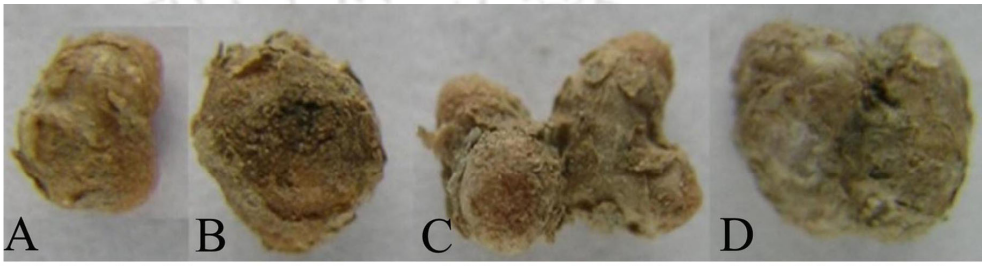
Shape				
Size (mm)	1.08 (L), 0.6 (B)	2.06	3.03 (L), 4.05 (B)	2.62 (L), 3.93 (B)
SE	0.05 (L), 0.07 (B)	0.04	0.03 (L), 0.04 (B)	0.04 (L), 0.09 (B)

Figure 5.1. Nodule shapes and size found in *P. pinnata* collected from study site.

A. small rod; **B.** spherical; **C.** amorphous; **D.** heart.

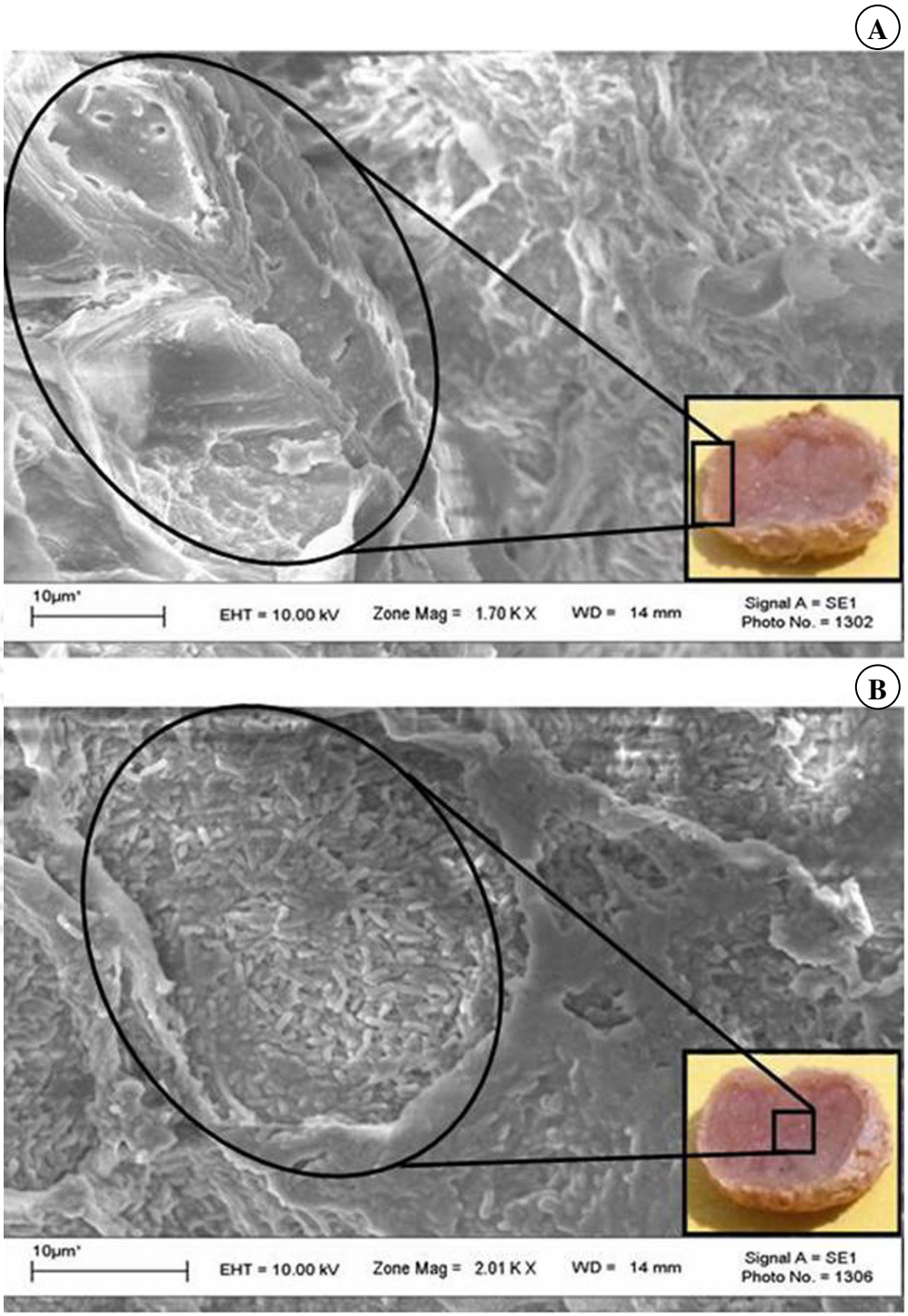


Figure 5.2. Scanning electron micrograph of *P. pinnata* root nodule.

A. nodule outer wall; **B.** central region of the nodule showing rod shaped bacteria.

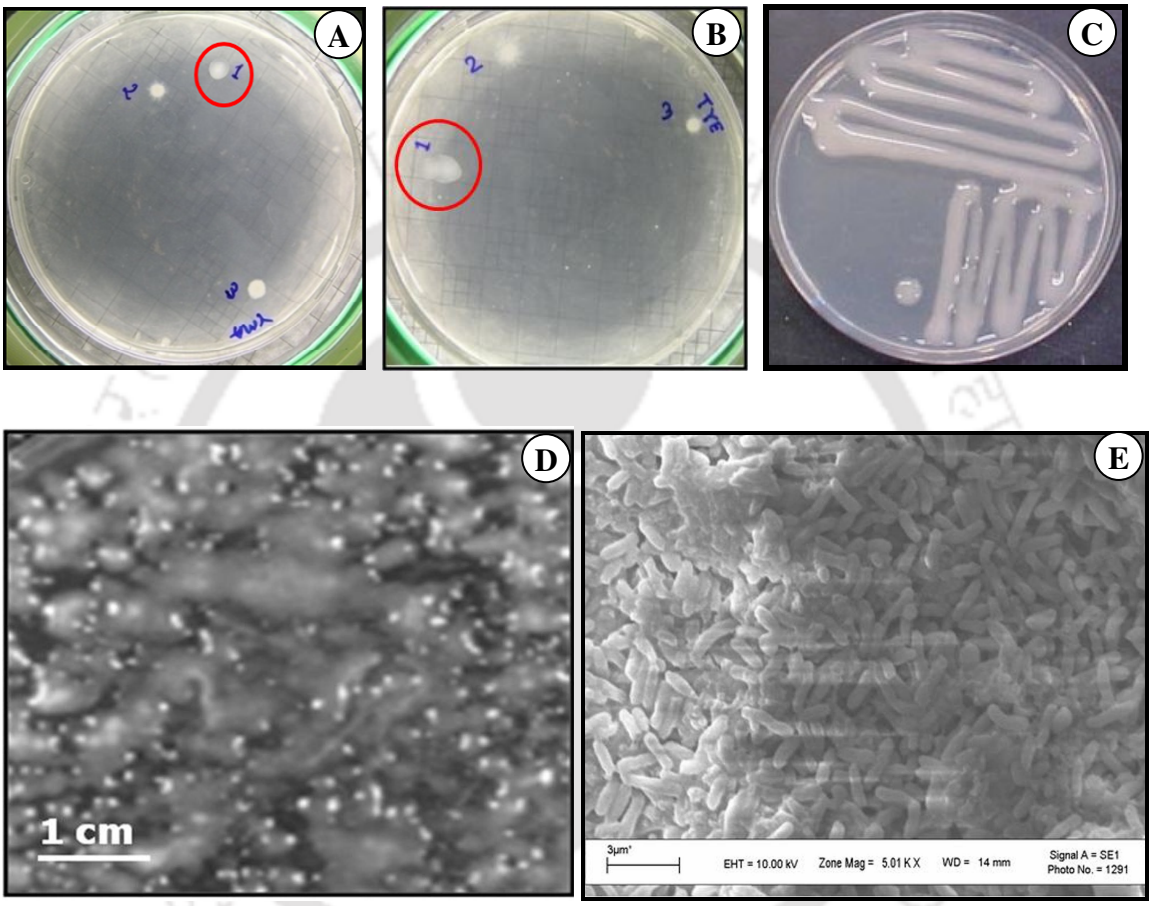


Figure 5.3. *Rhizobium pongamiae* VKLR01^{T}.

A-D. Colonies on Yeast extract mannitol (YEM) and trypton yeast extract (TYE) agar; **E.** Scanning electron micrograph showing rod shape cells.

Reports exists to show that trees are as often nodulated by fast-growing as by slow growing rhizobia (Dreyfus & Dommmergues, 1981; Zhang et al. 1991; Turk & Keyser, 1992; Moreira et al. 1998). In this work the strain VKLR01^T based on phenotypic features from native *Pongamia* trees were mainly fast-growing rhizobia strain forming colonies of 4-5.5 mm in diameter in 2 days time.

Gram stain and scanning electron micrograph confirms the strain VKLR01^T to be gram negative rods. Growth of the strain VKLR01^T occurs at temperature range of 25-30 °C (optimally at approx. 28 °C) and can tolerate upto 42 °C, but no growth recorded at 4 or 50 °C (Fig. 5.4A). Strain VKLR01^T can tolerate the salt concentration varied in the range of 1-4 % NaCl but no growth observed at 5 % NaCl concentration in the medium (Fig. 5.4B). Salt accumulation is a factor that may render soil unsuitable for agriculture. Salt concentration from 50 to 200 mM NaCl significantly limits productivity by interfering with legume growth (Craig et al. 1991; Delgado et al. 1993). Interestingly, the strain VKLR01^T was able to grow well in the presence of 4 % NaCl in YEM medium, which differentiates this strain from other species. Similar results were found with rhizobia that nodulate *Lotus corniculatus* (Baraibar et al. 1999). The strain also grew at pH 6.0 to pH 11.0 but no growth was observed at and below pH 5.0 (Fig. 5.4C). Rhizobia vary significantly in its acid tolerance. Vance & Graham (1995) reported that *S. meliloti* do not grow below pH 5.6. The identification of glutamate as a compatible solute in acid-stressed cells and the demonstration that cell membrane differences could influence pH tolerance, have been found by these authors as responsible for the acid pH tolerance. Nevertheless, field studies are important to verify the *in vitro* results. Optimum growth conditions for the strain VKLR01^T were; temperature 28-30 °C, pH 7.0-8.0 and 2 % (w/v) NaCl. Phenotypically, strain VKLR01^T could be separated from other strain of *Rhizobium* based on growth at adverse environmental conditions i.e. higher temperature (37 to 42 °C), salinity (upto 4 % NaCl) and alkaline pH (11). Fig. 5.5A-G & Table 5.3 shows that strain VKLR01^T was highly sensitive to 9 antibiotics viz., Gentamycin (10 µg), Streptomycin (10 µg), Tetracyclin (30 µg g), Cephotaxime (30 µg), Cephalexin (30 µg), Carbenicillin (100 µg), Amikain (10 µg), Kanamycin (30 µg), Tobromycin (10 µg) and resistant to 7 antibiotics viz. Co-Trimoxazole (25 µg), Nitrofurantoin (300 µg), Fuezolidone (50 µg), Lincomycin (2 µg), Methicillin (5 µg),

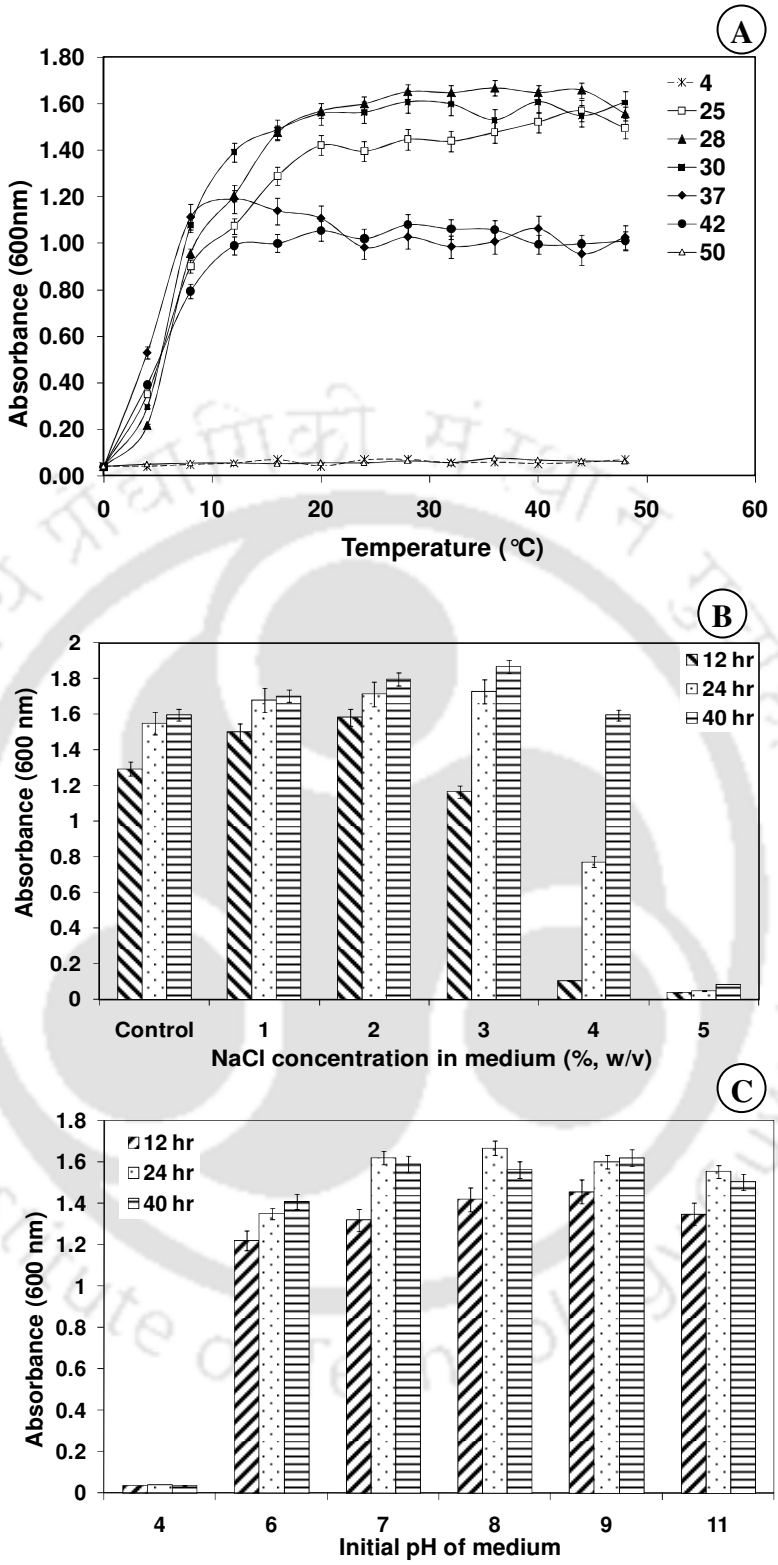


Figure 5.4. Growth characteristics of *Rhizobium pongamiae* VKLR01^T on Yeast extract mannitol (YEM) medium.

A. Effect of temperature; **B.** Effect of NaCl; **C.** Effect of pH.

Table 5.3. Susceptibility of *Rhizobium pongamiae* VKLR01^T and type strains of phylogenetically related *Rhizobium* species against reference antibiotics.

S. No.	Antibiotic	Notation	Octadisc	1	2	3
1	Ampicillin	A	I,III	+*	+	-
2	Ciprofloxacin	Cf	I,IV	+†	ND	ND
3	Colistin	Cl	I	+*	ND	ND
4	Co-Trimoxazole	Co	I, II	+	ND	ND
5	Gentamycin	G	I,III	-	ND	ND
6	Nitrofurantoin	Nf	I,IV	+	ND	ND
7	Streptomycin	S	I,IV	-	ND	ND
8	Tetracyclin	T	I,IV	-	ND	ND
9	Cephotaxime	Ce	II,III	-	ND	ND
10	Cephalexin	Cp	II	-	ND	ND
11	Chloromphenicol	C	II,III	+*	+	ND
12	Nalidixe	Na	II	+*	ND	ND
13	Fuezolidone	Fr	II	+	ND	ND
14	Norfloxacin	Nx	II,III,V	+†	ND	ND
15	Oxytetracyclin	O	II	+†	ND	ND
16	Carbenicillin	Cb	III,IV	-	+	ND
17	Co-Trimazine	Cm	III,IV	+	ND	ND
18	Oxacillin	Ox	III	+†	ND	ND
19	Amikain	Ak	IV	-	ND	ND
20	Kanamycin	K	IV,V	-	+	+
21	Cephaloridine	Cr	V	+*	ND	ND
22	Lincomycin	L	V	+	ND	ND
23	Methicillin	M	V	+	ND	ND
24	Oleandomycin	Ol	V	+	-	ND
25	Penicillin G	P	V	+	+	ND
26	Tobromycin	Tb	V	-	ND	ND
27	Cephalothin	--	--	ND	+	ND
28	Novobiocin	--	--	ND	+	ND
29	Erythromycin	--	--	ND	ND	+

Strains: 1, *Rhizobium pongamiae sp. nov.*; 2, *R. galegae* LMG 6214^T (Yoon et al. 2009); 3, *Agrobacterium tumefaciens* (*R. radiobacter*) (Tighe et al. 2000; Quan et al. 2005).

+, resistant; -, sensitive; +*, mild sensitive; +†, moderate sensitive; ND, data not available.

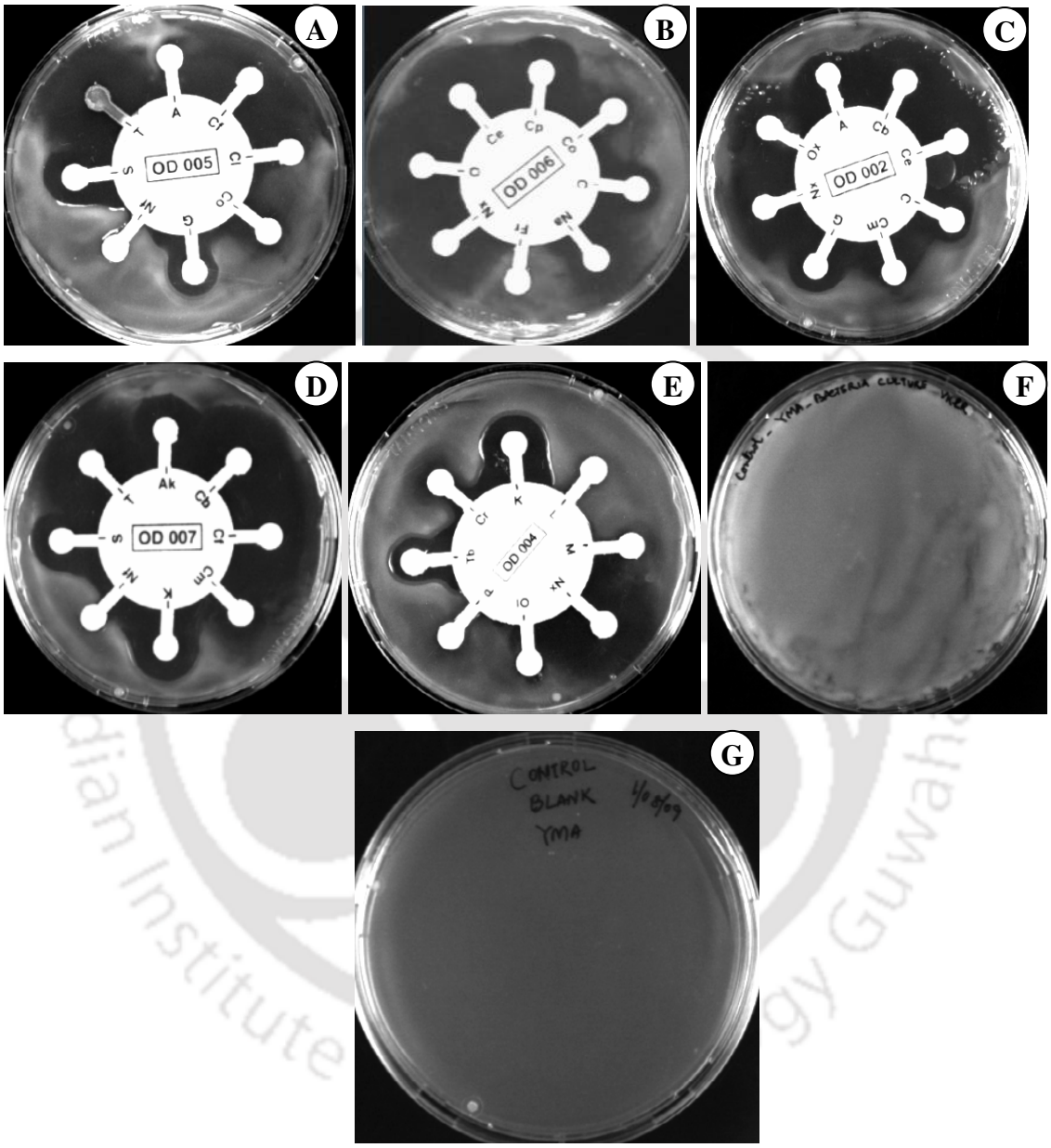


Figure 5.5. Antibiotic sensitivity of *Rhizobium pongamiae* VKLR01^{T} on Yeast extract mannitol (YEM) medium.

A. I; **B.** II; **C.** III; **D.** IV; **E.** V; **F.** positive control; **G.** negative control

Oleandomycin (15 µg), Penicillin G (10 µg) and for rest 10 antibiotics either mildly sensitive or moderately sensitive. Döbereiner et al. (1981) related the increased resistance of rhizobial strains to the presence of antibiotics in the soil as a consequence of microbial activities such as Streptomyces. This might have resulted in the isolation of rhizobial strains to a wide spectrum of antibiotics to be included in soil microbial analysis (Kremer & Peterson, 1982).

5.4.2 Biochemical characterization and FAME analysis

Strain VKLR01^T was positive for oxidase, catalase and nitrate reduction test however showed negative result for gelatin hydrolysis and methyl red tests. Gas is not produced from raffinose, sucrose, arabinose, mannitol, lactose and glucose. In the API 50 CH system, acid are produced from fermentation of sucrose, mannitol and lactose but not from raffinose, arabinoise, and glucose. Odee et al. (1997) reported that slow growth of rhizobia associated with woody tree species is related to alkali production and fast growth is related to acid producers.

R. pongamiae strain VKLR01^T contained ubiquinone-10 (Q-10), at a peak ratio of approximately 100 % as the predominant isoprenoid quinone. The most abundant fatty acids are summed feature 8 (65.92 %; comprising C $_{18:1}$ ω7c and/or C $_{18:1}$ ω6c) followed by C $_{16:0}$ iso (10.43 %), summed feature 2 (7.42 %, comprising C $_{14:0}$ 3OH/ C $_{16:1}$ iso I and an unidentified fatty acid with an equivalent chain length of 10.9525), C $_{16:0}$ 3OH (4.19 %), C $_{13:1}$ at 12-13 (2.80 %), C $_{19:0}$ cyclo ω8c (2.76 %), summed feature 3 (2.41 %; comprising C $_{16:1}$ ω7c/ C $_{16:1}$ ω6c). The following fatty acids are detected in small amounts; C $_{14:0}$ (0.75 %), C $_{18:0}$ 3OH (0.58 %), C $_{12:0}$ (0.55 %), C $_{17:1}$ ω8c (0.52 %), C $_{18:0}$ (0.66 %) and C $_{18:0}$ 10-methyl, TBSA (0.34 %). Yoon et al. (2009) also showed that CFA profile of 5 *Rhizobium* species (*R. soli* DS-42^T, *R. huautlense* LMG 18254T, *R. galegae* LMG 6214T, *R. loessense* CIP 108030^T and *R. cellulosilyticum* DSM 18291^T) contains $C_{18:1}$ ω7c as the major fatty acid, although there were differences in the proportion of some other fatty acids. The cellular fatty acid profile for strain VKLR01^T and other phylogenetically related *Rhizobium* species is shown in Table 5.4.

5.4.3 Nodulation assessment test

Nodulation test was performed by investigating the ability of the strain VKLR01^T to form

nodules on the young germinated seedlings of *P. pinnata* grown on a nitrogen free nutrient media. Nodules were observed after 2-3 months (Fig. 5.6) when an optimal dosage inoculation of the strain VKLR01^T was treated on the root hairs of germinated seedlings *of Pongamia* ascertaining that the rhizobial strain could nodulate the original host species CPT of *P. pinnata* raised on a nitrogen free nutrient media.

5.4.4 Molecular characterization

The almost complete 16S rRNA gene sequence of strain VKLR01^T determined in this study comprised 1428 nucleotides (approximately 95 % of the *Escherichia coli* 16S rRNA sequences) as shown in Fig. 5.7A. Sequence analysis of 16S rRNA gene showed that strain VKLR01^T was most phylogentically closely related to the genus *Rhizobium* sp. and *Agrobacterium*. In the phylogenetic tree constructed using the maximum-likelihood and maximum-parsimony algorithms, strain VKLR01^T fell within the clade encompassed by the genus *Rhizobium* (*Agrobacterium*). Strain VKLR01^T exhibited 16S rRNA gene sequence similarity values of 98 % to the type strains *Rhizobium* sp. 120, *R. galegae* Ca2 and *Agrobacterium tumefaciens* st. ORS 3405. The next most closely related type strains to strain VKLR01^T were *Agrobacterium larrymoorei* st. 2R46 and *Bradyrhizobium japonicum* PRY 65 with a sequence similarity of 97 % respectively. The phylogenetic reconstruction based on a neighbour-joining analysis for the strain VKLR01^T to closely related type strains is depicted in Fig. 5.7 B.

Table 5.4. Cellular fatty acid composition (%) of *Rhizobium pongamiae* VKLR01^T and type strains of phylogenetically related *Rhizobium* species.

Fatty acid	1	2	3	4
Straight chain fatty acid				
12:0	0.55	-	-	-
14:0	0.75	-	0.11	0.01
15:0	-	-	0.29	0.04
16:0	10.43	7.7	9.03	11.97
17:0			0.13	0.19
18.0	0.66	0.9	0.17	0.62
Unsaturated fatty acid				
13:1 at 12-13	2.80	-	0.49	-
16:1 ω5c	-	-	-	1.06

17:1 ω8c	0.52	-	-	-
15:1 ω8c	-	0.8	-	-
17:1 ω6c	-	-	-	0.15
17:1 ω8c	-	-	0.22	0.48
18:1 ω5c	-	-	-	0.02
18:1 ω7c	-	76.2	-	-
Hydroxy fatty acid	-	-	-	-
12:0 3-OH	-	-	-	0.04
13 : 0 2-OH	-	-	-	-
15:0 3-OH	-	-	0.03	-
15 : 1 3-OH iso	-	-	-	-
16:0 3-OH	4.19	2.0	4.76	-
17:0 3-OH	-	-	0.18	-
18:0 2-OH	-	-	-	-
18:0 3-OH	0.58	0.7	-	-
15 : 1 G iso	-	-	-	-
16:0 iso	0.67	-	-	-
10-methyl 18:0 TBSA	0.34	-	-	-
10-methyl 19:0	-	2.1	1.09	-
11-methyl 18:1 ω7c	-	-	0.23	1.91
17:0 cyclo	-	-	1.60	-
19:0 cyclo ω8c	2.76	3.9	18.78	1.16
20:2 ω6,9c	-	-	0.01	-
20:3 ω6,9,12c	-	-	0.33	-
Unknown (ECL 18.794)	-	-	-	0.01
Summed Features*	-	-	-	-
2	7.41	4.6	8.18	1.06
3	2.42	0.52	-	-
4	-	-	1.62	-
7	-	-	52.41	81.22
8	65.92	-	-	-

-, not detected

*Summed features 2 (12:0 aldehyde? and/or 16:1 iso I and/or 14:0 3-OH and/or unknown ECL 10.928 and/or unknown ECL 10.9525 and/or 15:1 iso H/I, 13:0 3-OH)

*Summed features 3 (16:1 ω7c and/or 16:1 ω6c and/or 15:0 iso 2-OH)

*Summed features 4 (iso-17: 1 I and/or anteiso-17: 1 B and/or 15:0 iso 2-OH, 16: 1 ω7c)

*Summed features 7 (18:1 ω7c and/or ω9 trans and/or ω 12 trans and/or 18:1 ω7c and/or ω9c and/or ω12trans)

*Summed features 8 (18:1 ω7c and/or 18:1 ω6c)

Strains: 1, *Rhizobium pongamiae sp. nov.*; 2, *R. galegae* LMG 6214^T (Yoon et al. 2009); 3, *Agrobacterium tumefaciens* (Tighe et al. 2000); 4, *Bradyrhizobium japonicum* (Tighe et al. 2000). Values are percentages of the total amount of fatty acid compounds present for that species. ECL, equivalent chain length

Figure 5.6. Nodulation by VKLR01^{T} on the root of host plant *P. pinnata* saplings. **A.** field sapling showing nodulation; **B.** test sapling showing nodulation; **C.** negative control.

A

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GCGATGGGCCCTCCTTACTCATCTACATGCGCGTCGAACGCCCCGCATTGGGAGTGGCTTACGGGTGAGTAACGCGTGGGAA
CATACCCTTTCCTGCGGAATAGCTCCGGGAAACTGGAATTAATACCGCATACGCCCTACGGGGGAAAGATTTATCGGGGAAG
GATTGGCCCGCGTTGGATTCGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATC
AGCCACATTGGGACTGAGACACGACCCACTCTACTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGAT
CCAGCCATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTCACCGATGAAGATAATGACGGTAGTCGGAGAAG
AAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGAATTACTGGGCGTAAACGCGC
ACGTAGGCGGATATTTAAGTCAGGGGTGAAATCCCGCAGCTCAACTGCGGAACTGCCTTTGATACTGGGTATCTTGAGTATG
GAAGAGGTAAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTTACTGG
TCCATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAAT
GTTAGCCGTCGGGCAGTATACTGTTCGGTGGCGCAGCTAACGCATTAAACATTCCGCCTGGGGAGTACGGTCGCAAGATTAA
AACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCT
CTTGACATTCGGGGTATGGGCATTGGAGACGATGTCCTTCAGTTAGGCTGGCCCCAGAACAGGTGCTGCATGGGCTGTCGTC
AGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCCTTAGTTGCCAGCATTTAGTTGGGCACTC
TAAGGGGACTGCCGGTGATAAGCCGAGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGTCCTTACGGGCTGGGCTACACAC
GTGCTACAATGGTGGTGACTGTGGGCCGCGAGACATACGATTTCAAGCTATTCTCCAAAAGCCATCTCAGTTCGGATTGCAG
TCTGCAACTCGAGTGCATGAGGTTGGAATCGCTAGTAATCGCAGATCAGCATGCTGCGGTGAATACGTTCCCGGGCCTTGTA
CACACCGCCCGTCACACCATGGGAGTTGGTTTTACCCGAAGGTAGTGCGCTAACCGCAAGGAGGCAGCTAACCACGGTAGGG
TCAGCGAGCTGGGGTGAAGTCGTAACAAGTAGCC
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B

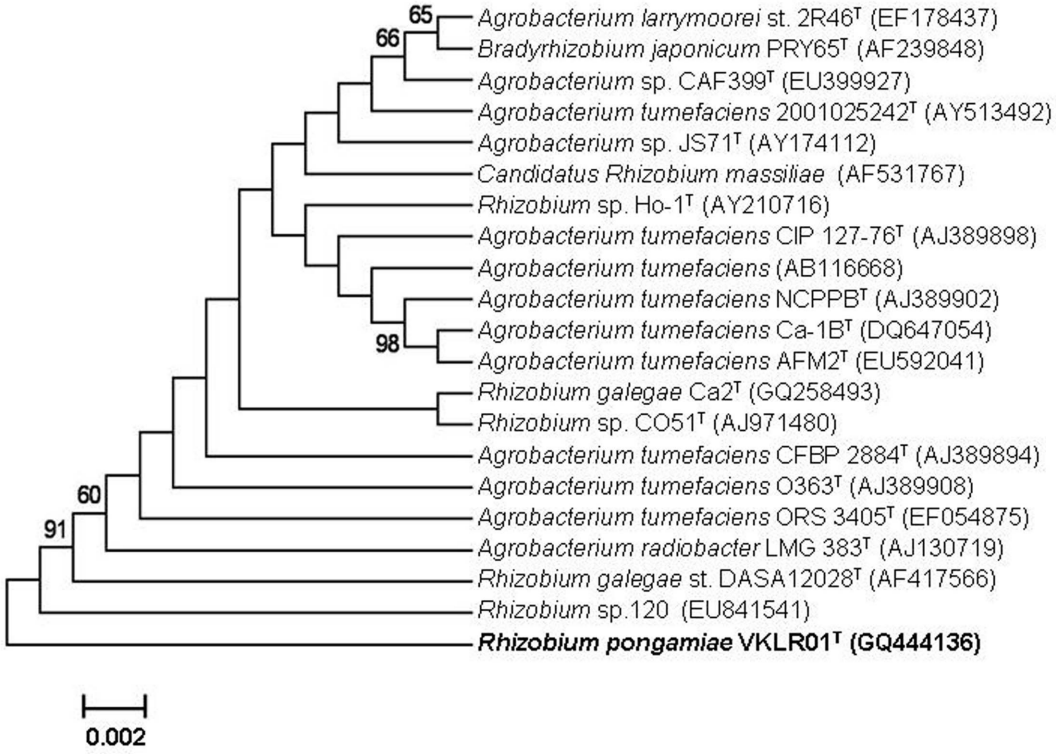

Figure 5.7. *Rhizobium pongamiae* VKLR01^T.

A. 16S rDNA sequence (1428 bp); **B.** Dendrogram depicting the phylogenetic relationships of strain VKLR01^T within the family *Rhizobiaceae* determined using 16S rRNA gene sequence analysis and generated with the MEGA 4.0 software as described in text. Bootstrap values based on 1000 replications are listed as percentages at branching points. Boostrap values below 50 were omitted from the dendrogram. Bar, 0.002 substitutions per nucleotide position.

The DNA G+C content of the type strain VKLR01^T is 59.1 mol % well within the range of values for the genus rhizobia (Chen et al. 1995; Jordan, 1984). However DNA G+C content of the type strain VKLR01^T was lower than that of the type strains *R. soli* DS-42^T (60.8 mol %), *R. galegae* LMG 6214^T (63.0 mol %), *R. loessense* CIP 108030^T (59.5 mol %), *R. tianshanense* 6 (63 mol %), and *R. tianshanense* A-1BS^T (61 mol %) but higher than that of *R. huautlense* LMG 18254^T (57 mol %) and *R. cellulosilyticum* DSM 18291^T (57 mol %) respectively (Chen et al. 1995; Yoon et al. 2009). DNA-DNA hybridization provides a useful strategy to establish the taxonomic place and identity of novel strain (Willems et al. 2001). Strain VKLR01^T exhibited mean DNA-DNA relatedness values of 51.9 % to the type strain of phylogenetically related *Rhizobium* species *Agrobacterium radiobacter* (*Rhizobium radiobacter*) DSM 30147^T (=AB247615). Since the *R. radiobacter* strain VKLR01^T shares DNA-DNA hybridization value of less than 70 % with reference strain DSM 30147^T, the strain VKLR01^T is regarded as a distinct *Rhizobium* species (Wayne et al. 1987). The phylogenetic distinctiveness, together with the DNA-DNA relatedness data and differential phenotypic properties, is sufficient to allocate type strain VKLR01^T to a species that is separate from the recognized *Rhizobium* species (Stackebrandt & Goebel, 1994). Differential features between the novel strain and closely related members of the genus *Rhizobium* are presented in Table 5.5. Thus, on the basis of the results of morphological, numerical taxonomy, 16S rRNA gene sequencing, DNA base composition, DNA-DNA hybridization and host infection it is proposed that the strain VKLR01^T from root nodule of CPT of *P. pinnata* belong to novel species for which the name *Rhizobium pongamiae* sp. nov. is proposed.

Table 5.5. Phenotypic characteristics of *Rhizobium pongamiae* VKLR01^T and type strains of phylogenetically related *Rhizobium* species.

Characteristic	1	2	3	4	5
Origin	Root nodule of *P. pinnata* (North Guwahati, Assam)	*Galega orientalis* (Finland)	ND	Soybean nodule (Florida)	*Ficus benjamina* (Florida)
Cell morphology (µm)	Rods (0.4 - 0.5 x 1.4 - 1.6)	Rods (0.9-1.0 x 1.5-1.8)[a]	Rods (0.6-1 x 1.5-3.0)	Rods (0.5-0.9 x 1.2-3.0)	Rods
Flagella	ND	1-2	Several, peritrichous	One polar, subpolar	Several, peritrichous
DNA G+C content (mol %)	59.1	57.0	57.1	62.8	ND
Nodulation	+	+	-	+	-
pH range	6-11	5.0-9.5 [a]	ND	4-9	ND
Growth at/in					
40 ºC	+	-	ND	-	-
1 % NaCl	+	+	+	+	+
2 % NaCl	+	-	+		+
4 % NaCl	+	-	-	-	-
Utilization as carbon source					
Sucrose	+	+	ND	+	ND
Arabinose	-	+	+	+	ND
Mannitol	+	+	+	+	ND
Lactose	+	-	ND	+	ND
Glucose	-	+	ND	+	ND
Maltose	+	ND	+	+	+
Melibiose	+	+	+	ND	ND

Strains: 1, *Rhizobium pongamiae* VKLR01^T; 2, *R. galegae* LMG 6214^T; 3, *Agrobacterium tumefaciens* (Biovar 1); 4, *Bradyrhizobium japonicum* I LMG 6138^T; 5, *Rhizobium larrymoorei* AF3-10^T. Data taken from Jordan (1984), Tighe et al. (2000), Bouzar & Jones (2001), Rivas et al. (2004), Quan et al. (2005), Yoon et al. (2009), and this study. [a]Unpublished data of E. T. Wang (personal communication) using the method of Wang et al. (1998). +, positive; -, negative; ND, data not available. All strains are Gram negative, aerobic, rod shaped and non spore forming. All strains are positive for oxidase, catalase and nitrate reductase tests.

5.5 Conclusion

Description of *Rhizobium pongamiae* sp. nov.

Rhizobium pongamiae (pon.ga.mi'a.e. N.L. gen. n. pongamiae of *Pongamia*, isolated from root nodules of *P. pinnata*, a legume biodiesel crop growing in North Guwahati, Assam.).

Cells are Gram-negative, straight rods (approximately 0.4 - 0.5 µm wide and 1.4 - 1.6 µm long), non-sore forming and motile. Colonies on YEM media are circular, convex, smooth, glistening, and creamy white with exopolysachharide production having a diameter of 1.5-3.5 mm after growth for 24 h at 28 °C. Metabolism is strictly aerobic and positive for oxidase, catalase and nitrate reduction test. Growth occurs at 25-30 °C (optimal growth occurs at 28 °C) and can tolerate the temperature upto 42 °C but no growth occurs at 4 or 50 °C. Grows at pH 6.0-11.0 (optimal growth occurs at pH 7.0). The production of exopolysachharide is more at alkaline pH (11) and no growth occurs at acidic pH (4). Growth occurs in the presence of NaCl concentrations of upto 4 % (w/v), but not at 5 % NaCl. Optimum growth occurs in the presence of 1-2 % (w/v) NaCl at 28 °C and at pH 7.0. Negative results for gelatin hydrolysis and methyl red tests. Gas is not produced from raffinose, sucrose, arabinose, mannitol, lactose and glucose. Acid is produced from fermentation of sucrose, mannitol and lactose but not from raffinose, arabinose, and glucose. Utilizes lactose, sucrose, mannitol, D-ribose, maltose, melibiose as sole carbon sources. The predominant respiratory quinone is Q10 (100%). The major fatty acids are summed feature 8 (65.92 %; comprising $C_{18:1}$ ω7c and/or $C_{18:1}$ ω6c), $C_{16:0}$ iso (10.43 %), summed feature 2 (7.42 %, comprising $C_{14:0}$ 3OH/ $C_{16:1}$ iso I and an unidentified fatty acid with an equivalent chain length of 10.9525), $C_{16:0}$ 3OH (4.19 %), $C_{13:1}$ at 12-13 (2.80 %), $C_{19:0}$ cyclo ω8c (2.76 %), summed feature 3 (2.41 %; comprising $C_{16:1}$ ω7c/ $C_{16:1}$ ω6c). The following fatty acids are detected in small amounts; $C_{14:0}$ (0.75 %), $C_{18:0}$ 3OH (0.58 %), $C_{12:0}$ (0.55 %), $C_{17:1}$ ω8c (0.52 %), $C_{18:0}$ (0.66 %) and $C_{18:0}$ 10-methyl, TBSA (0.34 %). The DNA G+C content of the type strain is 59.1 mol % (determined by HPLC). The *rhizobium* bacterium isolated in this study is significantly different in phenotypic characteristics from the bacterial standards. The type strain, VKLR01^T, was isolated from root nodules of CPT of *Pongamia pinnata*, a potential biodiesel crop growing in North Guwahati, Assam, India.


भारतीय प्रौद्योगिकी संस्थान गुवाहाटी
Indian Institute of Technology Guwahati

Chapter 6

Chapter 6

Awareness and extension studies in *Pongamia*

The significant findings of earlier chapter were consolidated and made available to the local people through an awareness program. This program was conducted at Sila Forest Office, North Guwahati, Assam in January 2010 with the help of forest officials of North Guwahati. The awareness program was done by display of charts and posters prepared in the local languages (Assamese). The responses of this program and the uses of *P. pinnata* by local people were also recorded.

6.1 Role of local people in large scale cultivation and sustainable utilization

The country today faces a challenge to produce enough biodiesel to meet at least 5 % of its requirement of automobile fuel. A mission program has been launched by the Department of Biotechnology (DBT) on the Biofuel plants. In the present scenario, the biodiesel industry based on tree borne oil seeds (TBOs) is speedily rising because of increasing energy demand, environmental concerns and depleting petroleum reserves on a global scale. CPTs of *P. pinnata* growing in non cultivable area or marginal lands under less favorable environmental conditions are one of major biodiesel feedstocks for developing country like India. A number of research institutes are contributing to meet the long term targets on the development of *P. pinnata* based biodiesel. However, the role played by the local communities can not be sidelined. Since local people are traditional holders of knowledge on the extensive utility of all parts of the tree species that might help in strengthening there local sustainable development. They know the values of tree species that is passed on through generations by oral traditions. They understand the economic, ecological value and association of the plant species. They have profound knowledge on the status, distribution, usage and regeneration mechanism of plants. As they have a clear concept of

phytogeographical conditions, they can efficiently and sustainably utilize and propagate CPTs of *P. pinnata*.

6.2 People in the study site

In the present study, *P. pinnata* was tagged and studied from Sila Forest Range, North Guwahati, Assam. In the awareness program held at Sila Forest Office, North Guwahati nearby rural communities and villagers having occupation of farming, fishing and local business participated. The awareness program organized through kind support from DST project funded to Dr. Latha Rangan, Principal Investigator (PI), Department of Biotechnology, IIT Guwahati also enable local villagers to get involved in plantation aspects within IITG campus and forest sites. Almost all the local men, women and children (above age group of 15 years) participated in awareness program enthusiastically. Initially, it was the informal interactions with the local people and forest officials and later on posters were displayed for making the programme simpler (Fig. 6.1 & 6.2).

6.3 Traditional uses

P. pinnata is commonly called karanja, pongam, honge or a derivation of these names.

Wood: *P. pinnata* is commonly used as fuel wood. Its wood is medium to coarse textured. However, it is not durable, is susceptible to insect attack, and tends to split when sown. Thus the wood is not considered a quality timber. The wood is used for cabinet making, cart wheels, posts, agricultural implements, tool handles and combs .

Oil: A thick yellow-orange to brown oil is extracted from seeds. The estimated annual production of oil from its seeds is about 50,000 t (Ahmad et al. 2003). Yields of 25 % of volume are possible using a mechanical expeller. However, village crushers average a yield of 20 % (Anbumani & Singh, 2009). The oil has a bitter taste and a disagreeable aroma, thus it is not considered edible. In India, the nonedible oil is traditionally and locally used as a fuel for cooking, and lightening the lamps (Source: Power Pods India Feb 2004, ITDG). The oil is also used as a lubricant, water-paint binder, pesticide, and in soap making and tanning

Figure 6.1. Posters prepared for awareness program at North Guwahati, Assam, highlighting the importance of conventional and biotechnological approach for large scale cultivation of *P. pinnata.*

Figure 6.2. Posters prepared for awareness program at Sila Forest Office North Guwahati, Assam, highlighting the importance of biodiesel and medicinal aspects of *P. pinnata*

industries (Ahmad et al. 2003; Reddy et al. 2007). The oil is known to have value in folk medicine for the treatment of rheumatism, as well as human and animal skin diseases (Meera et al. 2003). It is effective in enhancing the pigmentation of skin affected by leucoderma or scabies (Sugla et al. 2007; Burkill, 1966). The oil of *P. pinnata* is also used as a substitute for diesel. Oil cake can also be used as organic fertilizer and is rich in nitrogen and micronutrients (ICRISAT, 2007)

Fodder and feed: Opinions vary on the usefulness of this species as a fodder. The leaves are eaten by cattle and readily consumed by goats. However, in many areas it is not commonly eaten by farm animals. Its fodder value is greatest in arid regions. The oil cake after oil extraction from the seeds, is used as poultry feed, and fish poison (Cribb & Cribb, 1981; Bottoms, 2000).

Other uses: Dried leaves are used as an insect repellent in stored grains. The oil cakes, when applied to the soil, have pesticidal value, particularly against nematodes and also improve soil fertility (Scott et al. 2008). *P. pinnata* is often planted in homesteads as a shade or ornamental tree and in avenue plantings along roadsides and canals. It is a preferred species for controlling soil erosion and binding sand dunes because of its dense network of lateral roots. Its root, bark, leaf, sap, and flower also have medicinal properties (Duke, 1983). All parts of the plant have been used as a crude drug for the treatment of tumours, piles, skin diseases, itches, abscess, painful rheumatic joints, wounds, ulcers, diarrhea, cancer etc. (Chaurasia & Jain, 1978; Shoba & Thomas, 2001; Meera et al. 2003; Essa & Subramanian, 2006). The decoction of the leaves is given to children with cough and is also applied as a bath or fomentation to the rheumatic joints. The juice of leaves is used against itches, herpes, flatulency, dyspepsia and diarrhea (Duke, 1983). The juice of stem and root is similarly useful. A poultice of the leaves is used in ulcers infested with maggots. The young leaves are applied to bleeding piles. The flowers are used as a remedy or diabetes. The pulp of the seeds is applied in leprosy. The powdered seeds after decortication are given as a specific for whooping cough. The powdered seeds are also supposed to of value as a febrifuge and tonic in asthenic and debilitating conditions. The seed oil has antiseptic and stimulant healing properties and is applied to skin diseases, scabies, sores and herpes. In

addition byproduct of oil extraction which is the cake is evaluated as a source of plant nutrient for enhancing the productivity of rainfed systems.

6.4 Agro-practices

Sowing and Germination: *P. pinnata* is easily established by direct seeds or by planting nursery-raised seedlings or stump cuttings of 1-2 cm root-collar diameter (Duke, 1983; Kesari et al. 2008; 2009a, 2010; Mukta et al. 2010). The seeding season is April to June, and the seed yield per tree ranges from 10-250 kg per tree (NOVOD, 2009). Seeds, which require no treatment before sowing, remain viable for about a year when stored in air-tight containers. Seed germinates within two weeks of sowing and seedlings attain a height of 25-30 cm in their first growing season.

Transplantation: Transplanting to the field should occur at the beginning of the next rainy season when seedlings are 60 cm in height. Seedlings have large root systems. Soil should be retained around the roots during transplantaion. The spacing adopted in avenue plantings is about 8 m between plants. In block planting, the spacing can range from 2 x 2 to 5 x 5 m. *P. pinnata* seedlings withstand shade very well and can be interplanted in existing tree stands (NOVOD, 2008; 2009).

Management: *P. pinnata* should be grown in full sun or partial shade on well-drained soil. It has also been recognized to possess applications in agriculture and environmental management, with insecticidal and nematicidal activity (Chopade et al. 2008). A relatively low maintenance tree once established, is resistant to high winds and drought but is susceptible to freezing temperatures below 30 °F (Tomar & Gupta, 1985; Misra & Singh, 1987; Gilman & Watson, 1994). *P. pinnata* will show nutritional deficiencies if grown on soil with a pH > 7.5. Pests: No pests are of major concern, but caterpillars occasionally cause some defoliation. Diseases: No diseases are of major concern.

6.5 Results from the present investigation

In the present investigation, out of 50 tagged genotypes from North Guwahati, Assam, 10 CPTs were identified having high seed yield and oil content (Kesari et al. 2008). The studied germplasm showed variability for phenotypic traits (Kesari et al. 2008). There have been considerable progress in standardization of propagation techniques through conventional means using seed and stem cuttings and biotechnological tools, oil content and oil quality analysis for medicinal and fuel properties and screening of CPTs based on DNA fingerprinting studies (Kesari et al. 2009a,c). The crude oil from seeds were found to posses both antibacterial and antifungal activity against a range of pathogenic indicators (Kesari et al. 2009b). The tree being a legume has also importance in prevention of soil erosion, improvement and maintenance of soil fertility. The present thesis focused towards tree improvement of *P. pinnata* for developing high yielding varieties, germplasm enrichment identification of superior individuals and its value addition.

6.6 Impact

The local people at Rangia, Sila Forest Range, North Guwahati, Assam after half-day awareness program were impressed knowing the usage and economic value of plant in the current study. Interaction with them brought out some interesting facts to the understanding of our knowledge and belief. The participants were aware of the potential of the seeds of *P. pinnata* having fuel and medicinal properties. In addition, they have been using leaves and seed paste for the treatment of skin infection. This was an interesting piece of knowledge or information that was brought to our notice and attention. It is therefore no surprising that in our study crude oil from seeds showed positive response against the pathogen indicators (fungi, yeasts and bacteria) and confirms the medicinal importance of *P. pinnata*.

The important fact that they come to know from us is that planting of *P. pinnata* on their land helps to make their land more fertile as in local language this plant has fertilizer potential because of its nitrogen fixing capacity. Technology for rapid clonal propagation from the stem cuttings of CPTs transferred to Sila Forest office, Rangia Guwahati. Forest officials and local people decided to plant more of elite genotypes of *P. pinnata* in the unused lands, which would provide their livelihood security.

6.7 Conclusion of awareness program

Unless the value of plant genetic resources is known among local communities there may not be much interest in their sustainable maintenance and utilization. Therefore, value addition to a plant thereby strengthening the livelihoods of the local communities would be an effective method for promoting the large scale cultivation of *P. pinnata*. Awareness programme at Sila Forest, North Guwahati, was a step towards the sustainable management practices where in the results of the present investigations were explained to the people in the form of posters and informal presentation. The people actively participated in the discussions and they suggested similar studies in other unexploiated tree species such as *Mesua ferrea* which also grow naturally and profusely in North East. Innovative research on bio-diesel from *Pongamia* is not only ensuring energy, livelihood and food security to local villagers, but also reduces the use of fossil fuels, which in turn can help in mitigating climate change. Large scale cultivation of *P. pinnata* can bring the revenue to the villagers with small expenses as the plantation of this potential biodiesel tree require little water, can withstand stress and are inexpensive to cultivate. The unskilled labourers took care of the plants as their own and they were given the rights to harvest *Pongamia* trees. As a long term objective, Department of Biotechnology and Center for Energy, IIT Guwahati is making there efforts in contributing to the global biofuel revolution through its innovative research on non edible tree borne oil seeds in partnership with forest officials and local villagers without compromising on food security.

Summary

With depleting fossil fuel, soaring price hikes and adverse environmental impacts associated with the use of petro fuels, there is a renewed interest in alternative fuels from renewable resources. Plant sources for fuels that replace fossil fuels are a topical subject. As an anticipatory research to meet the global demand for diesel, genotypes with high oil content need to be selected so that a high seed yield gets translated into high oil production. *Pongamia pinnata*, oleaginous versatile nitrogen fixing tree species (NFTs) has primarily come to public attention because of the high oil content of the seeds. Although, these species are well adapted to harsh weather conditions, there is a need to domesticate them for cultivation under different production systems on degraded lands and community wastelands. In addition, information on seed characters such as seeds morphology, storage reserves and its composition is infancy. The research is being carried out in naturally occurring *Pongamia pinnata* (L.) Pierre from North Guwahati, Assam that covered a broad range of topics such as candidate plus trees (CPTs) identification based on morphometric markers, macro and micropropagation, genetic diversity studies, biochemical characterization of seed oil, seed developmental biology and root nodule symbiosis. The above works were achieved using tools of biotechnology by understanding the basic developmental and molecular aspects of *P. pinnata* that can be used to tease out for the traits of significance.

The work was carried out in six distinct phases; **first phase** starts with the collection of germplasm of naturally occurring *Pongamia pinnata* from North Guwahati, Assam and its evaluation for the identification of leading candidate plus trees (CPTs) based on morhometric traits and oil content. Owing to its huge market potential as a source of biodiesel, enormous planting material is required to raise *Pongamia* plantations. With this objective in mind propagation techniques (*in vitro* and *ex vitro*) were standardized and genetic stability of the micro-propagated plants was assessed through RAPD markers for the production of elite varieties of clones having high seed yield and high oil content throughout the year for afforestation programs and to raise its large scale cultivation in the wasteland or unproductive land (Kesari & Rangan 2007; Kesari et al.

2008; 2009a; 2010). **Second phase** involved standardization of new modified SDS method of DNA isolation protocol from seeds of CPT of *P. pinnata* (Kesari et al. 2009b) and teasing out genetic variability among CPTs using molecular markers (RAPD, SSR and AFLP). The knowledge on this type of variation would be useful for breeding purpose and genetic improvement especially in *Pongamia* were the desired ideo type is yet to be identified due to its wide adaptability and end use pattern. In the **third phase**, best reporting stable genotype was progressed further for oil extraction using different solvents (polar and non polar) and their azeotropic mixtures, quantification, analysis to compare the physico-chemical properties of the seed oil for its biodiesel aspect and to investigate the antimicrobial activity of the seed oil against referential bacterial and fungal strains (Kesari et al. 2009c). In the **fourth phase**, seed (collected at various stages of development) development were studied with respect to phenotypic traits, protein content, protein profile, localization of reserve materials using microscopic techniques (SEM and TEM) and transcript analysis of some of the fatty acid genes (FAD, SAD, oleosin protein) through semi-quantitative RT-PCR. In addition, comparisons were drawn for the SDS-PAGE profile of total soluble seed protein and localization of storage reserves during sexual seed maturation and germination stages. Data of SDS-PAGE were combined with those of microscopic techniques to acquire insight into the functions of the seed reserve. The findings of microscopic technique on oilseeds provides a tool for the identification of appropriate targets for biotechnological engineering of seed oils and lipid biosynthesis in *Pongamia* in future whereas expression analysis can be helpful in understanding the biosynthesis and in efforts to improve biosynthesis of TAGs (triacylglycerols) content in the mature seeds of potential biofuel crop like, *Pongamia*. The imminent study on seed biology of a potential unexplored biodiesel crop even becomes a timelier step in this global warming world providing new opportunities for comparative genomics and proteomics. **Fifth phase** involved isolation of *Rhizobium pongamiae* sp. nov. strain VKLR 01^T nodulating the roots of CPT of *P. pinnata* and its further characterization based on its phenotypic, biochemical, FAME and molecular studies (16S rDNA, G+C content and DNA-DNA hybridization). In the **sixth and final phase** an attempt was made to renew and revive the interest in local communities, holder

of traditional knowledge through means of awareness program on various applied aspects of *Pongamia* and its significance.

Such an in-depth analysis may establish a case study of genetic enhancement of biodiesel species *Pongamia* Genetic Resource Center (PGRC). The work has both theoretical and applied significance. Such studies will not only pave the way for designing a molecular breeding programme for oil traits in biofuel crops, and other such similar crops, but will also help to enhance our capacity to undertake precision breeding in biodiesel crops. Now *P. pinnata* is regarded as one of the most useful plants for legume study and researchers who have interests in biodiesel, nodulation and other aspects of legume biology use it worldwide. In long term, such a study will form the base for sustainable development in terms of techno-economics, environmental as well as socio-cultural considerations. The present research has been designed keeping in view the enormous economic, social and environmental benefits of *P. pinnata* due to its ingrained diverse applications and availability in study site, North Guwahati, Assam, India.

Future Scope

Theses are the potential works which can be explored on the basis of present work:

- Government of India has announced its plan of long term biodiesel production. So steps need to be announced regarding integrated cultivation and processing of *Pongamia* involving rural people in different districts.

- Development of SCAR markers linked for the oil traits in *Pongamia*.

- Investigation on the identity and function of over expressed seed storage proteins during late stage of seed maturation in *Pongamia*.

- The findings obtained in this study provide the basis for future investigation of *Pongamia* fatty acid genes in terms of regulation, expression pattern analysis, and evolution.

- cDNA library construction from seeds of identified and well characterized elite material of *P. pinnata* occurring in North Guwahati.

- Construction of gene for desired trait into cloning and mobilizing vectors.

- Nodulation diversity assay of novel *R. pongamiae* VKLR 01^{T}.

- Development of hairy root system in *Pongamia* to undertake functional genomic studies in root biology and nodule symbiosis.

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Publications

PUBLISHED (JOURNALS)

1. **Kesari V**, Vinod MS, Parida A, Rangan L. 2010. Molecular marker based characterization in candidate plus trees of *P. pinnata*, a potential biodiesel legume from North Guwahati, Assam. *Annals of Botany Plants* (In Press)
2. **Kesari V**, Rangan L. 2010. Development of *Pongamia pinnata* as an alternative biofuel crop- current status of plantations in India and scope. *Journal of Crop Science and Biotechnology* (In Press)
3. **Kesari V**, Das A, Rangan L. 2010. Effect of genotype and auxin treatments on rooting response in stem cuttings of CPTs of *Pongamia pinnata*, a potential biodiesel legume crop. *Current Science* 98(9): 1227-1230.
4. **Kesari V**, Das A, Rangan L. 2009. Physico-chemical characterization and microbial assay from seed oil of *Pongamia pinnata*, potential biofuel crop. *Biomass and Bioenergy* 33: 1724-1728.
5. **Kesari V**, Sudarshan M, Das A, Rangan L .2009. PCR amplification of the genomic DNA from the seeds of Ceylon Ironwood, *Jatropha*, and *Pongamia*. *Biomass Bioenergy* 33: 1116-1121.
6. **Kesari V**, Krishnamachari A, Rangan L. 2009. Effect of auxins on adventitious rooting from stem cuttings of candidate plus tree *Pongamia pinnata* (L.), a potential biodiesel plant. *Trees – Structure and Function* 23(3): 597-604.
7. **Kesari V**, Krishnamachari A, Rangan L. 2008. Systematic characterization and seed oil analysis in candidate plus trees of biodiesel plant, *Pongamia pinnata* (L.). *Annal of Applied Biology* 152: 357-404.

UNDER REVIEW

8. **Kesari V**, Vinod MS, Parida A and Rangan L. Genetic diversity analysis by RAPD markers in candidate plus trees of *Pongamia pinnata,* a promising source of bioenergy. *Biomass and Bioenergy*
9. **Kesari V**, Suman DK, Rangan L. *Rhizobium pongamiae* sp. nov., from root nodules of biodiesel crop (*Pongamia pinnata* L.). *International Journal of Systematic and Evolutionary Microbiology*

PUBLIC DOMAIN

1. **Kesari V**, Rangan L. 2009. LOCUS GQ444136 *Agrobacterium tumefaciens* strain VKLR 01 16S ribosomal RNA gene (1428 bp), TITLE Physiological and

phylogenetic characterization of *Rhizobium radiobacter* sp. nov. isolated from root nodules of a potential biodiesel crop - *Pongamia pinnata*

BOOK CHAPTER

5 **Kesari V**, Rangan L. 2007. *In vitro* propagation of potential biodiesel plant, *Pongamia pinnata* (L.) Pierre. *In: Biofuel plant cultivation, practices and seed bank* (S. K. Mehla ed.), ISBN 81-7132-514-6 (Pointer publishers), pp. 112-117

CONFERENCE ATTENDED

NATIONAL

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