Multiparametric investigations in socioeconomic non-edible oil crops

A thesis submitted by

RESHMI DAS

For the award of degree of

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Dedicated to my Parents and Family

INDIAN INSTITUTE OF TECHNOLOGY

DEPARTMENT OF BIOSCIENCES AND BIOENGINEERING

STATEMENT

I do here by declare that the research embodied in this thesis entitled **"Multiparametric investigations in socioeconomic non-edible oil crops"** is the result of experiments carried out in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India, under the guidance of Prof. 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 research.

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CERTIFICATE

It is certified that the work described in this thesis entitled **"Multiparametric investigations in socioeconomic non-edible oil crops"** by Reshmi Das (Roll No. 136106004) 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 Biosciences and Bioengineering, IITG. The work embodied in this thesis has not been submitted elsewhere for a degree.

November, 2018

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Reshmi Das

CONTENT

ABBREVIATIONS

LTR	Long Terminal Repeats
DNA	Deoxyribonucleic Acid
Zn	Zinc
Cd	Cadmium
Pb	Lead
Cu	Copper
Ni	Nickel
As	Arsenic
Cr	Chromium
Ag	Silver
N	Nitrogen
P	Phosphorus
K	Potassium
Ca	Calcium
Fe	Iron
Mn	Manganese
MTCC	The Microbial Type Culture Collection and Gene Bank
RNA	Ribonucleic Acid
TDZ	Thidiazuron
ISSR	Inter Simple Sequence Repeat
RAPD	Random Amplified Polymorphic DNA
AFLP	Amplified Fragment Length Polymorphism
SSR	Simple Sequence Repeat
WPM	Woody Plant Medium
MS	Murashige and Skoog medium
PGR	Plant Growth Regulators
cDNA	Complementary DNA
UV light	Ultra Violet light
PI	Propidium Iodide
rDNA	Ribosomal DNA
LMA	Leaf Mass per unit Area

TEs	Transposable Elements
ABA	Abscisic Acid
ROS	Reactive Oxygen Species
mRNA	Messenger RNA
RFO	Raffinose Family Oligosaccharides
ACC	1-aminocyclopropane 1-carboxylic acid
NGPP46	North Guwahati *Pongamia pinnata*
IITG	Indian Institute of Technology Guwahati
IGEPAL	Octylphenoxypolyethoxyethanol
RNase	Ribonuclease
DNase	Deoxyribonuclease
FACS	Fluorescence-Activated Cell Sorting
BD	Beckman Coulter
CV	Coefficient of Variation
MFI	Mean Fluorescence Intensity
FSC	Forward Scatter
SSC	Side Scatter
FITC	Fluorescein Isothiocyanate
ANOVA	Analysis of Variance
SPSS	Statistical Package for the Social Sciences
HCl	Hydrochloric acid
PVP40	Polyvinylpyrrolidone
FCM	Flow Cytometry
PDB	Paradichlorobenzene
OQ	8-hydroxyquinoline
MBN	1-bromonaphthalene
EtOH	Ethanol
DPX	Dibutylphthalate Polystyrene Xylene
CO_2	Carbon dioxide
RT	Room Temperature
RT	Reverse Transcriptase
RT-RH	Reverse Transcriptase-RNase H
dNTP	Deoxyribonucleotide triphosphate

PCR	Polymerase Chain Reaction
OD	Optical Density
$CaCl_2$	Calcium chloride
LB medium	Luria-Bertani medium
EDTA	Disodium Ethylene Diamine Tetra Acetate Dehydrate
NaOH	Sodium hydroxide
BLASTN	Basic Local Alignment Search Tool for Nucleotide
MUSCLE	Multiple Sequence Comparison by Log-Expectation
NCBI	National Center for Biotechnology Information
AT-GC	Adenine Thymine Guanine Cytosene
ORF	Open Reading Frame
MSA	Multiple Sequence Alignment
NJ method	Neighbor Joining method
PPTY	*Pongamia pinnata* Ty1-*copia* clones
JCTY	*Jatropha curcas* Ty1-*copia* clones
RCTY	*Ricinus communis* Ty1-*copia* clones
MFTY	*Mesua ferrea* Ty1-*copia* clones
RGR	Relative Growth Rate
RWC	Relative Water Content
BOD	Biological Oxygen Demand
Wf	Fresh weight
Wd	Dry weight
Wt	Turgid weight
$NaNO_2$	Sodium nitrite
$AlCl_3$	Aluminium chloride
$FeCl_3$	Ferric chloride
NDF	Neutral Detergent Fiber
ADF	Acid Detergent Fiber
CTAB	Cetyltrimethyl Ammonium Bromide
H_2SO_4	Sulfuric acid
DCFH-DA	Dichloro-dihydro-fluorescein diacetate

UNITS

cm	Centi meters
℃	Degree Centigrade
h	Hours
Kb	Kilo Base
m	Meter
M	Molar
Mbp	Mega base pairs
mg	Milligram
min	Minutes
mg/ml	Milligram per millilitre
mL	Millilitres
mM	Millimolar
ppm	Parts per million
pg	Picogram
g	Grams
w/v	Weight/Volume
v/v	Volume/Volume
wt	Weight
µg	Micrograms
µM	Micro Meter
µg/µl	Microgram per microlitre
µg/ml	Microgram per millilitre
µl	Microlitre

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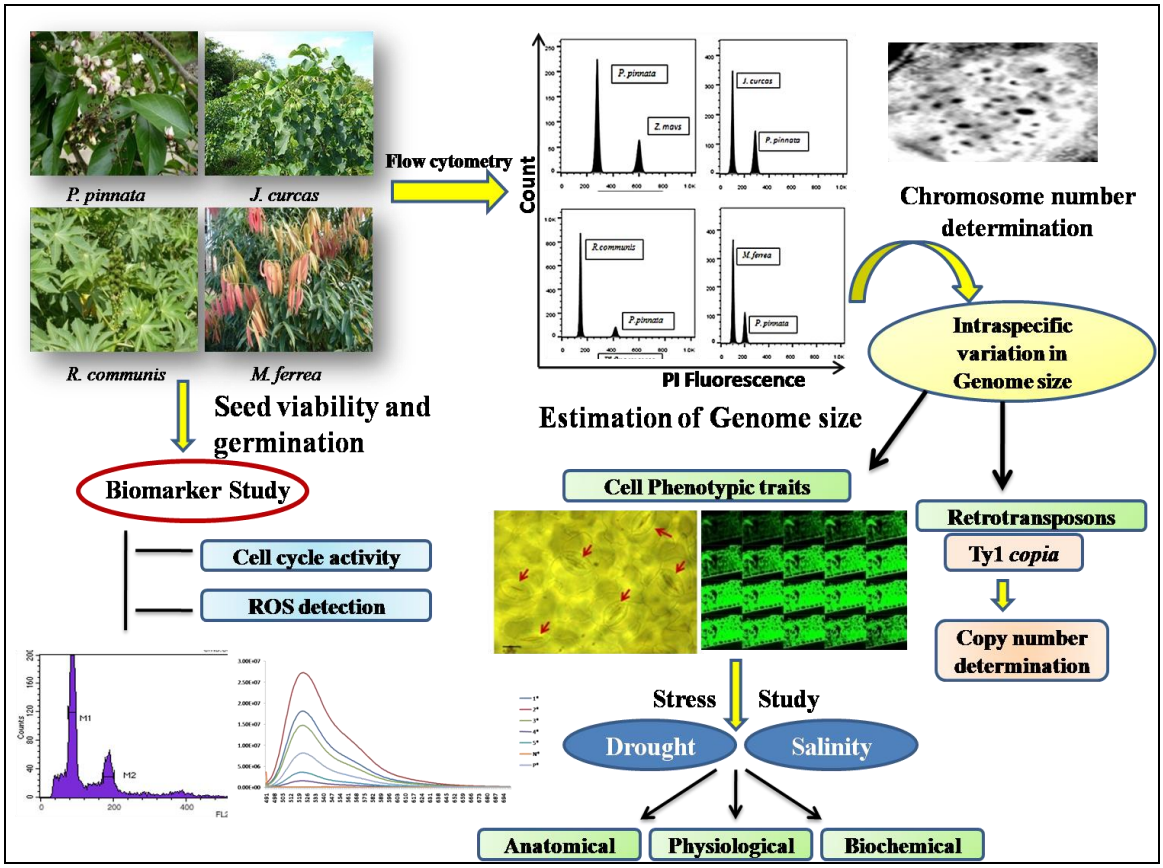
GRAPHICAL ABSTRACT



ABSTRACT

Many plants have been identified as potential biofuel crops to meet the increased demand for energy. As researchers attempt to exploit and improve the traits of significance, genome and phenotypic information becomes critical. In this study, flow cytometric investigation has been done to estimate the genome size of four socio-economic non-edible oil crops viz. *Pongamia pinnata*, *Jatropha curcas*, *Ricinus communis*, and *Mesua ferrea*. The genome size / 2C nuclear DNA content were in the following order: *J. curcas* (0.86 pg/2C DNA) < *R. communis* (1.01 pg/2C DNA) < *M. ferrea* (1.52 pg/2C DNA) < *P. pinnata* (2.49 pg/2C DNA). Intra-specific variations were observed in genome size whereas no such variations in the chromosome number were detected in plants collected from different eco-geographical regions of Assam. This is attributed to reverse *transcriptase-RNase H* (RT-RH) domains of Ty1-*copia* retrotransposons. Dot-blot analysis revealed that the Ty1-*copia* accounts for 2 % to 9.5 % of the total haploid nuclear genome for the studied plants and phylogenetic analysis showed that RT-RH sequences are heterogeneous that resolved into distinct groups. The results contribute to preliminary understanding about genome organization and evolution. Genome size is also known to affect various plant cellular traits, thus, the correlation was drawn by studying cell phenotypic characters as well. The correlation was, however, found to be growth form dependent. Genome size has been found to be influenced by environmental factors; hence, anatomical changes in leaves of *Pongamia, Jatropha, Ricinus and Mesua* were studied under drought stress and salinity stress and their significance was evaluated by numerical analysis. Some anatomical features related to leaf viz. stomatal length, pore size and average number of stomata and epidermal cell area were found to be important characters that varied in plants under stress and were statistically significant. Relative water content, root: shoot ratio, total chlorophyll content, and relative growth rate were measured and values were found to be lower in stressed plants compared to the control. The phytochemical study was carried out which concluded that although plants have exercised anatomical and physiological changes due to stress, there is no weighing down in biochemical properties of these versatile plants. Therefore studied plants are idle candidates to be grown under abiotic stress condition without much productive loss. Since seeds of these plants are the source for biodiesel production thus; seed loss during germination will hamper the economic sector. Biomarker using cell cycle activity and reactive oxygen species (ROS) accumulation intensity has been studied which not only will help to identify the seed germinating stage but also facilitate a better understanding of seed priming treatments.

Chapter 1

INTRODUCTION

This chapter gives a brief introduction of the present research, background, and motivation for the present study, highlighting the need for biofuel and the detailed significance of the four non-edible oil crops.

1. INTRODUCTION

Concerns of global warming in combination with the increased demand for fossil fuel supplies have spurred growing interest in renewable energy sources. Biofuel serve as the prospective renewable energy source that can mitigate the use of fossil fuel. The long standing pursuit for sustainable fuel has inspired the quest for biofuel crops. Some plants that can serve as the potential candidates to produce biofuel efficiently and economically, with minimal impact on food crops are *Pongamia pinnata*, *Jatropha curcas*, *Mesua ferrea*, and *Ricinus communis*. A lot of investigation on these plants has been carried out in various fields such as, oil characterization, phytochemistry, pharmacology, antimicrobial study, molecular diversity study and molecular marker study. (Scott et al. 2008; Kesari et al. 2008; Kesari et al. 2010; Naz et al. 2012; Pandey et al. 2012; Rao et al. 2012; Chahar et al. 2013; Muqarrabun et al. 2013; Aslam et al. 2014; Kanti et al. 2015; Aziz et al. 2016; Izaj et al. 2016; Peixoto et al. 2016; Rampadarath et al. 2016). As these plants have the potential for sustainable biodiesel production and the fact that they are able to adapt to a wide range of ecological conditions, suggest a considerable existence of genetic variability. Thus, to achieve full genetic characterization, a detailed study of their genome size is necessary. Genome information on biofuel crops can be helpful to search a potent trait in this field, helping the researchers in their attempt to exploit and improve the significant traits. The genome size information will be vital in providing information regarding genome organization and structure, understanding the evolution and conservation of the species, in-plant sequencing projects, plant breeding programs and also in exploring molecular markers. Unlike traditional methods and techniques such as, feulgen densitometry, flow cytometry has evolved as a method of choice to measure the nuclear DNA content in a cost and time effective way (Guo et al. 2015). In the earlier reports, with the estimation of genome size, both intra and interspecies variations have been reported (Zedek et al. 2010). Polyploidization was found to be one of the causes of genome variation, which occurs either due to chromosome doubling within a species or due to hybridization, followed by chromosome doubling. In the true diploid species, the variation in genome size exists either because of the repetitive elements or due to the environmental factors. The repetitive DNA content occupies a large portion of the nuclear genome, leading to variation in the genome size in different species thus, playing an

important role in the genome evolution. Genome size in plants differs as a result of variable amount of repetitive DNA, also known as transposons or mobile elements (Bennett and Leitch 2011). Among the transposon population, retrotransposons play a major role in affecting the genome size differences. The most abundant group of transposons are the long terminal repeat (LTR) retrotransposons, namely, Ty1-*copia* and Ty3-*gypsy* superfamilies, which have become exceptionally rich in plant genome over time (Zedek et al. 2010). Within a genome, these LTR retrotransposons occupy a large area by copying themselves through a process of replicative transposition and thus, increase the genome size in the host, unlike the DNA-mediated transposons which follow cut and paste mechanism (Cabanas and Izquierdo 2007). Till date, Ty1-*copia* retrotransposons have been studied and characterized widely in plants (Cheng et al. 2009). Ty1-*copia* group retrotransposons were reported to be present in maximum of the plant genomes with high copy number (Chen et al. 2012). Thus, calculating the copy number of Ty1-*copia* and studying its probable role in the genetic diversity will contribute enormously to the further understanding of retrotransposons in the genomic study. Additionally, environmental factors also affect genome size, which in turn affects the plant cellular traits. Thus, the knowledge of genome content and cell phenotypic characters makes it possible to infer the relationship between them. It has been hypothesized that phenotypic traits may be the primary determinant of genome size as their size affects functional efficiency in plants. Earlier studies on angiosperm have indicated that 2C DNA content has various correlations at the cellular level. Among the cellular level parameters, the stomatal traits along with the epidermal cell area and cell volume play a direct role in the plant growth and production. The genome size was positively correlated with the stomatal length and the epidermal cell area, but negatively with the stomatal density (Beaulieu et al. 2007; Hodgson et al. 2010).

Among the environmental stress factors, salinity and drought have the most adverse effect on plant productivity (Buchbauer and Baser 2010; Stanev 2010). In addition, alterations in the phenotype, leaf morphology and reduction in stomatal traits have also been reported due to climatic variation (Fraser et al. 2008). Tissue response of the plant to stress depends on their anatomical characteristics, which regulate the transmission of water to the cells. Thus, anatomical changes are good indicators of plants undergoing stress (Makbul et al. 2011). The anatomical and physiological changes in the plants occur

in order to protect the species during stress but, such changes also affect the yield of the plants. Thus, the objective in this thesis is to examine the effects of the abiotic stresses on several anatomical, physiological and biochemical parameters. This study is essential for a clear perception of the plant resistance mechanism to stress conditions. Since the plants under study have versatile importance, especially as biofuel energy crops, they are needed to be grown in a large scale for higher yield. Seeds are the protagonist for growing crops which comprises of germination and vigor and are essential for the successful establishment of the plant. Since seeds of the plants under study are having high economic value, seed loss during germination is not affordable. Thus, it is the need of the hour to identify a biomarker which can assist in identifying an ideal harvest time. Several promising markers have been reported so far for monitoring seed quality process (Kumar et al. 2016). Thus, for the four biofuel plants, identifying a biomarker for seed germination will be beneficial to decrease seed loss during sowing.

P. pinnata, belonging to Fabaceae family, is commonly known as Indian beech (English), Karanj (Hindi), Karchaw (Assamese), etc. The natural distribution of this species occurs along the coasts and river banks in India and Burma. It can be grown in non-arable lands and thus, it is considered as a sustainable biofuel source (Ramesh et al. 2014). It is a versatile oleaginous tree having multiple applications in the field of biofuel production and for the treatment of tumors, piles, skin diseases, rheumatic joint pain, etc. (Meera et al. 2003). Its application can be extended as animal fodder, timber and fish poison (Bottoms 2000). The non-edible oil extracted from *Pongamia* seeds are used as fuel, lubricant and in soap making.

J. curcas and *R. communis* belong to Euphorbiaceae family and are cultivated in the sub-tropical and tropical regions around the world. Common names of *Jatropha* include Barbados nut, Purging nut, Physic nut. It is a poisonous, semi-evergreen shrub or small tree, reaching upto a height of around 6 m (20 ft) (Nahar et al. 2011). The seeds contain oil that can be processed to produce a high-quality biodiesel fuel. The seeds are also a source of a highly poisonous compound, jatrophin (Achten et al. 2007). The seeds mature when the capsule changes from green to yellow, that contain around 20% saturated fatty acids and 80% unsaturated fatty acids (Achten et al. 2007). It has several uses not only in the field of biofuel but also in carbon dioxide sequestration; seed ashes are used as a salt substitute in the process. In the Indian system of medicine, *R. communis*

is given much importance. It is commonly known as Castor, Eri, and Ricin etc. The leaf, root and seed oil of *Ricinus* are used for the treatment of the inflammation and liver disorders, hypoglycemia, as laxative etc. (Joshi et al. 2004). Apart from its role as biofuel, leaves of this plant are used to feed and rear the larvae of muga and endi silkworms. Castor oil is an effective motor lubricant and has been used in internal combustion engines. It does not mix with petroleum products. However, it has been largely replaced by synthetic oils that are more stable and less toxic. Jewelry is often made of castor beans, particularly necklaces and bracelets, due to their ornamental appeal.

M. ferrea commonly known as Ceylon ironwood, Indian rose chestnut, or Cobra's saffron, is a species in the family Calophyllaceae. It is also known as Nag champa (Hindi), Nahor (Assamese), Nagesar (Bengali) and Naka (Tamil). This slow-growing tree is named after the heaviness and hardness of its timber. It is widely cultivated as an ornamental tree due to its beautiful shape, grayish-green foliage with a beautiful pink to the red flush of drooping young leaves, and large, fragrant white flowers. It is native to wet and tropical parts of Sri Lanka, India, southern Nepal, Burma, Thailand, Indo-China, Philippines, Malaysia, and Sumatra, where it grows in evergreen forests, especially in river valleys (Chahar et al. 2012). The flowers, leaves, seeds, and roots are used as herbal medicines in India, Malaysia, etc. (Chanda et al. 2013). As the seeds produce substantial oil, they were also used as household lighting source before the introduction of kerosene by the British in the North-eastern state of India, Assam (Basumatary 2013).

P. pinnata, J. curcas, R. communis, and *M. ferrea* are the major biofuel crops that grow abundantly in the North-eastern region of India. As they grow in non-arable lands and produce high oil-yielding seeds, they are considered as important pioneers of the second-generation sustainable biofuel crops. Another important attribute is their non-edible property. These four crops have been, considered as a good source for biodiesel production (Keseri et al. 2008; Achten et al. 2007; Saribiyiket al. 2010; Bora et.al. 2013). Other than the oil sector, much research has also been conducted on their environmental aspects, industrial applications, plant developmental and pharmacological aspects. However, no information related to genomic and cytogenetic study on these four plants is available till date. Cell phenotypic and genomic study with respect to environmental variance is critical. Also study on repeat elements and plant resistance to abiotic stress is

limited. Thus, based on the background information, the current study was focused on the following objectives.

Specific Objectives

1.4.1 Genome size estimation and cytological analysis in non-edible oil crops

1.4.2 Genome size correlation with cell phenotypic traits and mobile element

1.4.3 Stress evaluation in non-edible oil crops - anatomical, physiological and phytochemical approaches

1.4.4 Study of biomarkers for seed germination in non-edible oil crops

Chapter 2

REVIEW LITERATURE

This chapter includes a detailed review of the literature on the current status of non-edible oil crops. The review further illustrates the importance of genome size and its correlation with retroelements and phenotypic traits. Also, the review focuses on biomarker study for seed germination and viability.

2. REVIEW OF LITERATURE

2.1 INTRODUCTION

Sustainable energy or power is required to satiate the economic and technological development of a country. However, energy like biofuel, derived from renewable sources offer sustainable solution to counteract the imminent environmental hazards associated with the usages of fossil fuel reserves. Biofuel is produced through contemporary biological processes (anaerobic digestion and agriculture), unlike fossil fuel which is produced by geological processes. According to the biofuel production sources, biofuel is divided into four categories or generations based on their sources. First generation biofuels are produced from the food crops that are grown in arable lands (Naik et al., 2010). Second generation source stems from the non-edible oil crops, woody plants, lignocellulosic biomass, agricultural residues and waste plant materials (Dutta et al., 2014; Pandey et al., 2012). The third generation is also known as Algae fuel as their source is algae which yields more than 50% oil (Wagner, 2007). Fourth generation biofuel includes electrofuels and photobiological solar fuels (Aro, 2016). Generally, biofuels are derived directly from energy crops. Biofuels are produced by the chemical process known as transesterification, where alcohol is added to the extracted plant seed oil (Ho et al., 2014). Previously, soybean, rapeseed, palm oil and other cooking oils were used as feedstock for biofuel production. But at present, energy crops are grown specifically for the production of fuel which includes *Miscanthus*, Reed canary, Switch grass, Willows, Poplar, *Jatropha*, Castor, *Pongamia*, *Mesua*, Alfalfa, Napier grass and Johnson grass, etc**.** (Ho et al., 2014).

The sources of the second-generation biofuels are grown in non-arable lands unlike the first generation sources and thus, are safe from the perspective of food and agriculture. Many crops have been exploited so far and among them, *P. pinnata, J. curcas, R.communis*, and *M. ferrea* are the major biofuel crops abundantly found in the North-eastern region of India (Achten et al., 2007; Aydin et al., 2010; Bora et al., 2014; Kesari et al., 2008).

With advantages of being drought and varying environmental stress tolerant, as well as possessing high oil-yielding seeds, they are considered as pioneers of the second

generation sustainable biofuel crops. As these plants have a high socio-economic value, extensive research has been concentrated on these four plants.

2.2 Source of biofuel production

With the increasing need for petroleum products coupled with its diminishing availability, need for an alternative source of energy has become utmost importance. Currently, *Pongamia* is considered as an important tree species which can serve as a potential source for biodiesel production (Gresshoff et al., 2017; Kesari et al., 2008). Due to its high seed oil content (40%), it has the potential to provide an eco-friendly fuel, which can reduce the current diesel engine emissions. *Jatropha* oil is also considered to be a prospective feedstock for biodiesel production; yield 25 - 40% oil by weight (Pandey et al., 2012). Experiments have proven that upon treatment with methanol and using sulphuric acid as catalyst, the high free fatty acid content (14%) of *Jatropha* oil can be lowered below1%. This process yields biodiesel above 99%, which fulfills the American and European standards (Tiwari et al., 2007). Concerning sulfur content, the biodiesel produced from *Jatropha* oil was found to be superior to conventional diesel, with a higher density, lower cetane number and boiling point as compared to diesel (Barua, 2011). *Ricinus*, with its high oil-yielding seeds (30 - 50%), is also there in the list of biofuel crops (Ijaz et al., 2016). Reports suggest that the seeds of *Mesua ferrea* contain a large amount of oil (70-75%) and are processed to produce biodiesel. Their high oil content (70%), contains both saturated and unsaturated long-chain fatty acids (Konwar & Karak, 2011) which can serve as a better substitute for diesel derived from fossil fuel. As the biodiesel from the plants mentioned above can also reduce smoke opacity, biodiesel produced from such non-edible sources can serve as adequate substitutes for conventional fuels.

2.3 Environmental aspects

Several reports are available concerning environmental applications of these plants. *Pongamia* has been reported to be responsible for the accumulation and significant decrease in the concentrations of heavy metals in soil (Adithya et al. 2015; Pei et al., 2015; Sarma et al., 2005). *Jatropha* also provides an eco-friendly and cost-effective method to remediate polluted soil (Pandey et al. 2012). A study by Moursy suggests that *Jatropha* can remove Zn, Pb, Cd, and Cu effectively from 100% sewage sludge (Moursy

et al., 2014). *Ricinus* has been reported to have an exceptional ability to extract the majority of toxic metals like Cd, Pb, Ni, As, and Cu as well as some organic contaminants like pesticides (Bauddh et al., 2015). Additionally, *Ricinus* shows phytostabilization potential in fly ash polluted sites, thus limiting metal toxicity (Pandey, 2013). A study suggests that *Mesua* also can phytostabilise Cr in contaminated mining soil due to its high potential for bioaccumulation and subsequent translocation (Panda et al., 2016).

2.4 Industrial applications

The compost of *P. pinnata* is reported to improve the availability of macronutrients (N, P, K and Ca) and micronutrients (Fe, Mn, and Cu) and thus, help to increase the yield and the protein content of crops (Chaturvedi, 2011; Chaturvedi et al., 2009). The leaf extract is reported to have good sun-protecting activity; thus it is used in sunscreen based cosmetics (Patil et al., 2015). Also, Ag nanoparticles prepared from the fresh bark of *Pongamia* was found to have excellent antibacterial activity against Gram-positive and Gram-negative pathogens (Rajkumar et al., 2012). The organic manure obtained from *Jatropha* is utilized to remediate acidic soil (Sharma & Pandey, 2009). The biomass of *Jatropha* hulls can be used for the production of bioactive compost. The ash obtained upon burning of the fruit and seed cakes are used as soil fertilizer (Kumar & Sharma, 2008; Openshaw, 2000). The oil and aqueous extract from *Jatropha* have been used to control insect pests (Kaushik & Kumar, 2005). Charcoal is prepared from the pressed cakes which also serve as a valuable fertilizer to increase crop production (Kumar & Sharma, 2008). Even the terpenoids isolated from the plant are tremendously used in perfume and other cosmetic industries (Warra, 2012). Also, the high oil content has been utilized for the preparation of detergents, surfactants, and soaps, etc. on a commercial basis (Akbar et al., 2009; Openshaw, 2000). *Ricinus* also has multiple industrial applications; the oil has been used as starting material for making many industrial chemical products such as paints, inks, lubricants, coatings, etc. The oil extracted from seeds is used in cosmetics, as an antibacterial agent, etc. Moreover, the oil has been commercialized for hair growth treatment (Ogunniyi, 2006). The castor cake is also used as fertilizer in large-scale farming. Rhamnolipid, produced by *Pseudomonas aeruginosa*

(MTCC 7815) using *Mesua ferrea* seed oil, has high pharmaceutical-cosmetics values (Singh et al., 2013).

2.5 Plant development aspects

The cultivation of *P. pinnata* through seed is difficult due to low germination and high seedling mortality issues. Sujatha and Hazra have reported the dual role of Thidiazuron (TDZ) in the micropropagation of *Pongamia* from mature-tree-derived axillary meristems (Sujatha & Hazra, 2007). Besides, *in vitro* regeneration system for *Pongamia* has been successfully established from cotyledonary explant (Shrivastava et al., 2010). Nagar et al. developed the reproducible protocol using hypocotyl segments as explant, and multiple shoots were induced through direct adventitious shoot bud regeneration (Nagar et al., 2015). Moreover, studies on molecular marker technologies suggest clear and more direct information of the genetic polymorphisms, distinguishing the accessions of *Pongamia* (Biswas et al., 2011). *Jatropha*, due to its immense potential as a biodiesel plant, has drawn the attention of plant biotechnologists in recent times. Numerous protocols have been designed for the micropropagation of *Jatropha* through embryo, axillary shoot buds or nodal buds for generating clones, with a promising method for disease-free and healthy plantation (Mve et al., 2013; Purkayastha et al., 2010; Rathore et al., 2015). However, in recent years, organogenesis from hypocotyl segments, young and mature leaves have been accomplished in elite *Jatropha* plants for commercial plantation (Deore & Johnson, 2008; Gangwar et al., 2016). Genetic transformation through the use of RNA mediated gene silencing has been successfully employed for reduction or suppression of curcin gene transcript expression (Gu et al., 2015; Patade et al., 2014; Zong et al., 2010). Studies have demonstrated the effective use of Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) along with Simple Sequence Repeat (SSR), which can be exploited as a useful technique to differentiate between the varieties of *J. curcas* and to identify polymorphic markers (Pamidimarri et al., 2009). In *Ricinus*, the genetic improvement of the plant for yield and yield contributing traits was achieved through mutation breeding (Kallamadi et al., 2015). By recurrent selection, a genotype with the 10-fold reduction in ricin was developed (Auld et al. 2003; Kallamadi et al., 2015). Study of different molecular markers like AFLP, SSR, RAPD, and ISSR was used for estimating the extent of diversity among castor germplasm accession (Gajera et al.,

2010; Pecina et al., 2013; Vasconcelos et al., 2012; Vivodík et al., 2014). Saini et. al discovered that the leaf and nodal segment of *Mesua* are active explants for micro-propagation which showed maximum growth of callus in Murashige and Skoog medium (MS) as well as in woody plant medium (WPM) with different concentration of Plant Growth Regulators (PGRs) (Saini et al., 2014). Also, Jadhav and Deodhar standardized the protocol for micropropagation of *Mesua* through use of immature seed as explants (Jadhav & Deodhar, 2015).

2.6 Pharmacological aspect

Pongamia, *Jatropha*, *Ricinus*, and *Mesua* are also well known for their medicinal properties, besides being potent sources of biofuel. **Table 2.1** depicts the bioactivities of various parts of these plants.

Table 2.1: Bioactivity of different parts of *Pongamia pinnata*, *Jatropha curcas*, *Ricinus communis* and *Mesua ferrea*

	P. pinnata	***J. curcas***	***R. communis***	***M. ferrea***
leaf	• Antiviral (Rameshthangam & Ramasamy, 2007) • Anti-parasite (Singh et al., 2012) • Cytotoxicity (George et al., 2010) • Anti-ulcer (Giri et al., 2010) • Anti-inflammatory (Srinivasan et al., 2001)	• Larvicidal activity (Gutierrez et al., 2014) • Antibacterial (Omoregie & Folashade, 2013) • Antioxidant (Fu et al., 2014) • Antidiabetic (El-Baz et al., 2015) • Analgesic (Uche & Aprioku, 2008) • Anti-ulcer (Kannappan et al., 2008)	• Antioxidant (Jena & Gupta, 2012) • Boil, sores, and swellings (Rana et al., 2012) • Hepatoprotecti-ve activity (Jena & Gupta, 2012)	• Antibacterial (Mazumder et al., 2004) • Analgesic (Hassan et al., 2006) • Anti-venom (Chahar et al., 2013)

	P. pinnata	*J. curcas*	*R. communis*	*M. ferrea*
Stem/ bark	• Anti-inflammatory (Badole et al., 2012) • Anti-diabetic (Badole & Bodhankar, 2010) • Anti-pyretic (Kalidhar, 2002)	• Larvicidal activity (Gutierrez et al., 2014) • Antibacterial (Omoregie & Folashade, 2013) • Antidiarrhoeal (Sachdeva et al., 2012)	Analgesic activity (Rajeshkumar et al., 2013)	• Antibacterial (Keawsa-ard & Kongtaweelert, 2012) • Anticancer (Keawsa-ard & Kongtaweelert, 2012)
Seed	• Antioxidant (Vadivel & Biesalski, 2011) • Antibacterial (Kesari et al., 2010) • Antifungal (Kesari et al. 2010) • Anti-inflammatory (Prabha et al., 2009)	• Antibacterial (Devappa et al., 2012) • Antifungal (Shehab et al. 2013) • Wound healing (Sachdeva et al., 2011) • Antidiabetic (Patil et al., 2011) • Antioxidant (Boudjeko et al., 2013)	• Hair growth (Ross 2003) • Sexual disease (Ross, 2003) • Abortifacient affect (Nath et al., 2011)	• Anti-arthritis (Asif et al., 2017) • Antibacterial and antifungal (Parekh, 2007) • Anti-inflammatory (Garg et al., 2009) • Antioxidant (Rajesh et al. 2013)
Root	• Antioxidant and anti-inflammatory (Al et al., 2013) • Antibacterial (Khan et al. 2006)	• Antidiarrhoeal (Mujumdar et al., 2001) • Antioxidant (El et al., 2009)	• Abdominal disorder (Ross 2003) • Lumbago (Rana et al., 2012)	• Anti-inflammatory (Gupta et al., 2014)

Thus, it has been found that much research has been conducted concerning these four plants in various sectors including plant development, phytochemical and pharmacological study, etc. Literature study reveals that a lot of research, related to genome size estimation and chromosomal study has been conducted in many other plants. Moreover, researchers had focused on investigating the relationship between genome size

and transposons. Phenotypic traits like cell size and stomatal size were being studied intensely to draw some correlation with genome size variation. Study on plants undergoing abiotic stress has also got importance as abiotic stress affects the growth and yield of plants. Recent reports are available on seed markers for assessment of the quality and germination stage. These study and information thus available are important for understanding the proper growth and yield of the plants.

2.7 Nuclear genome content

Genome size is the total amount of DNA contained within one copy of a single genome. It is generally measured in picograms or as the total number of nucleotide base pairs, i.e., in megabases. One picogram equals 978 megabases. Commonly in diploid organism genome size is also referred to as C-value. It has been found that the organism's complexity is not proportional to the genome size as some single cell organisms were found to have more DNA than some of the multicellular organisms. Genome size plays a vital role in many genetic and molecular biological studies (Bennett & Leitch, 2005). Knowledge of the haploid nuclear DNA content or genome size is vital for both basic and applied studies involving genome organization, species relationships, gene expression analysis and germplasm improvement (Greilhuber et al., 2005). DNA C-value is a fundamental parameter in the study of biology and biodiversity as well. Genome size estimates are also crucial for constructing and screening genomic and cDNA libraries (Bennett et al., 2000). It is also necessary for developing linkage maps for genetic analysis, breeding purposes, in evaluating reproductive and somatic compatibility. Genome size data is also useful in various fields of plant biology, including systematics, evolution, and conservation (Bennett & Leitch, 2005). *Arabidopsis thaliana* was the first plant chosen for genome sequencing (Initiative, 2000). Bennett & Smith (1991) and Bennett & Leitch (1997) compiled estimates for the nuclear DNA amounts of more than 1000 angiosperm species. Ohri and Kumar determined the genome size of ten different legume plants by flow cytometry; nuclear DNA content was estimated to be 2C=3.60 pg in *Pongamia glabra* and 2C=4.0 pg in *Millettia ovalifolia* (Ohri & Kumar, 1986). In legumes, the 2C nuclear DNA value was reported to be in the range from 1.68 to 4.60 pg. Ramesh et al. developed Flow cytometric protocol and estimated genome size of *P. pinnata* to be 2C=2.66 pg (Ramesh et al., 2014). Later, Choudhury et al. estimated the

genome size of *in vitro*-regenerated *P. pinnata* with the help of flow cytometric analysis and reported 2C=2.51pg (Choudhury et al., 2014). For *J. curcas* flow cytometry indicates an average 2C value of 0.85 pg (Carvalho et al., 2008). The draft genome sequence of the oilseed species *R. communis* and flow cytometric estimation of genome size was found to be 1.04 pg (Chan et al., 2010; Houben et al., 2003). Also, flow cytometric nuclear DNA amounts for many plant species belonging to different families have been reported in Kew Royal botanical garden. The range of genome size in plants varies from 0.065 pg to 127.4 pg. Genome size variation plays a significant role at cellular, tissue and organismal levels. It also influences the phonological and ecological behavior of organisms.

For the determination of the 2C nuclear DNA content, the following approaches have been reported so far:

1. The measurement of individual nuclei.
2. The analysis of DNA extracted from a large number of cells.

Measurement of individual nuclei, is technically more demanding, but it offers much higher precision (Dolezel & Bartos, 2005). Chemical analysis and reassociation kinetics are examples of the second approach. It is possible that the sample may contain cells at different phases of the cell cycle, having different DNA amounts. Thus chemical analysis does not estimate the accurate 2C nuclear DNA amount. Also, after the reassociation kinetic study, the *Cot* curve obtained is hard to interpret regarding C- values because of the presence of repetitive elements present in the sequences. Earlier, measurements of individual nuclei were dependent on the absorption of UV light by the DNA molecule. Later, the nuclei were stained with the help of Feulgen method, which was considered specific for DNA (Feulgren & Rossenbeck, 1924), and the quantification of the absorbed visible monochromatic light was done (Swift, 1950). To eliminate the errors caused due to irregularly shaped nuclei, micro-spectrophotometry was developed (Deeley, 1955) followed by DNA image cytometry (Vilhar et al., 2001). Due to inherent limitations of the above techniques concerning time, expense and sample size, a new technique called flow cytometry was developed for analyzing microscopic particles in fluid suspension. Samples are passed through a fluid stream through the focus of intense light. Optical sensors convert the collected fluorescence scattered light into electric current, and the particles are analyzed individually at high speed. Thus, large populations can be measured in a short time, and the presence of subpopulations is also detected. Due to the ease of

sample preparation and the ability to estimate DNA in large populations, flow cytometry has been considered nowadays in the alternative to micro-spectrophotometry.

Flow cytometry provides an accurate and simple means to determine nuclear DNA contents within plant homogenates (Galbraith, 2009). Buffer choice, staining period and PI concentration has been shown to have a statistically significant effect on nuclear DNA content estimates, altering the 1C-values (Dolezel et al., 2007). Nuclei isolation buffer protects the nucleus from degradation, provides a suitable environment for stoichiometric staining of the nucleus and minimize the negative effect of the cytosolic compounds on the DNA (Loureiro et al., 2007). Various lysis buffer, i.e., Galbraith's buffer (Galbraith et al., 1983), LB01 (Dpoolezel et al., 1989), Otto's buffer (Loureiro et al., 2006), Tris.$MgCl_2$ (Marie & Brown, 1993) were used to prepare the sample from leaf tissues of plant species. However, the effect of the cytosolic compounds present in the leaves was finally minimized by the modified hypotonic PI buffer (Ramesh et al., 2014). Estimation of plant nuclear DNA content is usually performed using young leaves; however, seed can be used as an alternative material because they are easy to transport and can be stored for prolonged periods. The measurements of relative fluorescence intensity of stained nuclei are performed using the linear scale, and 10000 nuclei are analyzed for each sample (Ramesh et al., 2014). The following formula estimated the 2C nuclear DNA content:

Genome size of the sample = (Sample G1 peak mean/ standard G1 peak mean) × Genome size of the standard.

The DNA amounts in the unreplicated nuclear genome of an organism characterize the C-value of that organism. Thus, the estimation of DNA content in the G0/G1 stage is said to be 2C-value as two copies of the chromosome.

Till date, several experiments were performed to estimate genome size in plants using randomly selected angiosperms. Variation in the procedure was detected after replicate experiments performed with the same plants. This leads to the concept of intraspecies variation (Suda et al., 2007). Generally, for studying genome size variation, a minimum of three plants should be analyzed, each of them three times (Greilhuber & Obermayer, 1998). Other conditions which are necessary to fulfill for estimating genome size are: the isolated nuclei must be intact, not degraded and must be in sufficient

quantity, the DNA staining must be specific for both the target and standard nuclei, and lastly, the genome size of the reference standard must be known.

Across the angiosperms, nuclear DNA content varies approximately 1000 fold (Fay et al., 2005). The sample used for estimation of plant nuclear DNA content is usually young leaves; however, seeds can also be used as an alternative sample which will help in easy transport and also for prolonged storage (Sliwinska, 2006).

2.8 Chromosome study

Chromosome number determination plays an important role in understanding the cytogenetical constitution of a species. Chromosomal survey not only helps in studying the relationship between taxa but also provides a base for future improvement programmes (Kumar & Rao, 2002). At the end of 19th century, Strasburger for the first time counted chromosome number in orchids (Felix & Guerra, 2005). Cytological studies are also well documented across Zingiberaceae family (Yu et al., 2010). As per record, the chromosome number determined for genus *Pongamia* indicates the presence of both 2n=20 and 2n=22 chromosomes (Atchison, 1951; Patel & Narayana, 1937; Sarbhoy, 1977). Later Ramesh et al. have reported the chromosomal count to be 2n=22 corresponding to diploid level X=11 for *P. pinnata* (Ramesh et al., 2014). This indicates that there exist two races of *P. pinnata* with chromosomal number X=10 and X=11. Till date, ten species of the genus *Jatropha* including *J. curcas* were studied for the chromosomal determination. Seven species along with *J. curcas* exhibited chromosome number to be 2n=22 i. e. X=11. Whereas, *J. villosa. villosa* and *J. villosa ramnadensis* showed 2n=20 chromosomes, i.e., X=10. In this genus also the study concluded the occurrence of two kinds of haploid chromosome numbers X=10 and X=11. In *Jatropha curcas*, cytological investigation exhibited natural and complete pairing in metaphase I and equal separation of the chromosome in anaphase was found, which indicated that the course of meiosis was normal (Sasikala & Paramathma, 2010). The estimated chromosome number for *Ricinus communis* was 2n=20 corresponding to the diploid level X=10. Chromosomal length determination was done in previously *Allium cepa* (Bandaru et al., 2011). Bandaru has also drawn a comparison between chromosome number and genome size. Information on genome size along with the determination of chromosome number will help in understanding the organismal complexity and development.

2.9 Genome size in correlation with phenotypic characters

Environmental factors affect genome size which in turn affects the phenotypic cell characters. Eukaryotic nuclear DNA amount (genome size) ranges nearly five orders of magnitude. Early reports on genome size variation study have noted various correlations at the cellular level. This includes a positive correlation with cell volume and nuclear volume (Jovtchev et al., 2006) and a negative correlation with cell cycle duration (Beaulieu, 2008). A broad study on animal kingdom has consistently reported a strong positive relationship between genome size and cell size (Hardie & Hebert, 2003; Organ et al., 2007). For plants, studies were conducted on interspecies comparison across varying ploidy series which concluded with the fact that ploidy cells are larger than their progenitor cells (Diallo et al., 2016). However, studies on the large taxonomical group did not reveal any consistent result on correlation study so far. Correlation study is species dependent (Grime et al., 1997). Moreover, correlation studies on genome size and cell size have been carried out on limited herbaceous angiosperms. The relationship between genome size and cell size often serves as the basis for testing genome size-dependent variation in higher phenotypic scales (Beaulieu et al., 2008; Beaulieu et al., 2007; Knight et al., 2005). A large-scale comparative analysis was made between genome size and cell size across 101 species of angiosperms of varying growth forms. Guard cell length and epidermal cell area were used as the two matrices for studying the correlation (Beaulieu et al., 2007). Stomatal length has also been associated with nuclear DNA content (Tatum et al., 2006); larger the nuclear DNA content, greater is the overall guard cell length appears (Rayburn et al., 2009), increasing length of stomata (Khazaei et al., 2010). Genome size could potentially be an ultimate consequence of stomatal size simply because guard cell osmoregulation is dependent on endogenous protein synthesis, particularly the enzymes of malate synthesis pathway that regulate the accumulation of osmotica. Larger guard cell needs more of this metabolic machinery, and more copies of the rDNA gene sequences coding for ribosomes, and thus are indeed associated with larger eukaryotic genomes. Gruber et al., studied the relationship between genome size and root meristem growth rate, with the hypothesis that the root growth rate might have significant consequences on genome size expansion in plants (Gruber et al., 2010). Also, the study was conducted with pollen DNA content using flow cytometry (Kron & Husband, 2012). This might help to understand the pollen grain development and to detect unreduced gametes. Knight et al.,

in 2010 studied the relationship of pollen size with genome size in angiosperm and gymnosperm. Other reports are available concerning the relationship between genome size and variation in leaf mass per unit area (Beaulieu et al., 2008). Thus, it was hypothesized that the genome size could be a genetic driver for LMA. However, the relationship between genome size and LMA was found to be weak across 274 species and mostly driven by divergences of basal taxonomic groups (Beaulieu et al., 2007). Thus, this relationship has been assumed to influence the phenotype but, it is species dependent. Furthermore, a strong positive relationship between genome size and seed mass was also suggested (Beaulieu et al., 2007; Grime et al., 1997; Knight & Ackerly, 2002). Whereas, after analyzing 1222 angiosperm species, Beaulieu *et al*. (2007b) discovered that this relationship was not as strong as reported earlier. Though these results have created doubts about the genome size and cell size relationship, it has also been stated that mutations on phenotypic traits might have broken down genome size scaling effects (Knight & Beaulieu, 2008; Otto, 2007). Also, the first phylogenetically informed analyses of the genome size and cell size relationship were reported for vertebrates (Organ et al., 2007) and diatoms (Connolly et al., 2008), confirming the positive relationship. Till date, many researchers have documented relationships between genome size and environmental conditions (temperature, water availability, latitude, and elevation) (Knight & Ackerly, 2002). These environmental predictors of genome size might be a consequence of genome size change. The main reason for the significant variation in plant genome size within species is still largely unknown. Thus, studies had been conducted in great interest to link this intraspecies genome size variation to plant phenotypic traits.

2.10 Retrotransposons in correlation with genome size

Transposable elements (TEs) are found to be present in all eukaryotic genomes (Wicker et al., 2007). Transposable elements are divided into DNA transposons and retrotransposons according to their mode of movement in the genome. Retrotransposons are again subdivided into long terminal repeat (LTR) retrotransposons and non-LTR retrotransposons. The most abundant group of TEs is LTR retrotransposons, namely, the Ty1-*Copia* and Ty3-gypsy superfamilies. By the process of replicative transposition, LTR retrotransposons copy themselves in the genome and are thus able to increase the genome size of the host (Boeke & Corces, 1989; Wilhelm & Wilhelm, 2001). Genome size

considerably varies due to the activity of retrotransposon. They occupy around 50% of the plant genome, move by copy-paste mechanism and thus lead to genome obesity (Du et al., 2010; Feschotte et al., 2002). Study of retrotransposons can be helpful to draw the relationship between their copy number patterns with genome size, hence are the excellent tool for the study of evolution, gene tagging, diversity, genome organization and marker-assisted selection (Kalendar et al., 2011). Almost all the types of retrotransposons have been detected in plant genomes (Mehmood et al., 2013). Their ubiquitous presence in the plant nuclear genome suggests that they are of very ancient origin (Bennetzen, 2000). Retrotransposons have been found in a variety of angiosperms. Till to date the Ty1-*copia* group is the best-characterized and understood retrotransposon group and are so numerous that they comprise significant fractions of the genome (Kumar & Bennetzen, 1999, 2000). Gene expression and function is directly affected by the insertion of retrotransposon (Hollister & Gaut, 2009; Kashkush et al., 2003; Lockton & Gaut, 2009). Recombination among retrotransposons can cause large chromosomal rearrangements, including inversions, translocations, deletions and duplications (Lonnig & Saedler, 2002; Vitte & Panaud, 2005). Thus, retrotransposons act as the primary source of genetic and phenotypic diversity in plants (Grandbastien et al., 2005; Kumar & Bennetzen, 1999; Mansour, 2007). In plant genomes, LTR retrotransposons are found to be epigenetically silenced and throughout the evolutionary process undergoes strong purifying selection pressure (Baucom et al., 2009; Navarro-Quezada & Schoen, 2002; Okamoto & Hirochika, 2001). LTR retrotransposons proliferation is much faster than their active removal from the genomes of host species (Vitte & Panaud, 2005). They also respond to various stress stimuli, such as environmental changes and polyploid formation (Baumel et al., 2002; Capy et al., 2000; Comai et al., 2000; Mansour, 2007; Parisod et al., 2010). Till date, only a few retrotransposable elements were reported to populate the plant genome successfully. For example, in *Oryza australiensis*, only three families were found responsible for doubling the genome size (Piegu et al., 2006), and in case of barley, nearly 10% of the genome was reported to be occupied by BARE-1 *copia*-like elements (Soleimani et al., 2006).

In addition, genome size variation has always been correlated with changes in repetitive DNA content and ploidy level. It has been observed that the genome size of plant changes due to an activity of retrotransposons copy number. Generally, plant

genome size occupies different magnitude ranging from *Utricularia gibba* (82 Mbp) to *Paris japonica* (148.8 Gbps) (Pellicer et al., 2010; Ibarra-Laclette et al., 2013). In *Oryza* and *Gossypium* also an increase in the copy number of retrotransposon was reported (Hawkins et al., 2006; Piegu et al., 2006). Within the intraspecific cultivars of *Pisum sativum*, 1.29-fold genome size variations were observed (Greilhuber & Ebert, 1994). Naito et al., reported an increase in copy number of transposons in a few rice strains (Naito et al., 2009). A positive correlation of Ty1-*copia* population with $C_{/n}$ values was reported in the *Eleocharis* genus.

Interestingly, an increase in the copy number of Ty1-*copia* was responsible for the evolution of *Eleocharis* spp. which indicates polyploidy and symploidy (Naito et al., 2009; Zedek et al., 2010). A similar case was observed in the *Oryza* genus where genome size variation was detected due to polyploidization and Ty1-*copia* replication. Due to the proliferation of three LTR families, *Oryza australiensis* is subjected to genome size expansion, which led to the accumulation of more than 90,000 retrotransposon copies in the rice genome (Piegu et al., 2006).

Retrotransposons activity is responsible for somaclonal variation, expressional change in genes, mutation and genome organization in plants genome (Teo et al., 2005). They can acts as important tools for the study of gene tagging, mutation, expression, transformation, evolution, diversity, and marker-assisted selection (Kumar & Bennetzen, 1999). Retrotransposon study is important in view to understand the evolution, genome organization, and genome size. This states that retroelements are the major contributors of plant genome expansion and evolution. Understanding genomics and physical distribution of retrotransposons are vital to understand their evolution and significance (Heslop-Harrison et al., 1997). The isolation and characterization of different families of retrotransposons can be helpful to draw the evolutionary relationship between the plants. Isolated retrotransposons can be used in future to study the differential expression pattern of these elements against different stress and their evaluation for the adaptive characters.

2.11 Abiotic stress evaluation in non-edible oil crops

The productive potential and yield of plants are strongly affected by the environment. Harsh environmental stress adversely affects their growth, metabolism, and yield. Among all the environmental stress factors, salinity and drought have the utmost hostile impact

on plants (Anjum et al., 2011; Golldack et al., 2014; Negrao et al., 2017), that can directly influence the growth and yield (Buchbauer & Başer, 2010; Stanev, 2010). It can directly influence growth and crop production, particularly under certain weather conditions (Stanev 2010; Baser & Buchbauer 2010). Increasing salinization, which is a growing phenomenon worldwide, especially in arid and semi-arid regions, is caused by climatic conditions and agricultural irrigation (Carillo et al., 2011). The responses of plants to these stresses are highly complex. The immediate effects of salinity on plants include decreased osmotic potential, nutritional imbalance (due to high ionic concentration), and the toxic effects of certain ions, especially chlorine and sodium (Flowers, 2004; Munns, 2002; Willadino & Camara, 2010). Soil salinity results in reduced biomass accumulation because of the expense of huge metabolic energy in adapting to salt stress conditions. The increase in osmotic pressure of the soil solution and the reduction in the water infiltration rate results in water limitation for plants (Taiz & Zeiger, 2009), which, in turn, has a negative effect on the growth and development of most plant species. Water limitation has a negative effect on the growth and development of most plant species. Drought, as abiotic stress, also affects plants at various levels of their organization. In fact, under prolonged drought, many plants dehydrate and die. Drought stress in plants reduces the plant cells' water potential and turgor, which elevates the solutes' concentrations in the cytosol and extracellular matrices (Fathi & Tari, 2016). As a result, cell enlargement decreases, leading to growth inhibition and reproductive failure. This is followed by the accumulation of abscisic acid (ABA) and compatible osmolytes like proline, which cause wilting. At this stage, overproduction of reactive oxygen species (ROS) and the formation of radical scavenging compounds such as ascorbate and glutathione further aggravate the adverse influence. Drought not only affects plant-water relations through the reduction of water content, turgor, and total water but also affects stomatal closure, limits gaseous exchange, reduces transpiration and arrests photosynthesis rate. Negative effects of mineral nutrition (uptake and transport of nutrients) and metabolism lead to a decrease in the leaf area and alteration in partitioning among the organs. Alteration in plant cell wall elasticity and disruption of homeostasis and ion distribution in the cell has also been reported. Synthesis of new protein and mRNAs associated with the drought response is another outcome of water stress on plants. Under water stress, cell expansion slows down or ceases, and plant growth is retarded. However, water stress influences cell enlargement

more than cell division. Plant growth under drought is influenced by altered photosynthesis, respiration, translocation, ion uptake, carbohydrates, nutrient metabolism, and hormones. In sunflower, drought stress was reported to cause early aging of leaves, reduction in the number of leaves, leaf area and also grain yield (Yegappan et al., 1982). Dihllon and Sidhu had reported that the lack of irrigation in sunflower caused a reduction in grain yield up to about 21% and also affected oil content (Hemmati & Soleymani, 2014). Safari had also reported that the droughts stress reduced grain numbers and oil yield in sunflower (Hemmati & Soleymani, 2014). Drought and salinity stress affects total chlorophyll content, root: shoot ratio, relative water content and relative growth rate, which are useful means for determining the physiological status of plants (Abbasi et al., 2013; Mane et al., 2011). During the limitation of water availability caused by salinity or drought, the root: shoot ratio of plants increases. This increase is caused because the roots are less sensitive to growth inhibition than shoots by low water potentials (Anjum et al., 2011). Also, in the case of total chlorophyll content, a significant decline was observed in soybean under drought stress. The same result was reported in other plant species, and it was concluded that the chlorophyll pigment is sensitive to the increasing environmental stress (Amirjani, 2011; Ashraf & Harris, 2013; Terzi et al., 2010). In soybean, some anatomical and physiological changes were seen to occur under drought stress (Makbul et al., 2011). Anatomical changes induced by stress conditions in higher plants are better-observed indicators. It is well known that leaf water status always correlates with stomatal conductance. Thus, there is stress-induced root-to-leaf signaling, which is promoted by soil drying through the transpiration stream, resulting in stomatal closure. Earlier it has been reported that leaves are undergoing stress usually have smaller stomata than leaves under well-maintained conditions (Guerfel et al., 2009; Makbul et al. 2011). Tissues exposed to environmental stress have generally shown a reduction in cell size and stomatal parameters (Guerfel et al., 2009; Munns, 2002). The effect in physiological and anatomical parameters due to stressed condition often leads to lower the growth, development, and productivity of the plants.

Phytochemicals are essential for plant defenses against stress. Plant-derived phytochemicals are considered as one of the parameters for the yield of the plants and could be used for commercial production of bio-products. The diverse uses of plants in the treatment of various diseases are attributable to the presence of the phytoconstituents

(Lekhak & Yadav, 2012). Flavanoid supplements and fungicides containing flavanoid are available in markets (Scalbert et al., 2005; Williams & Spencer, 2012). Industrially, tannin is considered an important ingredient in the process of tanning leather. It is also used as wood adhesives, anti-corrosive primer and to increase the wine flavor (Khanbabaee & van Ree, 2001). Phenol is used in disinfectant, plastic processing, wood processing, in the chemical industry and also sold as a dietary supplement (Martin & Appel, 2009; Prior et al., 2005). Saponin is used as antifungal agent and detergent and is also seen to appear in beverages and cosmetics as emulsifier (Oleszek & Hamed, 2010). Terpenoid has the aromatic quality and hence used in citral, menthol, and camphor. Moreover, it has high demand in pharmaceutical, cosmetics, perfume and food industries (Leavell et al., 2016). It is also considered as starting material for synthesis of Vitamin A supplement (Dewick, 2002). Cellulose is used to produce paperboard, paper and textile ingredients like rayon (Reddy & Yang, 2005; Yamanaka et al., 1989). Hemicellulose is fermented to ethanol, xylitol, lactic acid which has important applications in pharmaceutical and food industry as a non-caloric sugar sweetener (Philippe, 2012). Many earlier reports are available where studies have been conducted in different angiosperms undergoing stress. Differences in the values of phytoconstituents content present in the leaves were reported in comparison to the leaves of the control plants (Durai et al., 2016; Santhi & Sengottuvel, 2016; Siddhuraju & Becker, 2003).

2.12 Study of seed markers during development and germination

Seed provides nutrition and most importantly conserve the species. Seed is considered as the main agricultural product; however, an increase in population and high demand for quality food is the prime importance. Thus, quality seeds are necessary for successful growth and crop improvement. Seed quality comprises of seed health, germination capability, vigor and genetic purity (McDonald, 1998). Quality seeds germinate faster as compared to normal seeds as their seedlings are little sensitive to climate change (Jeevan Kumar et al. 2015). However, it is the need of the hour to identify reliable biomarkers to evaluate the seed performance in field conditions. Generally, at commercial scale, seed maturity is evaluated visually by the expert growers. So far, several physiological tests were applied which not only suffers lack of preciseness but also leads to variability between the seeds lots. Therefore, by unrevealing the mechanisms behind the various

physiological processes, seed quality and performance can be understood. Recent studies have confirmed some facts as potential markers at biochemical and molecular levels. Antioxidants, membrane integrity, ethylene production, raffinose family oligosaccharides, and cell cycle study are the reliable indicators of seed quality for proper germination, seed maturity and seed priming treatments (Corbineau, 2012). For seed viability, membrane integrity is one of the crucial factors (McDonald, 1999). Aging of seeds leads to loss of membrane integrity by causing the leakage problem. This problem reduces the quality of membrane for solute diffusion and seed vigor, thus more the leakage lesser will be the quality of seeds (Corbineau, 2012). However, in the case of seeds containing high sugar and oil content, membrane integrity test may not serve as a good indicator (Hampton & Tekrony, 1995). Ethylene synthesis is another good indicator of membrane conditions in higher plants. The synthesis takes place through 1-aminocyclopropane 1-carboxylic acid (ACC) oxidase activity (Espindola et al., 1994). Less synthesis of ethylene is a sign of membrane damage which was earlier reported in *Araucaria angustifolia* embryos (Corbineau, 2012; John et al., 2012). Also, in a few other species, seed vigor is correlated with ACC dependent ethylene production (Abdellaoui et al., 2013; Bogatek & Gniazdowska, 2012). Raffinose Family Oligosaccharides is one of the seed makers which is in high demand of researchers (Hill et al., 2005). During seed maturation, RFO gets synthesized and is involved in maintaining the intracellular contents in a glassy state. This helps to restrain the aging effect in seeds (Corbineau, 2012; Farrant et al., 2012). Seed dehydration also depends on the accumulation of its soluble sugar. This might act as a good indicator of seed drying conditions. A positive correlation between the sucrose accumulation and electrolyte leakage has been reported in pea (Corbineau et al., 2000). Much research has been conducted in this field which suggests that the RFO family sugars content could be a good indicator for seed storability (Corbineau, 2012; Dolezel & Bartos, 2005; Jeevan et al., 2015).

Antioxidants and Reactive oxygen species (ROS) are two of the best biomarkers studied for seed sowing stage determination. ROS synthesis takes place during any metabolic process. Thus, maintenance of seed quality is difficult. ROS comprises peroxide, superoxide, hydrogen peroxide, hydroxyl radical and singlet oxygen which has detrimental effects on lipids, DNA, RNA, and proteins, leading to cell death. The condition could be more deteriorative if the seed contains a high amount of oil like the

seeds of biofuel crops. Through lipid peroxidation, ROS affects the permeability of cell and damages the integrity of cell membranes (Avila et al., 2007). The same effect of ROS was seen in soybean seeds which is rich in oil content (Ishibashi et al., 2012). Antioxidant is used to control the increase in ROS concentration is controlled, hence reduction of ROS concentrations could act as a second messenger in seed germination, seed maturation, endosperm and more profoundly linked to seed longevity (Kumar et al., 2015; Sano et al., 2015). Bailey has reported that due to enzymatic activity the ROS scavenging potential was observed to increase in the developing bean seeds (Bailly et al., 2001). Research with sunflower seeds revealed that the scavenging potential of enzymatic activities is directly associated with seed germination rate (Bailly et al., 2004).

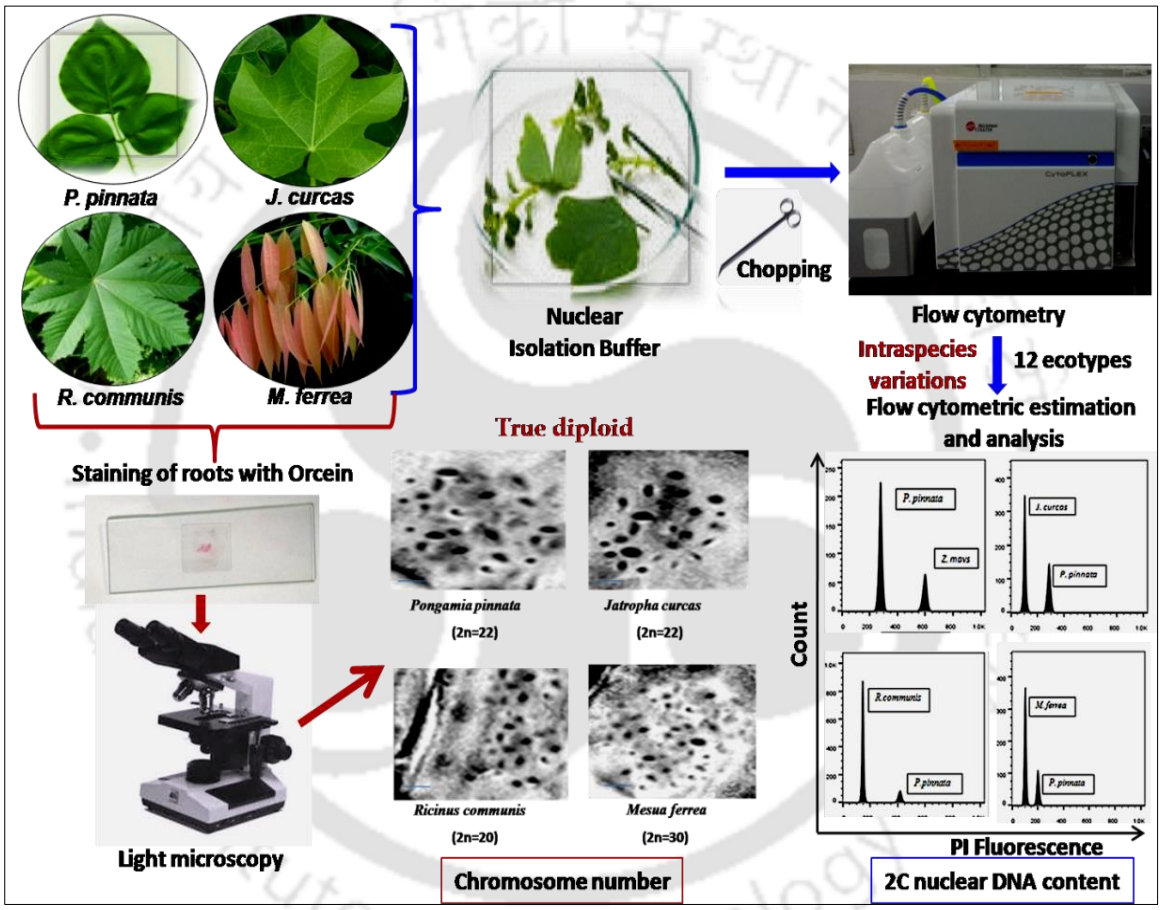
For the proper development of the embryo, nourishment is required which is fulfilled by the endosperm. At different developmental stages, mitotic and endoreduplication takes place in seeds. In an embryo, after the replication phase, i.e., S phase, a typical proliferative cell undergoes mitosis where two n is duplicated to four n conditions. Unlike diploid plants, endosperm cells of some polyploids undergo endoreduplication where repeated rounds of DNA replication occur in nuclei without the mitotic stage leading to the formation of endopolyploid cells. This interesting fact could be studied through flow cytometry (Sliwinska, 2009). DNA replication study could help in knowing the seed vigor because the low-quality seeds require a longer time for DNA repair (Sliwinska, 2009). Thus, the 4n/2n ratio is considered as a useful marker for seed germination, and quality check as active cells need a short span to complete its cell cycle (Kumar et al., 2015). In the early stages of the seeds, cell cycle activity occurs at a faster rate in the embryo. For example, reports are available in neem, sugarbeet, pepper, and soybean, where the ploidy of 4 n conditions is attained at 45%, 30%, and 15-20% respectively (Portis et al., 1999; Sacande et al., 2000; Sliwinska, 1998). In sugarbeet endosperm to embryo, ratio is about 3:2 and 9:1 at 21 days old developing and 36 (mature) day-old seeds respectively (Sliwinska, 1998). Similarly, in cucumber seeds 45% at 21 days after pollination followed by 13% and 8% at harvest and processing stages were reported respectively (Sadowski et al., 2008). These indicate that in the early stages of seed development endosperm and embryo cells actively divide and maintain the ploidy condition. Whereas, during cell maturity, the embryo and endosperm nuclei undergo G0 phase and endoreduplication process, thus reduction in ploidy condition of embryo nuclei in comparison to endosperm

nuclei (polyploidy) is observed. Reports are available where the seed maturity could be monitored effectively with the ratio of embryo/endosperm cell number along with DNA contents of corresponding nuclei (Rewers & Sliwinska, 2012). Hence, knowledge on endosperm to embryo nuclei would help in monitoring seed maturity and quality.

Hence, from literature review, it has been concluded that in the four plants under study, less information is available about their genomic and cytogenetic study. Also, no study had been conducted on their cell phenotypic traits and transposable elements so far. Abiotic stress related study is also critical. Thus, estimation of nuclear genome content followed by a correlation study with cell phenotypic traits and mobile elements has been included in the present study. Also, the focus has been given on studying the role of abiotic stress on these four plants and on identifying a biomarker for seed germination.

Chapter 3

GENOME SIZE ESTIMATION AND CYTOLOGICAL ANALYSIS IN NON-EDIBLE OIL CROPS



This chapter describes the nuclear genome estimation of *P. pinnata*, *J. curcas*, *R. communis* and *M. ferrea* collected from different geographical locations of Assam. Furthermore it also deals with the determination of the somatic chromosome number of the mentioned plants.

GENOME SIZE ESTIMATION AND CYTOLOGICAL ANALYSIS IN NON-EDIBLE OIL CROPS

3.1 INTRODUCTION

Depleting reserves of fossil fuel has increased the demand for biofuel; owing to the fact that biofuel is biodegradable, renewable, nontoxic and safe to store. Many crops, both edible and non-edible have been explored so far for the production of biofuel. Among the non-edible crops, *P. pinnata, J. curcas, R. communis,*and *M. ferrea* are emerging as potential sources for sustainable biodiesel production. These plants are considered as strategic crops for developing countries because of their diverse applications. The fact that these crops can be cultivated on marginal lands with minimal maintenance make them potential candidates for biodiesel production. Additionally, their ability to adapt to a wide range of ecological conditions suggests the considerable existence of genetic variability, which needs to be exploited for potential realization. While these potential biofuel crops are being harvested in many parts of the world with the purpose of crop improvement, very less information is available about their nuclear DNA content and chromosome number. To achieve full genetic characterization of these species, a detailed study of their genome size is obligatory. Nuclear DNA content is a specific karyological feature that is useful for systematic purpose and evolutionary consideration (Moscone et al., 2003). However, extensive DNA content measurements for these crops do not exist. Analysis of genome is vital to understand the structure, organization, and composition, lack of which serve as the bottleneck in the genetic characterization of the species. In addition, knowledge of genome size is more than a practical necessity in large-scale sequencing programs.

The estimation of the amount of DNA was possible in plant nuclei for over 50 years, and since the key role of DNA in biology was discovered in 1953, such research has increased in each successive decade (Bennett & Leitch, 2005). Earlier, Fuelgen densitometer was used to estimate the nuclear DNA content; nowadays Flow cytometry, a biophysical laser-based instrument, has become the predominant technique for establishing plant genome size. It is a powerful technique for analyzing and sorting cells, nuclei, and chromosomes efficiently and accurately. This technique has provided relevant information for biodiversity, ecological, evolutionary and taxonomy studies (Bennett &

Leitch, 2005). This can further help to conduct breeding programs, sequencing projects and research using molecular markers.

In cytotaxonomic studies, chromosome number plays the dominant role and contributes principally to the delineation of taxonomic units at different hierarchical levels. Chromosome count is always found to be constant for a species. Due to the difference in ploidy level, variations in chromosome count have been reported. Kanellos (2009) has reported that polyploidization results in a better harvest of biodiesel crops. Cytogenetic studies have revealed the chromosome number of *P. pinnata* to be 2n=20 or 22 and nuclear DNA content of 2.56 (Ramesh et al., 2014). Another promising biofuel crop, *Jatropha curcas*, has a chromosome number of 2n=22 and an estimated nuclear DNA content of 2C=0.85 pg (Dahmer et al., 2009).Interest in genome size has been fuelled by the fact that nuclear DNA content can affect various plant characteristics at the cellular, tissue and organism levels and can have important ecological and evolutionary consequences (Knight et al., 2005). Hence, the flow cytometry technique combined with cytogenetic study will promote better understanding of the plant genome.

Although *Pongamia*, *Jatropha*, *Ricinus*, and *Mesua* are being harvested as important sources of non-edible oil with the overall purpose of crop improvement, no information is available with regard to its genome content, organization and structure. Estimation of nuclear DNA content and chromosome number could be helpful in understanding genome organization and evolution. Thus, in this study, an attempt has been made to use flow cytometric analysis to determine the nuclear DNA content and chromosome number in plants collected from different locations of Assam, India. Also, an attempt was made to see if any correlation existed between that of the nuclear DNA content and temperature in twelve individuals of the four biofuel crops. This knowledge will facilitate the use of available germplasm resources, enabling the development of optimized breeding strategies towards improvement in yield and production particularly as it relates to the oil trait.

3.2 MATERIALS AND METHODS

3.2.1 Plant material

Seeds were collected from twelve different geographical locations of Assam, India (different geographic environments or ecotypes) **(Table 3.1).** The collected seeds of the

four plants under study were washed several times with tap water and then, rinsed twice in ethanol (25% v/v). To enhance the seed germination, pre-treatment was carried out. Various seed treatment methods were attempted for improving the germination (Kumar et al., 2007). Among them, hot water treatment at 60 °C for 30 mins followed by soaking in cold water for 4 hours was found to be best for germination and breaking seed dormancy of *P. pinnata*. To soften or break the seed coat of *R. communis* and *M. ferrea*, seeds were soaked in cold water for 12 and 24 hours respectively. Cold water treatment did not improve the germination capacity of *J. curcas* seeds, and hence, cow dung slurry was utilized to budge the hardness. For this purpose, *Jatropha* seeds were soaked in the cow dung slurry for upto 4 hours, which provided better germination as compared to cold water treatment or nicking of the seed coat. The seeds of the four plants collected from each ecotype were, then, washed thrice with distilled water and were plated between the papers for germination. All the seed samples were maintained in an incubator at 27±3 °C. One seed per polythene bag (half filled with sand and clay in the ratio of 1:4) were placed in a mist chamber at 28±2°C and 70-80% relative humidity. The seeds were kept well hydrated until germination that occurred within 7-15 days. Seedlings were planted in the open after two to three months when they reached a height of 30-40 cm. Proper sunlight was provided for hardening of the plants before they were transferred to bigger pots. Young tender leaves from raised seedlings were used for flow cytometric analysis. *P. pinnata* (NGPP46), *Oryza sativa*, *Pisum sativum*, and *Zea mays* were selected as the reference plants for this study **(Table 3.2).** *P. pinnata* (NGPP46,2C = 2.66 pg) (Ramesh et al., 2014) from IITG campus and *Zea mays* (CE-777, 2C = 5.43 pg) supplied by Jaroslav Dolezel (Experimental Institute of Botany, Czech Republic) were finally used as the reference standards.

Table 3.1 Study site characteristics

Individuals	Collection site	Coordinates	Temperatures (ºC)	Height from sea level (m)
Mf-a	Dispur	26.1838º N, 91.7633º E	28	55
Mf-b	Tezpur	26.6300º N, 92.8000º E	28	73
Mf-c	Jorhat	26.7500º N, 94.2200º E	22	116
Mf-d	Dibrugarh	27.4800º N, 95.0000º E	22	118
Mf-e	Silchar	24.8200º N, 92.8000º E	28	22
Mf-f	Tinsukia	27.5000º N, 95.3700º E	22.2	117
Mf-g	Kokrajhar	26.4000º N, 90.2700º E	27	37
Mf-h	Halflong	25.1800º N, 93.0300º E	20	680
Mf-i	IIT Guwahati	26.1872º N, 91.6917º E	31	55.5
Mf-j	Amingaon	26.1883º N, 91.6783º E	31	55.5
Mf-k	Mangaldoi	26.4300º N, 92.0300º E	28	59.61
Mf-l	Changsari	26.2800º N, 91.7000º E	31	54

Table 3.2 Reference plant used in experiment along with their genome size

Species	Cultivar	2C DNA content (pg)
Pongamia pinnata	NGPP-46	2.66
Zea mays	CE-777	5.43
Pisum sativum	Ctirad	9.09
Oryza sativa	R-36	1.00

3.2.2 Sample preparation and isolation of nuclei

Fully grown tender leaves were used for the preparation of the suspension of intact nuclei. Five different buffers namely Propidium iodide/Hypotonic citrate buffer (Krishan, 1975), Galbraith's buffer (Galbraith et al., 1983), LB01 (Dolezel et al., 1989),Woody plant buffer (Loureiro et al., 2007) and General Purpose buffer (Loureiro et al., 2007) were tested in the preliminary experiments for the extraction and staining of the cells. Among all the tested buffers, Krishan's buffer (1975) was preferred with little modification in composition as per Ramesh et al., (2014), because it gave clear histograms with minimum nuclear disruption and background noise. The tender leaves ($1cm^2$) were chopped using surgical scissor in 1ml of propidium iodide (PI) hypotonic citrate buffer following the protocol described by Ramesh et al., (2014).The propidium iodide (PI) hypotonic citrate buffer contains 0.3% v/v detergent IGEPAL CA-630 (Sigma–Aldrich) and 0.1% w/v sodium citrate (Sigma-Aldrich). Staining was done using PI (25 mg/L; Sigma–Aldrich) in a solution containing DNase–free RNase (2mg/mL; Sigma–Aldrich). Nylon mesh of 50 µm (Swedesboro, NJ) was used to filter the suspension and used for flow cytometric analysis**.** Estimation of nuclear DNA content was performed with BD FACSCalibur flow cytometer (BD Biosciences, New Delhi, India). Repeated set of experiment was performed with different reference plants. Histograms obtained were analyzed using FlowJo v. 7.6.5 (FlowJo, TreeStarInc, Ashland, OR) for estimating mean fluorescent intensity (MFI) and coefficient of variation (CV). Nuclear DNA content was calculated by comparing the MFI of the nuclei of the sample material with that of the reference standard and obtained by multiplying the nuclear DNA content of standard species by the ratio of their fluorescence intensities. The nuclear DNA content (picograms) was converted to base pairs by considering that 1 pg of DNA corresponds to 978 Mb (Dolezel et al., 2003). The analysis was repeated to check if the coefficient of variation of the sample was >5.0.

3.2.3 Flow cytometric analyses

The estimation of 2C nuclear DNA content was performed with BD FACS Calibur flow cytometer (BD Biosciences, New Delhi, India). PI-stained sample was excited with 15mW argon ion laser at 488 nm. Fluorochrome fluorescence was collected through a 645 nm dichroic long-pass filter and a 620 nm band pass filter. Throughout the experiments, the voltage and gain were kept constant and three dot plots first FSC vs. SSC (for

understanding size and granularity of the nucleus), second SSC vs. FL2A (to spot the signals due to intact fluorescence nuclei) and third FL2A vs. FL2W (for discriminating singlets and doublets) were plotted. To compare the mean position of the sample peaks relative to the internal standards, a histogram plot frequency vs. FL2A was generated. For each sample, 10,000 nuclei were analyzed at the rate of 20-50 nuclei per seconds. The test samples and standards were processed using internal, external and pseudo-internal standardization procedures. External standardization involved processing of sample and reference plant separately in nuclear isolation buffer but the starting material was kept constant. Internal standardization involved co-processing of the sample and standard leaves in the same buffer and pseudo-internal standardization experiment was performed where the test material and reference were processed separately and then mixed before feeding the sample to FACS machine. The mean fluorescence intensity (MFI) of nuclei of the samples and standards were obtained and results were acquired using BD Cell Quest Pro software (version 6.0, BD Biosciences). For estimating MFI and coefficient of variation (CV), the histograms obtained were analyzed using FlowJo v.7.6.5 (FlowJo, TreeStar Inc, Ashland, OR), followed by suitable gating to measure the parameters only for intact nuclei in a heterogeneous population. Autofluorescence was removed by selecting its fluorescence region in FITC channel and PI fluorescence profile. Gating procedure was followed to reduce the level of debris and disintegrated nuclei. The nuclei were gated in PI fluorescence channel vs. SSC dot plot for reducing debris; whereas, doublets and clumps were eliminated by gating on fluorescence width and fluorescence area profiles. Nuclei of interest can clearly be identified by their defined fluorescence intensity. Thus, the zone of interest was defined such that only single intact nuclei were included in the fluorescence histogram which corresponds to G0/G1 and G2/M phases of the cell cycle. The gating procedure followed in this study is represented in **figure 3.1.** The experiment and analysis were repeated if the CV of the sample was >5.0. The MFI of G0/G1 peak channel resembling unreplicated haploid genome was considered for estimating genome size. The nuclear DNA content was estimated using the following formula:

$$Sample\ 2C\ nuclear\ DNA\ content\ (pg) = \frac{Sample\ 2C\ peak\ mean}{Reference\ standard\ 2C\ peak\ mean} \times Reference\ standard\ 2C\ peak\ mean$$

The nuclear DNA content calculated in picograms were converted to base pairs by considering that 1 pg of DNA corresponds to 978 Mb (Ramesh et al., 2014). Genome size was estimated for the four plants under study collected from different geographical locations and a mean value was reported.

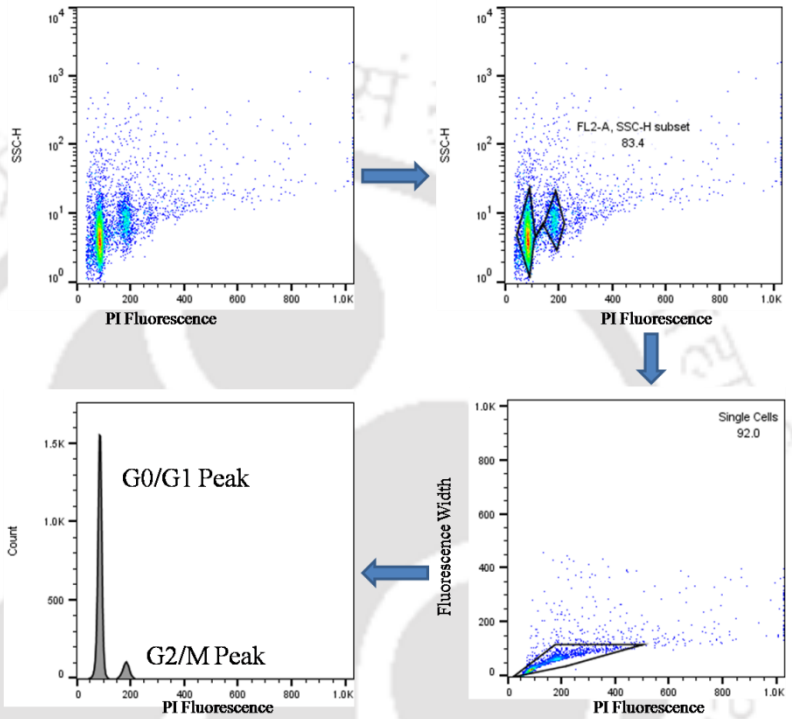


Figure 3.1: Step-by-step gating regime during standardization

3.2.4 Statistical analysis

To compare the genome size of *P. pinnata, J. curcas, R. communis* and *M. ferrea* plants collected from different geographical coordinates, they were analyzed in triplicates. To confirm the reproducibility of the experiments, replicate measurements were carried out on different days. The data obtained during the experiments were analyzed using one-way ANOVA. Significance test was performed using Student's t-test to determine exactly which groups were different ($p<0.05$). A linear regression analysis and a Pearson correlation were performed between the MFI of test and standard nuclei. All statistical analyses were carried out using SPSS software (IBM Corporation, Somers, NY, USA).

3.2.5 Cytological analysis

3.2.5.1 Collection and storage of root tips

Mature seeds of *P. pinnata, J. curcas, R. communis,*and *M. ferrea* collected from study sites as mentioned in **Table 3.1** were raised in pots containing moist soil in the Greenhouse of Biosciences and Bioengineering Department, IITG, Assam, India. The 3-4 weeks grown plants were transferred to hydroponics to obtain fresh root tips (Ramesh et al., 2014). The roots (approx. 1 cm in length) were collected at different times during morning hours (9AM to 11:30AM) followed by washing with tap water for 3-4 times and finally with distilled water. The experiments were conducted by taking 5 replicates of each sampling from different places and were used for cytological studies.

3.2.5.2 Pre-treatment and fixation

Pretreatment of excised roots was carried out in 0.02 % 8-hydroxyquinoline (BDH, India) at 4 °C for 4 hours followed by washing with distilled water for several times. Fixation of the pre-treated roots was done using freshly prepared modified Carnoy's fluid containing absolute alcohol, chloroform, glacial acetic acid,and methanol (7:3:1:1) for 48 hours. After 48 hours, the roots were washed with 70% alcohol for 3-4 times and preserved in 70% alcohol for future use.

3.2.5.3 Hydrolysis and staining

The protocol for hydrolysis was optimized for all four plants under study. The roots of *P. pinnata,* after fixation, were hydrolyzed with a 1:1 mixture of 1N HCl and absolute ethanol for 5 mins at 25 ˚C. In case of *J. curcas* and *R. communis,* the fixed roots were treated with 1N HCl at 60˚C for 12 mins. Due to their hardness, the hydrolysis of the roots of *M. ferrea* was done with 1N HCl for 30 mins at 50 ºC. The roots of the four plants were then transferred to a slide and few drops of a mixture of 2% aceto-orcein and 1 N HCl (in a ratio of 10:1) were put on them, followed by gently heating of the slides. The roots were then immersed in 2% aceto-orcein (staining) solution for 5 mins.

3.2.5.4 Slide preparation, squash, and observation

The stained roots were cut gently into small pieces towards the root tip, with a razor blade and dissecting needle. A coverslip was placed over the tissue to remove air bubbles and pressed down firmly by the thumb. Excess of stain was soaked with filter paper followed

by gentle heating to soften the tissue. With a small cork stick, the cells were spread into a monolayer. The uniformly spread stained segment of root tips was observed under a compound light microscope (Olympus CX 31, India). Mitotic metaphase stages with good chromosome spread from two to three slides were used for counting the chromosome number of each plant.

3.3 RESULTS AND DISCUSSION

In this chapter, the nuclear DNA content of 12 individuals each of *P. pinnata, J. curcas, R. communis* and *M. ferrea*from different locations of Assam were reported. Efforts were also put to observe if any intraspecific variation was present among the individuals belonging to twelve ecotypes. Mitotic chromosome analysis was also performed in the four biofuel crops under study.

3.3.1 Buffer optimization

Estimation of nuclear DNA content is based on the suspension of intact nuclei stained with a DNA-specific fluorochrome. Choice of isolation buffer is crucial as it protects the nucleus from degradation by providing an appropriate condition for specific staining of the nucleus and by minimizing negative effects of some cytosolic compounds on DNA staining (Loureiro et al., 2007). Although many different nuclear isolation buffers have been reported, none of them work well with all species with different types of tissues and different nuclear genome sizes (Bainard et al., 2010). Therefore, it was important to optimize the appropriate buffer and staining period so as to have correct estimates of nuclear DNA content (Choudhury et al., 2014). In the preliminary experiments, different buffers viz. Galbraith's buffer, LB01, Hypotonic citrate/PI and Woody plant buffer were used for the extraction and staining of the cells. Among them, Hypotonic citrate/Propidium iodide buffer was found to be the best for isolation of nuclei for the four plants under study. This buffer consisted of detergent, which resulted in a low coefficient of variation in comparison to other buffers, which failed to separate the nucleus from the cytosol. Modified hypotonic citrate/PI buffer contained non-ionic detergent IGEPAL CA-630, which was used to facilitate the release of nuclei from the cell, prevent nuclei clumping and attachment of debris. The plant nuclei suspension often contains a high concentration of calcium oxalate, which blocks the fluidics system of flow cytometry;

thus, sodium citrate was used as a chelating agent. PI was used as a DNA selective fluorochrome, which binds with the double-stranded DNA without base dependent bias and was suitable for estimating nuclear DNA content. For reducing the interference of secondary metabolites, using the antioxidant, β-mercaptoethanol, resulted in the consistency of nuclear DNA content. Though the exact mode of action of inhibitors is unknown, it was reported that phenolic substances generally possess active hydroxyl groups, which, in the reduced state form hydrogen bonds (reversible) with the carboxyl group of DNA (Greilhuber et al., 2007). When hydroxyphenols are oxidized, it forms quinines (quinone structure), that are highly reactive and forms covalent bonds (irreversible) with carboxyl groups, affecting the fluorescent dye accessibility to DNA. β-mercaptoethanol was used to prevent the formation of quinone structure (Endres, 1961).To maintain the reaction in the reversible state, PVP40 (Polyvinylpyrrolidone) was added. The free amide groups of PVP40 compete with DNA and manage to bind with inhibitors, thus blocking their available DNA binding sites. Thus, the addition of β-mercaptoethanol along with PVP40 in nuclear isolation buffer helped to remove the inhibitors from the nucleus. Buffer versatility has been demonstrated earlier for other species also, including those belonging to the category of biodiesel plants (Ramesh et al., 2014).

3.3.2 Reference standards for flow cytometry analysis

DNA reference standard ideally should have genome size close to the target species to avoid the risk of nonlinearity; it should be genetically stable with constant genome size, easy to use and available in sufficient quantities (Dolezel & Greilhuber, 2010).The mentioned criteria for selecting DNA reference standard is difficult to satisfy; thus, many reports are available where researchers have also used standards other than plant species like domestic chicken (Galbraith et al., 1983) and human (Lysak et al., 2000). As the genome size in plants ranges over thousand-fold (http://www.rbgkew.org.uk), a set of reference standards is needed for estimating the genome size (Dolezel & Bartos, 2005).

In this study, the plants used as reference standards for estimating the genome size were *P. pinnata* (NGPP46) for *J. curcas, R. communis,* and *M. ferrea* and corn (*Zea mays*) for *P. pinnata*. Other well-known reference standards such as *O. sativa* and *P. sativum* were also used. The main reason for selecting the above plants as the reference standards were its short germination time and easy maintenance under greenhouse conditions. On

the account of overlapping nature, *O. sativa* was ruled out as the standard for estimating nuclear DNA content. Though *P. sativum* clearly demarcated G0/G1, G2/M, and standard G0/G1 peaks, it was not selected for this study as a reference standard since its nuclear DNA content is more than 4-fold of that of G0/G1 peaks of *P. pinnata, J. curcas, R. communis,* and *M. ferrea*. A reference standard should have a genome size larger than that of the genome size of the sample, but not more than 4 times (Suda & Leitch, 2010). These criterions were very well satisfied by *Z. mays* for *P. pinnata.* Histogram G0/G1 peak of *Z. mays* was about 4-fold to that of the other three plants' G0/G1 peak whereas *P. pinnata* (NGPP46) was about 1-fold and thus appeared to be the most suitable reference standard plant for estimating genome size. The histogram was considered on account of the sharp peak of high nuclei count of standard and samples.

3.3.3 Estimation of Nuclear DNA content

Once the initial experiments were performed to optimize the nuclear extraction buffer and to confirm the robustness of the reference standard used, the next step was to estimate the genome size. The reliability of the estimated nuclear DNA content was compromised by the presence of inhibitors. Thus, many researchers have reported the practice of estimating genome size by the above mentioned three standardization procedures (as reported in section 3.2.3) (Choudhury et al., 2014; Rayburn et al., 1997). It is apparent that the simultaneous processing of tissues of the sample and the reference species are absolutely necessary to obtain reliable DNA content estimate. This helps to minimize the effect of inhibitors which greatly affect the fluorochrome fluorescence. The internal standardization exhibited the least variation, in comparison to external and pseudo-internal standardization. Earlier published reports of nuclear DNA content using the internal standardization method are in strong agreement with our results (Choudhury et al., 2014; Ramesh et al., 2014).The study also establishes that the compounds in samples greatly inhibit the PI fluorescence of the standard nuclei in both internal and pseudo-internal standardization procedures. However, it was observed that during the pseudo-internal standardization procedure of *P. pinnata*, the PI fluorescence of sample was inhibited by the compounds of the reference standard. A reported study confirms that the greatly exaggerated differences in fluorescence of the fluorochrome may not be due to differences in nuclear DNA content (Price et al., 2000). As observed from **Figure 3.2,** PI fluorescence

obtained by following external standardization procedure for *P. pinnata, J. curcas, R. communis,*and*M. Ferrea* was at 275, 150, 180 and 125 respectively. PI fluorescence for reference standards, *P. pinnata* (NGPP46) and *Z. mays*, were 200 and 600 respectively. Taking into consideration the minimum shift of MFI of the standards, the nuclear DNA content estimated for test samples by external, internal and pseudo-internal standardization was calculated. Clearly defined histograms for accurate determination of 2C values were obtained following FCM analysis of intact leaf nuclei of test samples using modified PI/ hypotonic citrate buffer. By comparing the G0/G1 peak values of test samples and references from external standardization procedure, the relative mean 2C DNA content of *P. pinnata, J. curcas, R. communis,* and *M. ferrea* was estimated to be 2.47, 0.85, 0.94 and 1.45 pg respectively. The mean 2C DNA value estimated by internal standardization procedure was 2.49, 0.85, 1.00 and 1.40 pg for *P. pinnata, J. curcas, R. communis* and *M. ferrea*respectively **(Figure 3.3)**. Similarly, the genome size estimated using pseudo-internal standardization procedure was found to be 2.44, 0.86, 0.99 and 0.54 pg for *P. pinnata, J. curcas, R. communis* and *M. ferrea* **(Figure 3.4)** respectively. The low average CV value associated with the fluorescent G0/G1 nuclei peak of this study indicates that a critical number of nuclei were reached during the extraction procedure used.

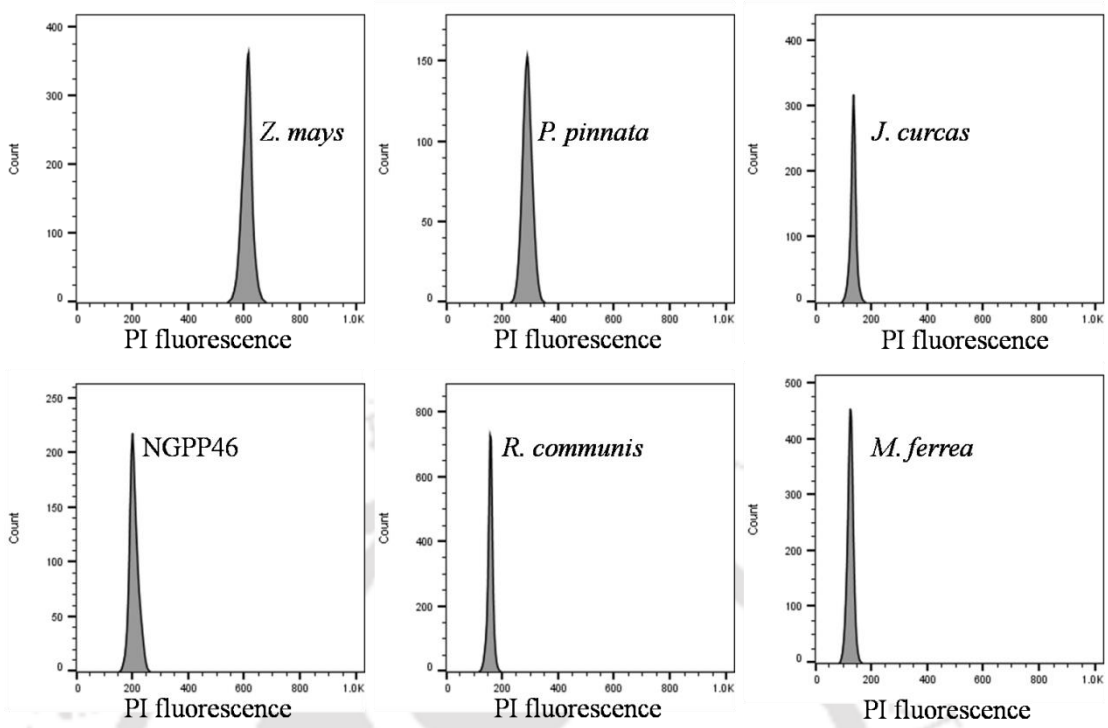


Figure 3.2: External standardization procedures for *Z. mays*, NGPP46, *P. pinnata*, *J. curcas*, *R.communis*, and *M. ferrea*

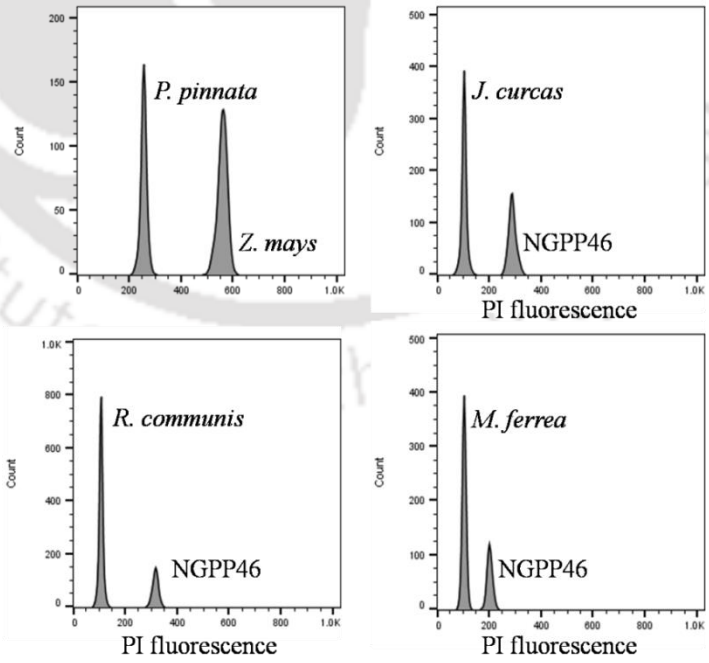


Figure 3.3: Internal standardization procedures for *P. pinnata*, *J. curcas*, *R.communis* and *M. ferrea* using *Z. mays and* NGPP46 as the reference standard

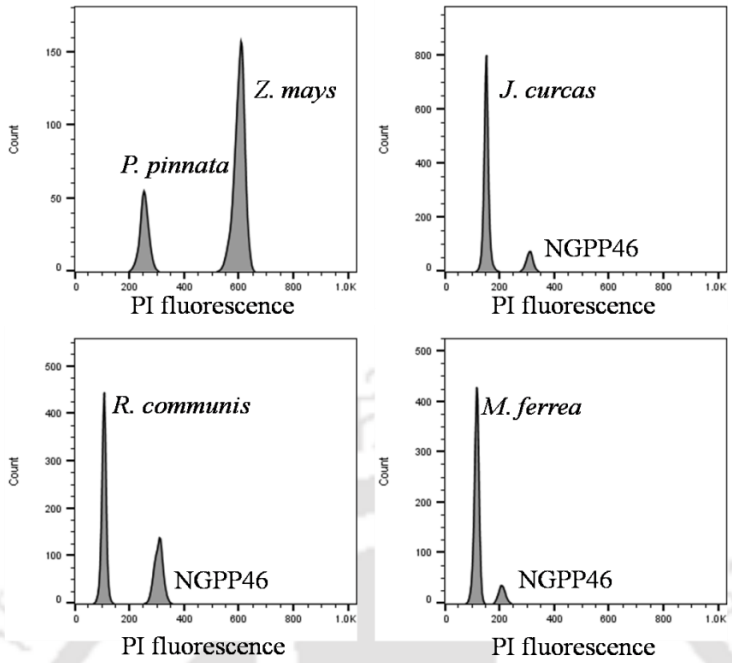


Figure 3.4: Pseudo-Internal standardization procedures for *P. pinnata, J. curcas, R.communis* and *M. ferrea* using *Z. mays and* NGPP46 as the reference standard

Considering the results of internal standardization procedure, replicate measurements within each individual belonging to twelve ecotypes were found to be highly consistent. The standard deviation of genome size ranged from 0 to 0.14 % for *P. pinnata,* 0 to 0.1 % for *J. curcas,* 0 to 0.02 % for *R. communis* and for *M. ferrea* 0 to 0.1 %. The mean 2C nuclear DNA content were in range of (2C = 2.41pg min to 2.56 pg max) for *Pongamia,* (2C = 0.75 pg min to 0.89 pg max) for *Jatropha,* (2C = 0.85pg min to 1.12pg max) for *Ricinus* and (2C = 1.3 pg min to 1.52 pg max) for *Mesua*. Thus for *P. pinnata, J. curcas, R. communis,* and *M. ferrea* this corresponds to a 5.11%, 15.73%, 24.10% and 14.47 % differences respectively in genome size between the individuals procured from different geographical regions of Assam. The mean genome size was 2C = 2.49 ± 0.04 pg, 0.85 ± 0.03 pg, 1.00 ± 0.07 pg and 1.40 ± 0.09 pg for *P. pinnata, J. curcas, R. communis,* and *M. ferrea* respectively **(Table 3.3** to**3.6)**. Intraspecific variation in nuclear DNA content has been observed in all the four plant samples under study. Walker ad also reported intraspecific variation in legume species (Walker et al., 2006). Similarly, intraspecific variation in nuclear DNA content has been well studied in *P. pinnata* collected from five different geographical locations of India (Ramesh et al., 2014). Molecular investigations on plants have shown that the variation in genome size within species is associated with

the difference in transposable elements present in the genome, with the chromatin condensation of the plant genome and with the increase in leaf furanocoumarins (Bennetzen et al., 2005; Biradar & Rayburn, 1994; Walker, 2006; Flavell et al., 1974; Temsch et al., 2010). Intraspecific variation of order 27% to 43% has been reported in genotypes of many species so far (Ebert et al., 1996; Keskitalo et al., 1998). Considering, these findings, the intraspecific variation revealed in the plants under study should be regarded as limited; however, it is in agreement with the findings of a number of recent studies which showed genome size variation in species with rather small genomes; for example, *A. thaliana* (Schmuths et al., 2004) and *Chenopodium quinoa* (Kolano et al., 2012).

Table 3.3 Estimation of the genome size of *P. pinnata* with different standardizing methods with *Z. mays*

Sl no.	**Sample (*P. pinnata*)**	**External standardization**	**Internal standardization**	**Pseudo-internal standardization**
1	Dispur	2.43±0.07	2.54	2.30
2	Tezpur	2.38±0.06	2.49	2.44±0.11
3	Jorhat	2.53	2.49	2.56
4	Dibrugarh	2.43	2.48±0.005	2.42±0.01
5	Silchar	2.54	2.56	2.54
6	Tinsukia	2.30±0.05	2.48	2.30
7	Kokrajhar	2.44	2.54	2.53
8	Halflong	2.42±0.15	2.41	2.4
9	IIT Guwahati	2.49	2.49	2.6±0.05
10	Amingaon	2.47	2.44±0.14	2.43
11	Mangaldoi	2.44	2.49±0.01	2.49
12	Changsari	2.49	2.49	2.44
Mean		2.47	2.49	2.44
Standard deviation		0.04	0.04	0.09

Table 3.4 Estimation of the genome size of *J. curcas* with different standardizing methods with *P. pinnata*

Sl no.	Sample (*J. curcas*)	External standardization	Internal standardization	Pseudo-internal standardization
1	Dispur	0.9±0.008	0.89±0.1	0.92±0.07
2	Tezpur	0.84±0.01	0.86	0.88±0.2
3	Jorhat	0.93±0.1	0.83	1.1
4	Dibrugarh	0.96±0.1	0.84	0.82±0.02
5	Silchar	0.91	0.89	0.9
6	Tinsukia	0.92	0.84	0.93
7	Kokrajhar	1.00±0.02	0.86	0.93±0.1
8	Halflong	0.72	0.75	0.73
9	IIT Guwahati	0.88	0.87	0.84
10	Amingaon	0.87	0.87	0.78
11	Mangaldoi	0.88	0.87	0.84
12	Changsari	0.78	0.87	0.83
Mean		0.85	0.85	0.86
Standard deviation		0.07	0.03	0.11

Table 3.5 Estimation of the genome size of *R. communis* with different standardizing methods with *P. pinnata*

Sl no.	Sample (*R. communis*)	External standardization	Internal standardization	Pseudo-internal standardization
1	Dispur	0.99±0.03	1.04±0.01	1.02±0.03
2	Tezpur	1.002±0.1	1.01	1.08±0.2
3	Jorhat	0.95±0.1	1.01±0.02	1.1
4	Dibrugarh	1.01±0.01	0.96	1.01±0.02
5	Silchar	0.94±0.01	1.12±0.01	0.92±0.005
6	Tinsukia	0.92	1.01	0.95
7	Kokrajhar	1.01±0.02	1.11	0.99±0.1
8	Halflong	0.83	0.85	0.85
9	IIT Guwahati	1.002	1.03	1.03
10	Amingaon	0.97	1.03	0.99
11	Mangaldoi	0.89	1.03±0.01	1.03
12	Changsari	1.04	1.03	1.01
Mean		0.94	1.00	0.99
Standard deviation		0.07	0.07	0.07

Table 3.6 Estimation of the genome size of *M. ferrea* with different standardizing methods with *P. pinnata*

Sl no.	Sample (*M. ferrea*)	External standardization	Internal standardization	Pseudo-internal standardization
1	Dispur	1.66±0.05	1.45±0.11	1.69±0.11
2	Tezpur	1.54±0.01	1.37±0.01	1.58±0.01
3	Jorhat	1.47±0.05	1.37±0.11	1.45±0.11
4	Dibrugarh	1.43±0.01	1.33	1.43
5	Silchar	1.72±0.02	1.52	1.75±0.01
6	Tinsukia	1.47	1.33±0.11	1.43±0.01
7	Kokrajhar	1.68±0.02	1.52	1.72±0.10
8	Halflong	1.02±0.002	1.30	1.24±0.01
9	IIT Guwahati	1.64±0.11	1.41±0.01	1.59
10	Amingaon	1.52	1.42±0.11	1.58
11	Mangaldoi	1.64±0.01	1.4	1.58
12	Changsari	1.37	1.38	1.53
Mean		1.45	1.40	1.54
Standard deviation		0.07	0.09	0.06

The nuclear DNA content estimated in the twelve individuals of four plants under study collected from different geographical locations of Assam correlated positively with both the average temperature of the city and with the height from the sea level by Pearson's product moment correlation **(Figure 3.5)**. The highest nuclear DNA content was observed for the individuals Kokrajhar and Silchar, which are located 37 m and 22 m above the sea level with the average temperature of 27 ºC and 28 °C respectively. The lowest nuclear DNA content was observed for ecotype from Halflong which has the lowest temperature. Genome size and chromosome numbers are considered among the important cytological characters that significantly influence various organism traits in higher plants. Thus, in the present study genome size variation was observed to be in a linear relationship with temperature change and aninverse relationship with the height from the sea level. This might be because of the differences in chromatin condensation

and arrangements of repetitive sequence elements (Jedrzejczyk & Sliwinska, 2010). In case of *P. pinnata* the environmentally induced genome size variations might also be because of leaf furanocoumarins, which is known to accumulate to a greater extent at higher temperatures in legumes as reported by Walker et al., (2006) for legume *Bituminaria bituminosa* populations. Walker's report also suggests that the temperature plays a significant role in the nuclear genome determination. Further experiments in the four plants under study is warranted for the proper reasoning of the above assessment related to the intraspecies variations in genome size and its relationship with temperature.

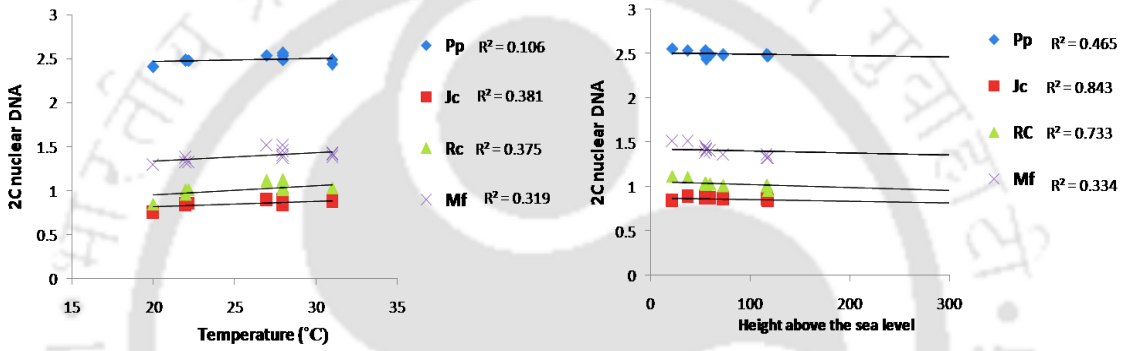


Figure 3.5: Correlation between genome size with the geographical distribution of *P. pinnata, J. curcas, R. communis* and *M. ferrea* in Assam

In this study, the nuclear DNA content of all the four plants collected from 12 different altitudes of Assam was estimated using FCM. Based on the plant DNA C-values database (Bennett & Leitch, 2010), the genome size of *P. pinnata* falls in the upper end of C-value distribution of its family Fabaceae with 683 records ranging from 0.60 pg (*Leucaena macrophylla*) (Hartman et al., 2000) to 54.79 pg (*Viciafaba*) (Bennett & Leitch, 1997). The 1C-value of *P. Glabra* showed a comparable value (1.8 pg) (Ohri & Kumar, 1986) to the 1C-value of *P. pinnata.* In a similar way, Ramesh et al (2014) have obtained the 1C value (1.33 pg) in *P. pinnata*. However, in this study, the estimated 1C-value was found to be 1.24 pg. As per the database, the 1C-value of *P. pinnata* has been found to be analogous with 16 different angiosperms such as *Lotus corniculatus* (Temsch et al., 2010), *Geranium tuberosum* (Veselyet al., 2011) and *Malus ioensis* (Tatum et al, 2005), etc. The 1C-value estimated for *J. curcas* (0.43 pg) in the present study was found to be

comparable with the result obtained by Carvallo (Carvalho et al., 2008; Ohri et al., 2004). Three of the earlier reports on *R. communis* reflecting 0.23 pg, 0.52 pg and 0.54 pg values of 1C collected from UK and Germany (Bennett & Leitch, 1997; Houben et al., 2003; Ohri et al., 2004) showed little variance with the 1C-value estimated in the present study i.e. 0.50 pg. The variation obtained within the species is presumably due to the variation in environmental factors. Within the 50 members of Euphorbiaceae, *J. curcas* and *R. communis* come in the upper end of 1C-value ranging from *R. communis* (0.23 pg) (Bennett & Leitch, 1997) to *Euphorbia polygona* (14.35 pg) (Bennett et al., 2000). Finally, the 1C-value estimated for *M. ferrea* was 0.7 pg. Surprisingly, there were no reports for the genus *Mesua*, hence, it was scientifically significant to broaden the search to Clusioid clade comprising of five families including Calophyllaceae (Ruhfel et al., 2011). Based on the 6 records ranging from 0.6 pg to 3.13 pg, the genome size of *M. ferrea* falls between the genome size of *Hypericum canariense* (0.51 pg) (Suda et al., 2003) and *H. perforatum* (0.78 pg) (Temsch et al., 2010). To the best of our knowledge, this is the first attempt to study different individuals of *P. pinnata, J. curcas, R. communis,* and *M. ferrea,* oil yielding tree species from Assam, which constitutes an extremely valuable source of feedstock for the biodiesel industry.

3.3.4 Cytological analysis

3.3.4.1 Collection of root tips

To check the ploidy level, chromosome count in different individuals of *P. pinnata, J. curcas, R. communis,* and *M. Ferrea* collected from various parts of Assam was carried out. The best time for collection of root tips from the 12 individuals belonging to the four plant species under study was found to be best in the morning hours. For collection of *P. pinnata* roots, the best time optimized was between 10.00 and 11.00 am, for *J. curcas* and *R. communis* it was 9.00 am to 9.30 am and for *M. ferrea* it was 11.00 - 11.30 am as per the GMT+5:30 (Kolkata/India zone); during this time ample metaphase stages are clearly visible for karyological study. The midday period is widely followed in the field of cytology as cell division in many plants is at a peak and thus yields the highest number of metaphases when fixed for cytological observation.

3.3.4.2 Pretreatment

Pretreatment is necessary for cytological studies as it arrests the mitotic cycle at the metaphase stage by inhibiting spindle fiber formation and revealing more cells in metaphase. Various pre-treatments and staining chemicals like PDB, 1-bromonaphthalene (MBN) and 8-hydroxyquinoline (OQ) have been used (Nair & Sasikumar, 2009; Ramesh et al., 2014). However, in the current investigation 8-hydroxyquinoline alone was effective in treating *P. pinnata, J. curcas, R. communis* and *M. ferrea* giving a high percentage of metaphase cells as it helps to block the mitotic cycle at metaphase stage by inhibiting spindle fiber formation revealing more number of cells in metaphase.

3.3.4.3 Hydrolysis and staining

The hydrolysis was performed to soften the root tissue. The roots of trees and shrubs are harder and larger; therefore, it was important to soften the pretreated roots for a specified period in acid (1N HCl). In the current study, the hydrolysis step was optimized for 5mins at 25 ^{o}C for *P. pinnata*, at 60 ^{o}C for *J. curcas* and *R. communis* and for *M. ferrea* it was optimized at 30 min at 50 ^{o}C. For staining the roots, 2% aceto-orcein dye was used. Heating for a few seconds in the acid-dye mixture intensified the staining of the chromosomes. Thus clearer pictures of chromosomes were observed as pink dots under a light microscope.

3.3.4.4 Slide preparation and observation

Squashing and spreading of the cells uniformly is a crucial step which results in proper visualization of the slides. The slides revealed the well-spread metaphases under low power (10X) and high power (40X) magnifications of the compound light microscope. Some of the cells were found to be in different growth stages like late prophase or anaphase stages. All of the 12individuals collected from different locations of Assam revealed chromosome number 2n=2x=22 for *P. pinnata* and *J. curcas* which were in accordance with the report of Ramesh et al., (2014) and Carvalho et al.,(2008) respectively. The chromosome number 2n=2x=20 determined for *R. communis* matches with the previous report of (Houben et al., 2003) and (Ohri et al., 2004) and for *Mesua* 2n=2x=30 was determined without any polyploidy; thus the plants are true diploids **(Figure 3.6).** The chromosome spectrum of Fabaceae family ranges from 2n=12 to 2n=96,

Euphorbiaceae family ranges from 2n=12 to 2n=196 but for Callophylaceae family, no information was available (http://www.rbgkew.org.uk). No intraspecific variation in the chromosome number was detected both from the results obtained and the literature search, though variations in genome size were observed among the 12 individuals belonging to the four studied plants. Although these species propagate sexually, the planting materials were confined to a few districts of Assam; therefore the chromosome number which is constant might be due to the lack of recombination.

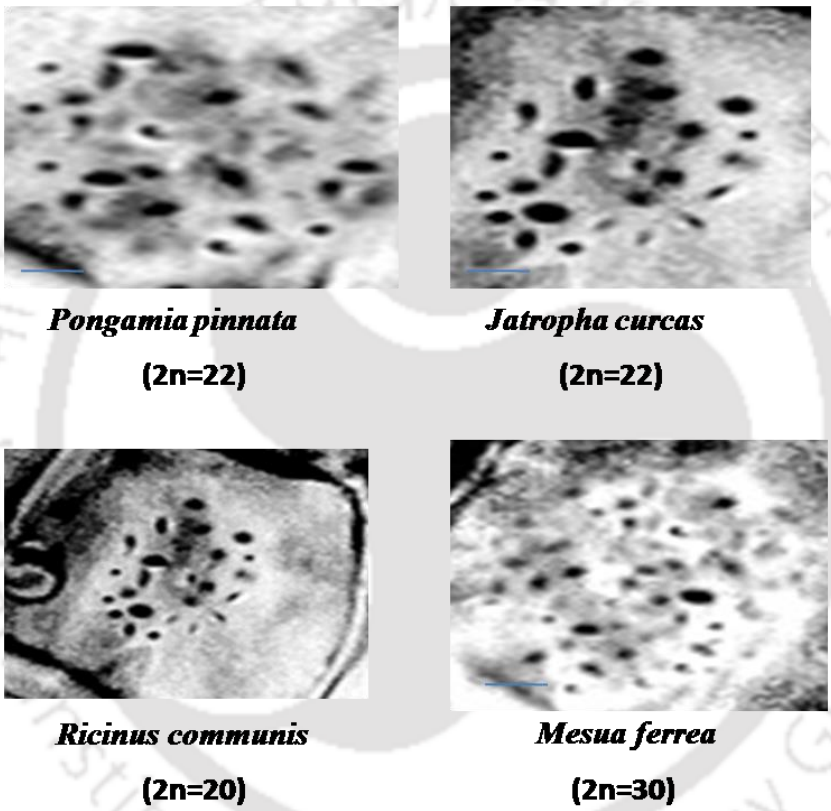


Figure 3.6: Somatic metaphase chromosome in *P. pinnata, J. curcas, R. communis,* and *M. ferrea*

The basic idea of incorporating the cytological information stems from the importance of chromosome number in cytotaxonomic studies. Chromosome number plays a dominant role in contributing to the delimitation of taxonomic units at different hierarchical levels. Genome size, along with chromosome numbers, are important cytological characters that significantly influence various organismal traits (Skornickova et al., 2007). Thus, it was necessary to understand that the genome size variation obtained

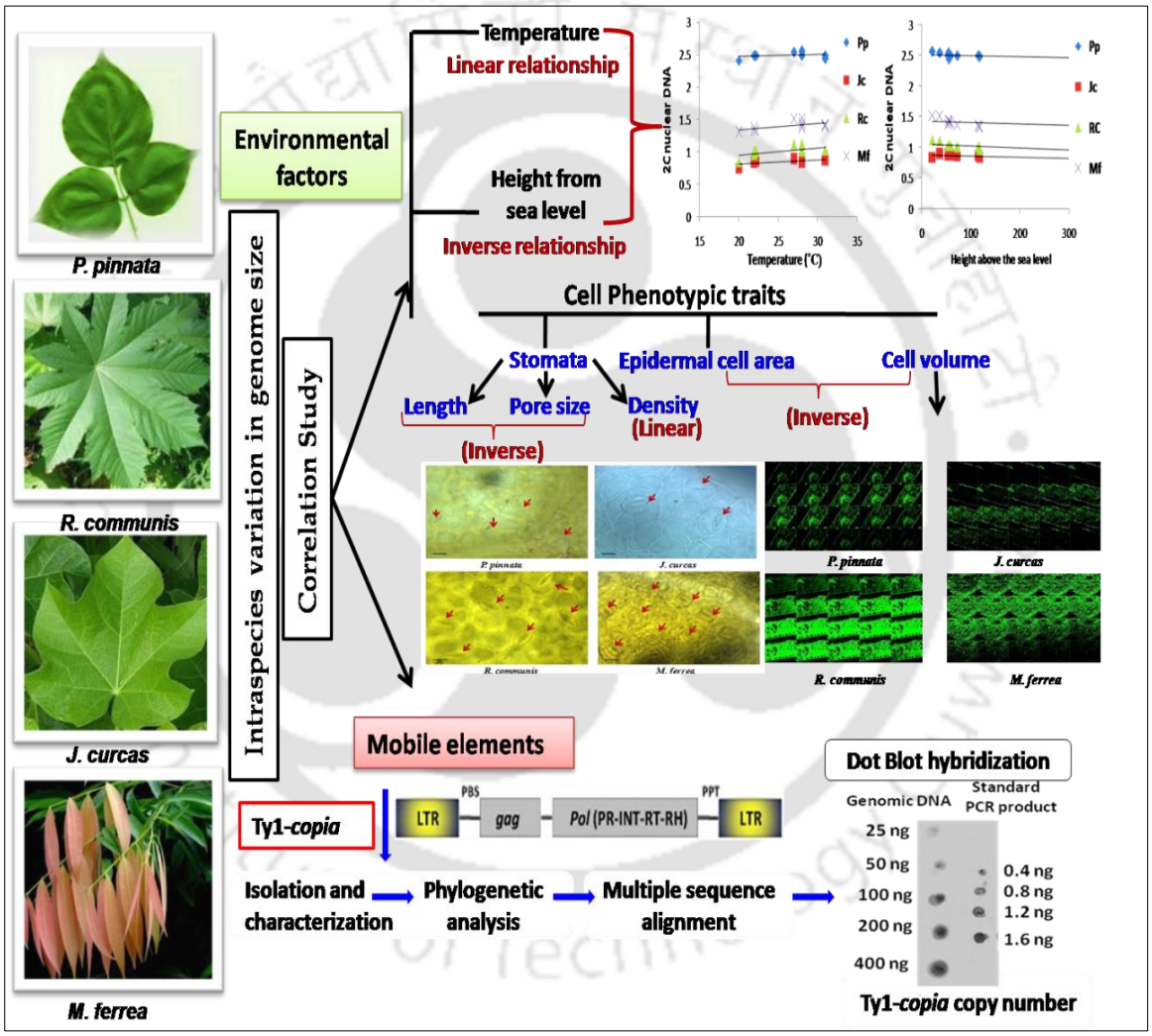
within species in order to understand the effect of polyploidization or variation due to environmental factors.

3.4 CONCLUSION

The nuclear DNA content was estimated for *Pongamia*, *Jatropha*, *Ricinus*, and *Mesua* collected from different geographical locations of Assam using the flow cytometry. It was revealed that the 2C value was consistent with repeated experiments. The genome size was in the order of *J. curcas* (0.85 pg/2C DNA), *R. communis* (1 pg/2C DNA), *M. ferrea* (1.4 pg/2C DNA) and *P. pinnata* (2.49 pg/2C DNA). Intraspecific variation was observed in the genome size of all the studied plants collected from different geographical locations. Whereas no such variation was detected when chromosome number was estimated and which indicated the absence of polyploidy. The variation in genome size collected from different geographical locations of Assam, India was found to have a positive correlation with the temperature and height above the sea level. This indicates that the variation of genome size might be due to the variation in environmental factors. The information reported here may be helpful in genome research as well as studying the relationship of DNA content to plant physiology and ecology.

Chapter 4

GENOME SIZE CORRELATION TO CELL PHENOTYPIC TRAITS AND MOBILE ELEMENT



This chapter deals with the study of cell phenotypic traits and mobile elements. Genome size correlation study with cell phenotypic traits and Ty1-*copia* copy number was performed to understand the cause of genome size variation.

GENOME SIZE CORRELATION WITH CELL PHENOTYPIC TRAITS AND MOBILE ELEMENT

4A CORRELATION OF GENOME SIZE WITH CELL PHENOTYPIC TRAITS

4A.1 INTRODUCTION

At present, the depletion of nonrenewable resources and increasing demand for energy is one of the major global concerns. Thus, researchers are focusing on developing more efficient biofuels with properties like non-toxicity, biodegradability and reduced of greenhouse gas emission. With this aspect, crops having high oil content are promoted, with little genetic screening on agronomic data as biofuel crops. Majority of oil-yielding crops are edible which limits the usage for biodiesel production. Thus, the focus is on producing biofuel using non-edible seeds. Over past few decades, *P. pinnata, J. curcas, R. communis,* and *M. ferrea*, have emerged as promising candidates for biofuel production due to their potential oil yielding capacity (Achten et al., 2007; Kesari et al., 2008). While biofuel crops are high on demand, much research has been conducted in the field of medicine, phytochemistry and oil sector but very little is known about its 2C nuclear DNA content and cell anatomy. In addition, these plants can adapt to a wide range of ecological conditions, which suggests a considerable existence of genetic variability. Thus in Chapter3, the genome size of *P. pinnata, J. curcas, R. communis,* and *M. ferrea* were estimated which shows the existence of intraspecies variation among these plants. Among plants, nuclear DNA content, or genome size ranges nearly four orders of magnitude (Bennett & Leitch, 2011). Individuals belonging to thesame genus have awide range of variations in their genome size. Variations were also observed in individuals of thesame species collected from different geographical locations (Bennett & Leitch, 1997). The reasons for genome size variations within the species might be due to polyploidization, environmental factors or due to repetitive elements.

Environmental factors affect genome size, which in turn affects the cell phenotypic traits responsible for plant growth and yield (Beaulieu et al., 2008a). Genome size was found to have various correlations at thecellular level as well. In addition, it has been hypothesized that cell phenotypic traits might be the primary determinant of genome size as

their size affects functional efficiency in plants. Knowledge of genome content and cell phenotypic characters makes it possible to infer the relationship between them and may lead to the genome evolution. So far, the genome size has been linked to nuclear volume, cell volume, cell cycle, seed mass, photosynthetic rate (Beaulieu et al., 2007), leaf cell size, stomata density (Hodgson et al., 2010) and pattern of genome size evolution (Leitch et al., 2005). Another relationship with genome size involves the stomatal parameters (Knight & Beaulieu, 2008a). Stomata play an important role in growth and development of plants. It consists of small pores at the leaf surface, each bounded by two guard cells. The principal mechanism of stomata is to control the exchange of gases, mainly the influx of carbon dioxide and the efflux of water vapor between the interior of the leaf and the atmosphere (Hetherington & Woodward, 2003; Raven, 2002; Woodward, 1998). Carbon dioxide plays the main role in photosynthesis. Taking in carbon dioxide and restricting the water loss, appears to be an important function of stomatal size (Aasamaa et al., 2001; Allen & Pearcy, 2000; Hetherington & Woodward, 2003). The change in the stomatal pore regulates the gaseous exchange. The length of its associated guard cells primarily determines the maximum size of the stomatal aperture. This stomatal length is in turn, constrained by genome size (Beaulieu et al., 2008b). Because of their greater structural uniformity, stomata might show a more reliable allometric association with genome size than that observed for seed mass (Beaulieu et al., 2007). Epidermal cells form the boundary between the plant and the external environment protecting the plant against water loss. It also secretes metabolic compounds and absorbs water and mineral nutrients in plants. It has been reported that species with small genome size had small epidermal cell areas while species with large genome size had large epidermal cell areas in some of the angiosperms (Knight & Beaulieu, 2008b). Cell volume also plays role in maintaining metabolic rate, intrinsic growth rate, nutrient acquisition, and light absorption. For unicellular diatoms, a significant relationship between genome size and cell volume was reported so far (Connolly et al., 2008). Thus, in the current study, six cell phenotypic traits viz, stomatal length, stomatal density, stomatal pore size, epidermal cell area, and cell volume were taken into consideration as these parameters are directly responsible for plant growth and yield.

The present research aimed to study the cell phenotypic traits responsible for the growth, development, and yield of the candidate plants collected from different geographical locations of Assam. This study reveals the correlation of genome size with

stomatal length, stomatal density, stomatal pore size, epidermal cell length and cell volume for *P. pinnata, J. curcas, R. communis, and M. ferrea*.

4A.2 MATERIALS AND METHODS

4A.2.1 Plant material and culture

Maintenance of plant material was done as reported in the previous chapter (section 3.2.1).

4A.2.2 Leaf cell measurements

4A.2.2.1 Estimation of stomatal parameters and epidermal cell area

For microscopic observation, fully-grown plant leaves were collected from each of the twelve individuals of *P. pinnata, J. curcas, R. communis,* and *M. ferrea*, belonging to the different geographical locations of Assam. Leaves were washed 3-4 times with distilled water and were dried gently using blotting paper. Leaf was sectioned ($1cm^2$) from the top, middle and bottom part using surgical blades. Sections of both adaxial and abaxial surfaces of the leaves were then taken on glass slides with 2-3 drops of 80% chloral hydrate solution. The samples were covered with coverslips followed by 3-4 times of short heating to remove the chlorophyll content. Both abaxial and adaxial surface of bleached leaves were observed under a light microscope. After brightening with 80% chloral hydrate, stomatal length, stomatal pore size, and epidermal cell area were estimated with a microscope (Olympus CX31, India) using an objective with 40X magnification corresponding to a visual field of 0.53 mm^2. In order to understand the dynamic stomatal movement, we considered only stomatal length, as stomatal breadth is more sensitive to environmental perturbations. Length and breadth measurement was carried out using the Qpro.7 software. Stomatal density was measured based on the number of count per 0.53 mm^2 section of a single leaf. All the experiments were performed in triplicates and the mean value has been calculated.

4A.2.2.2 Cell volume estimation

4A.2.2.2.1 Sample preparations

Fully-grown leaves from the candidate plants were collected and were washed 3-4 times with distilled water, pet dried and sectioned (1 cm^2) using surgical blades. Permanent slides were prepared using the protocol as discussed below.

4A.2.2.2.2 Fixation

This step is necessary to preserve biological tissues or cells as close to its natural state as possible in the process of preparing tissue for examination. Fixatives cover the cells or tissues with a soluble film that protects cell morphology. In this study, the leaf sections of *J. curcas* and *R. communis* were incubated in absolute ethanol (EtOH): glacial acetic acid (3:1) solution for 4 hours at 4 °C. The incubation period for *P. pinnata* and *M. ferrea* were optimized to12 hours at 4 °C.

4A.2.2.2.3 Dehydration

The fixing solution was removed by alcoholic dehydration. The fixative should be replaced by a liquid, which is immiscible with water and paraffin solution. Thus, the samples were subjected through a series of ethyl alcohol concentrations. The sections of *P. pinnata,J. curcas,* and *R. communis* were dehydrated in increasing concentrations of ethyl alcohol (50%, 70%, 90% and 100%), with an incubation of 20 mins in each. The dehydration steps were the same for *M. ferrea* with an optimized period of 2 hours for each step.

4A.2.2.2.4 Infiltration and embedding of paraffin

The dehydrated samples were passed through a mixture of xylene: EtOH

1:3 Xylene: EtOH for 15 mins

1:1 Xylene: EtOH for 15 mins

3:1 Xylene: EtOH for 15 mins

1:0 Xylene: EtOH for 15 mins

100% Xylene for 30 mins

To replace xylene with paraffin, the paraffin wax was melted at 60 °C and the following steps were carried out in a vacuum oven set at 58 °C.

2:1 Xylene: Paraffin for 15 mins

1:1 Xylene: Paraffin for 15 mins

1:2 Xylene: Paraffin for 15 mins

100% Paraffin for 2 hours

100% Paraffin for overnight

The steps involving paraffin should not exceed 60 °C in temperature as it will degrade the paraffin polymers and will make it brittle. The samples from the liquid paraffin embedded

in fresh new paraffin and the paraffin blocks containing tissues were kept at 25 °C for hardening.

4A.2.2.2.5 Sectioning and staining

Sectioning of the paraffin blocks (10 µm thick) containing the samples was carried out using a vibratome series (Leica RM 2245, Germany). Thin wax layers containing samples were incubated in a water bath for 1min at 40 °C and were stored in xylene to wash out the paraffin followed by 1 min incubation in absolute ethanol. Sections were removed from ethanol and were attached to glass slides. For *P. pinnata,* the staining procedure was followed for 10 mins using 10-100 µg/ml aqueous acridine orange in absolute ethanol, 5 mins each for *J. curcas* and *R. communis* and 30 mins for *M. ferrea* and was mounted with DPX. Prepared glass slides were then observed under a confocal microscope (ZEISS lsm 510, US) at 20 X resolution using the Z-stacking procedure. Laser-based sectioning resulted in a three-dimensional representation of the specimens by creating twenty optical sections. The volume of the cells was then determined using ImageJ software.

4A.2.2.3 Statistical analysis

To compare the estimated values of stomatal length, stomatal pore size, stomatal density, epidermal cell area and cell volume of the twelve ecotypes collected from different geographical locations belonging to the four biofuel crops, all experiments were conducted in triplicates. Replicate experiments were performed on different days to confirm the repeatability. Using one-way ANOVA the data obtained were analyzed and Student's t-test was performed for the significance test ($p<0.05$). Between the 2C nuclear DNA content and the cell phenotypic traits, linear regression analyses were performed. SPSS software (IBM Corporation, Somers, NY, USA) was used for all statistical analyses.

4A.3 RESULTS AND DISCUSSION

4A.3.1 Estimation of cell phenotypic traits

Anatomical or cell phenotypic study of leaves have played a significant role in plant taxonomy, particularly at the generic levels. Studies in this field have helped plant taxonomists to resolve taxonomic conflicts in different groups of the plant (Sonibare et al., 2014). In the present study stomatal dynamics, epidermal cell areaand cell volume

have been determined for *P. pinnata, J. curcas, R. communis* and *M. ferrea* belonging to different geographical locations of Assam. These parameters play important role in plant growth and yield and thus were considered for the correlation study with genome size. Microscopic analyses of the leaves were performed and the adaxial surface was found to be full of palisade cells. Thus, it does not give a clear view of stomata. However, the Abaxial surface of the leaves was more suitable for our study as it gives a clear visualization of the stomata and epidermal cells. Majority of the woody plants studied were found to bear amphistomatous leaves i.e. leaves bearing stomata on both surfaces. This is because such species typically occur in a sunny environment where CO_2 may limit photosynthesis. This feature facilitates easy diffusion of CO_2 and helps in leaf cooling (Hsie et al., 2015). In Fabaceae family, a diverse range of stomatal types was reported, with maximum species bearing amphistomatous leaves except for *Phaseolus vulgaris*, *Erythrina indica* and *Glycyrhiza glabra* etc., which bear hypostomatous leaves like *P. pinnata* (Tripathi & Mondal, 2012). The other three candidate plants also bear amphistomatous leaf but the stomata present in the adaxial surface of leaves were very less in comparison to the abaxial surface and were hard to visualize because of high palisade cell. Thus, stomata present in the abaxial surface of leaves were considered for further study. All the four plants were found to be rich in a paracytic type of stomata which is very common among trees and shrubs (Tripathi & Mondal, 2012). However, some studies have documented other types of stomata, *i.e.*, anisocytic and anomocytic, anomocytic only or paracytic and anisocytic in case of *J. curcas* and *R. communis* (AS, 2009; Hsie et al., 2015; Raju & Rao, 1977). There is no clear explanation for these differences but it can be speculated that this could be due to the natural variation that is expected to occur among geographically widespread species. Nonetheless, paracytic stomata are the most prevalent and considered as a standard type in Fabaceae, Euphorbiaceae and Callophylaceae family (Hsie et al., 2015; Kadiri & Adeniran, 2016; Srivastava & Misra, 2018; Stevens, 2007). The stomatal length ranges from 12.38-35.91 µm, 31.92-53.93 µm, 33.96-49.07 µm and 15.21-37.42 µm in *P. pinnata, J. curcas, R. communis,* and *M. ferrea* respectively. Average stomatal length in mature leaves was 24.92 µm in *P. pinnata* with stomatal density approx. 4 in number per 0.53mm^2.Stomatal length estimated for *J. curcas* was 39.80 µm along with stomatal density 3 in number. The average length of stomata calculated was highest in case of *R. communis*; 42.37 µm

with stomatal density 4 in number per 0.53mm^2 and lowest in case of *Mesua ferrea*; 24.63 µm with density 11 in number per 0.53mm^2. Smaller leaves showed higher stomatal densities, which could be due to the packing of the epidermal cells and the reduction in epidermal cell expansion. This is in agreement with the observations of Beerling and Chaloner in *Quercus robur* leaves (Hsie et al., 2015), where widely spaced stomata were reported along with the lower stomatal density. Similarly, in case of stomatal pore size the range estimated was 2.24-2.7 µm, 6.7-7.71 µm, 6.1-6.71µm and 2.01-2.61 µm for *P. pinnata, J. curcas, R. communis,* and *M. ferrea* respectively. Mean stomatal pore size was calculated for all the twelve individuals. *J. curcas* was estimated to have 7.17µm, which is the highest, and *M. ferrea* to have 2.30 µm, which was the lowest among the four plants. Stomatal pore size for *R. communis* and *P. pinnata* were measured as 6.35 µm and 2.52 µm respectively. Shrubs generally were observed to have larger stomatal length and stomatal pore size in comparison to trees (Carpenter & Smith, 1975). Small stomatal cells, small stomatal pore size, and high stomatal density were found in trees that may have adaptive significance. Also, increase in stomatal density is linked with greater stomatal conductance and transpiration rates, which are considered to be necessary for the transport of water and nutrients through the longer xylem and phloem pathways of a tree (Beaulieu et al., 2008a). Additionally, smaller stomata allow greater stomatal resistance and stomatal control during stress conditions (Hetherington & Woodward, 2003). The estimation of epidermal cell area was also carried out using the abaxial leaf surface of the four plants under study belonging to twelve different ecotypes. For *P. pinnata* the epidermal cell area was in the range of 1119.82 - 1244.91 µm^2, for *J. curcas* the range was from 1736.91-1766.07 µm^2, for *R. communis* it was in the range of 1418.63 - 1477.56 µm^2 and the range was estimated to be 437.21 - 491.11 µm^2 for *M. ferrea*. The mean values estimated for epidermal cell area were in a decreasing order of *J. curcas* 1751.63 µm^2, *R. communis* 1446.9 µm^2, *P. pinnata* 1206.26 µm^2 and *M. ferrea* 460.58 µm^2. **Figure 4A.1** represents the microscopic analysis of the abaxial leaf surfaces showing stomatal parameters and epidermal cell area.

For cell volume estimation, the samples belonging to twelve ecotypes were analyzed under a confocal microscope using laser scanning sectioning. Twenty sections of each sample were done **(Figure 4A.2),** converted to a 3D image using ImageJ software and the cell volume was calculated by measuring the length, breadth, and height of the

cells. The estimated values were in the range of 40969.56 - 41197.66 µm^3, 57911.66 - 57952.74 µm^3, 4849.78 - 48621.01 µm^3 and 6879 - 6918.78 µm^3 for *P. pinnata, J. curcas, R. communis,* and *M. ferrea*; the mean value was estimated to be 41126.51 µm^3, 57929.08 µm^3, 41298.82 µm^3 and 6898.82 µm^3 respectively. *J. curcas* was observed to have highest cell volume followed by *R. communis*, *P. pinnata*, and *M. ferrea*. The mean values of all the parameters calculated from all the individuals belonging to the plants under study are tabulated in **Table 4A.1.**

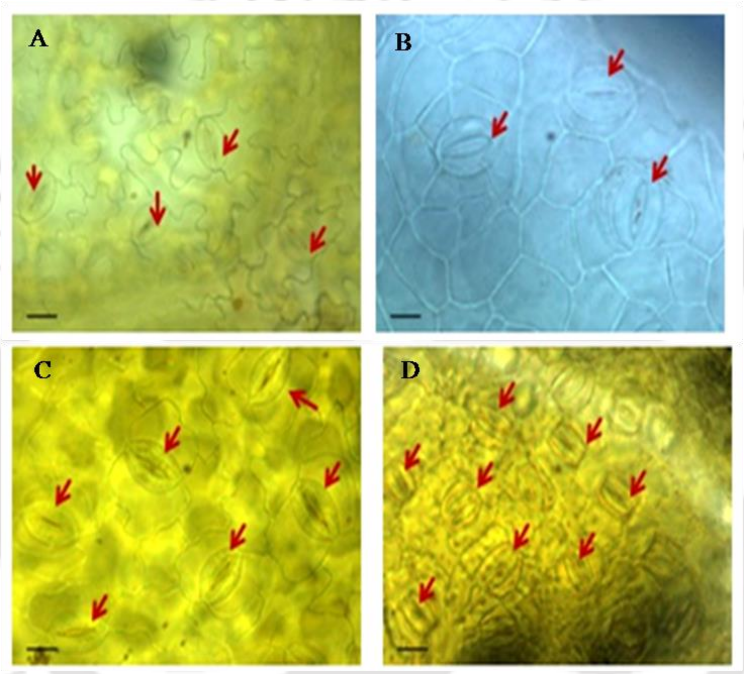


Fig. 4A.1: Microscopic analysis of abaxial leaf surface showing stomatal parameters (length, pore size, density) and epidermal cell area of (A) *P. pinnata,* (B) *J. curcas,* (C) *R. communis* and (D) *M. ferrea.* Images were taken at 40X magnification, Bar 6 µm

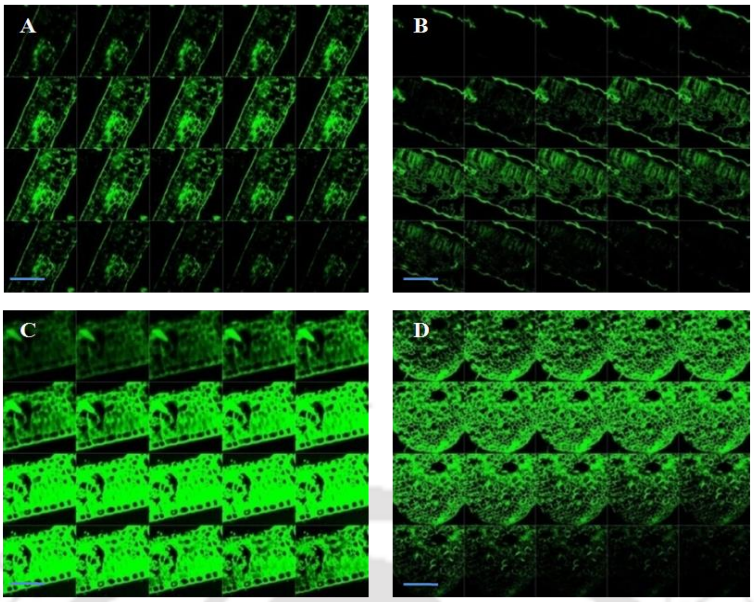


Fig. 4A.2: Microscopic analysis of leaf surface showing Z stacking for estimating cell volume in (A) *P. pinnata,* (B) *J. curcas,* (C) *R. communis* and (D) *M. ferrea.* Images were taken at 20X magnification, Bar 6µm

Table 4A.1 Mean value of stomatal parameters, epidermal cell area and cell volume of *P. pinnata, J. curcas, R. communis, and M. ferrea* collected from different geographical locations

Sl. no.	Plant	No.of stomata/ 0.53mm^2	Stomata length (µm)	Stomatal pore size (µm)	Epidermal cell area (µm^2)	Cell volume (µm^3)
1	***P. pinnata***	4	24.92±1.06	2.52±0.1	1206.26±0.18	41126.51±1.21
2	***J. curcas***	3	39.80±0.92	7.17±0.5	1751.63±0.57	57929.08±0.21
3	***R. communis***	4	42.37±1.19	6.35±0.1	1446.91±0.68	41298.82±1.45
4	***M. ferrea***	11	24.63±0.51	2.30±0.7	460.58±0.13	6898.82±0.73

All the cell phenotypic traits were observed to be higher in shrubs in comparison to trees except stomatal density which was in accordance with the study conducted by Beaulieu in 101 angiosperms of varying growth forms (Beaulieu et al., 2008b).

4A.3.2 Correlation of genome size and cell phenotypic traits

According to the literature, genome size was found to have correlations with environmental factors leading to cellular correlation with cell phenotypic traits (Beaulieu et al., 2008a). Environmental factors affect the cell phenotypic traits which, in turn, influence the genome size (Hodgson et al., 2010). Thus, a study was carried out with specific cell phenotypic traits like stomatal dynamics, epidermal cell area, and cell volume. The 2C nuclear DNA content or genome sizes of the four plants under study collected from different geographical locations of Assam were estimated in the previous Chapter 3. The results of the internal standardization procedure were taken into consideration to avoid the effect of inhibitors in the fluorescence fluorochrome. The estimated genome size of all the individuals of four plants was tabulated in **Table 4A.2** for better understanding. Variations in genome size were observed within the same species, thus, a correlation study of genome size with the cell phenotypic traits will possibly help to infer the reason for intraspecies genome size variation. Previously with regards to cell phenotypic study, researchers have reported stomatal length, density and epidermal cell area in association with nuclear DNA content (Tatum, Nunez, Kushad, & Rayburn, 2006). Upon comparing, a significant correlation between nuclear DNA content and cell phenotypic traits was observed in the present study as well ($P < 0.05$). Increasing stomatal length, pore size, epidermal cell area, and cell volume were associated with a decrease in nuclear DNA content, whereas nuclear DNA content was directly proportional to the stomatal density **(Figure 4A.3)** in the candidate plants. The results from the present study contradict from an earlier report on *Pteridium caudatum***,** where it had been reported that the nuclear DNA content was proportional to the overall guard cell length (Thomson & Alonso-Amelot, 2002). In addition, investigators have focused on angiosperm, where nuclear DNA content is significantly and positively correlated with stomatal length and epidermal cell area but negatively correlated with stomatal density (Beaulieu et al., 2008a; Hodgson et al., 2010). A strong positive association was reported between genome size and epidermal cell area, with genome size accounting for 59% of the total variation in the area. This relationship was linear, with no

indication of threshold effects. Species with small genome size had small epidermal cell areas, and species with large genome size had large epidermal cell areas (Knight & Beaulieu, 2008b). Connolly (2008) conducted a study in unicellular diatoms with 16 species. A significant positive relationship was found that explained 69% of the variation in cell volume for these diatoms. These results, combined with similar trends in the animal world, suggest that cell volume scaling with genome size is a general phenomenon for all life (Connolly et al., 2008). The results obtained from the present study thus contradict with the previous studies where genome size had a linear relationship with guard cell length and epidermal cell area. Such a contradiction can be possibly attributed to the inter-specific variation of genome size and cell phenotypic traits (Beaulieu et al., 2008b). In the present study, the strength of this relationship between genome size and cell phenotypic traits were found to be growth form dependent i.e., no significant results within trees and shrubs ($P \geq 0.05$). Despite the non-significant association between trees and shrubs, trees were characterized having greater genome size and smaller guard cell size compared to shrubs. There was a considerable range of guard cell size, among the four biofuel crops belonging to different ecotypes. The analysis across all the four species showed that 2C DNA content was significantly associated with cell phenotypic characters.

The main purpose of this study was to examine the relationship between genome size and cell phenotypic traits within biofuel crops using four potential species by a comparative approach. Therefore, results from the present study provide support for the general assumption that genome size has a correlation with stomatal parameters and cell traits, along with having a strong prediction of the minimum size of any given cell type. Among four different biofuel crops viz; *P. pinnata, J. curcas, R. communis and M. ferrea* belonging to two different growth forms (trees and shrubs), the striking result is the relationship set between the parameters. Additional factors such as the influence of individual genes and environmental perturbations also play an important role in determining the cell traits, perhaps only by modulating the final cell size from the minimum set by DNA content. Further investigation is required to examine whether it is true for other cell types. Further, how genome size responds to climatic catastrophes could be studied for our experimental plants to provide insight into the tempo of genome size evolution.

Table 4A.2 Estimation of the genome size of *P. pinnata*, *J. curcas*, *R. communis*, and *M. ferrea* collected from different geographical locations

Sl no.	Sample	Internal Standardization			
		P. pinnata	***J. curcas***	***R. communis***	***M. ferrea***
1	Dispur	2.54	0.89±0.1	1.04±0.01	1.45±0.11
2	Tezpur	2.49	0.86	1.01	1.37±0.01
3	Jorhat	2.49	0.86±0.02	1.01±0.02	1.37±0.11
4	Dibrugarh	2.48±0.005	0.84	0.96	1.33
5	Silchar	2.56	0.89	1.12±0.01	1.52
6	Tinsukia	2.48	0.84	1.01	1.33±0.11
7	Kokrajhar	2.54	0.86	1.11	1.52
8	Halflong	2.41	0.75	0.85	1.30
9	IIT Guwahati	2.49	0.87	1.03	1.41±0.01
10	Amingaon	2.44±0.14	0.87	1.03	1.42±0.11
11	Mangaldoi	2.49±0.01	0.87	1.03±0.01	1.4
12	Changsari	2.49	0.87	1.03	1.38
	Mean	2.49	0.85	1.0	1.4
	Standard deviation	0.04	0.03	0.07	0.09

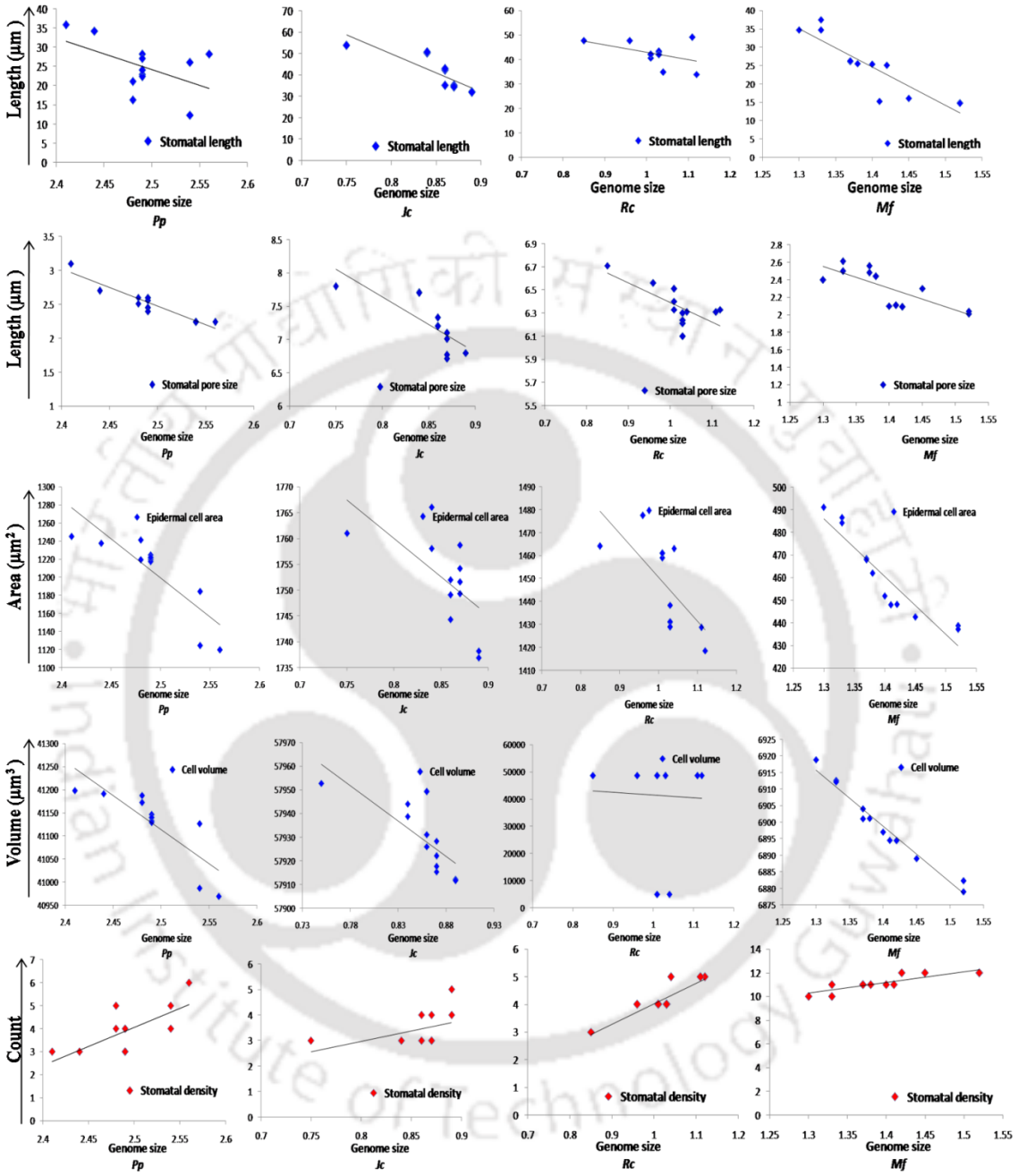


Fig. 4A.3: Genome size correlation with cell phenotypic characters in *P. pinnata*, *J. curcas*, *R. communis* and *M. ferrea* ecotypes, inverse relationship with stomatal length, pore size, epidermal cell area, and cell volume, whereas the linear relationship with stomatal density

4A.4 CONCLUSION

The main purpose of this study was to examine the relationship between genome and cell phenotypic traits within the potential biofuel crops that have been taken into consideration. Different cell phenotypic traits were studied and were selected according to their role in plant growth and development. Genome size was found to have an inverse relationship with epidermal cell area, guard cell length, stomatal pore size, and cell volume. However, it showed a linear relationship with stomatal density. The results confirmed the correlation of genome size with cell phenotypic characters, thus, it is being concluded that the intraspecies variation in genome size could be because of the environmental variations. This also provides an insight into the role of genetics in determining the ecological and evolutionary strategies of a species. This relationship will help to deduce genome size from fossil plant specimens as well. Leaf impression fossils with distinct guard cells are common in the fossil record for plants, and thus, the results presented here might help in deducing genome sizes for early land plants belonging to the family of the candidate plants. All the chosen plants are resilient to environmental extremities (drought and salt tolerant) which are of great potential for revealing genetic base unique to naturally stress-tolerant plants. Therefore, genome size estimates and study of phenotypic characters will also help us to select a suitable candidate for plant genome sequencing projects, which will be carried out in the future targeting different abiotic stress.

4B CORRELATION OF GENOME SIZE WITH MOBILE ELEMENT

4B.1 INTRODUCTION

From the perspective of molecular biology, an insight into genotype-phenotype relationship aids in deciphering the functionality of the species. In eukaryotes, the variation in genome size and genetic and phenotypic relations can be drawn through genetic elements known as transposons or transposable elements. Transposons (repetitive elements or mobile elements) are DNA sequences that can change their location within a genome; this sometimes alters the cell's genetic identity and genome size by creating or reversing mutations. Transposition often results in duplication of the same genetic material. These are the repetitive DNA, which occupies a large proportion of the nuclear genome leading to the variation of genome size in different plants. They have divided into Class I or retrotransposons and Class II or DNA transposable element based on the structure and their type of propagation in the genome. Class I or retrotransposons are sub-grouped into LTR and non-LTR retrotransposons based on the presence and absence of LTR at both ends of Class I element. LTR retrotransposons are further sub-divided into Ty1-*copia* and Ty3-gypsy element solely based on the differences in the arrangement of *Pol* genes. Among the transposons population, retrotransposons play a major role in genome size variations in plants and this variation can be ascribed to its differential accumulation (Kidwell, 2002). Through a process of replicative transposition, the LTR retrotransposons increase their copy number and are thus able to increase the genome size of the host (Boeke & Corces, 1989; Wilhelm & Wilhelm, 2001). Insertion of a retrotransposon into genes greatly affects gene expression and function (Hollister & Gaut, 2009; Kashkush et al., 2015; Lockton & Gaut, 2009). Recombination between retrotransposons can cause inversions, translocations, deletions and duplications; and in some cases large chromosomal rearrangements (Lonnig & Saedler, 2002; Vitte & Panaud, 2005). Hence, retrotransposons are an important source of genetic and phenotypic diversity in plants (Grandbastien et al., 2005; Kumar & Bennetzen, 1999; Mansour, 2007). In order to minimize the damaging effects caused by the retrotransposons, the plant undergoes restrain process in their genome thereby causing an epigenetic silencing of LTR retrotransposons (Okamoto & Hirochika, 2001) and experience some strong purifying selection pressure in the process of evolution (Baucom et al., 2009; Matsuoka &

Tsunewaki, 1999; Navarro & Schoen, 2002). The process of removing LTR retrotransposons from the genomes of host species is slower than retrotransposon proliferation (Vitte & Panaud, 2005). Various stress stimuli like environmental changes and polyploid formation affect retrotransposition (Baumel et al., 2002; Capy et al., 2000; Mansour, 2007; Parisod et al., 2010). Usually, the genome population is successfully populated by only restricted families of LTR retrotransposons. For example, in case of *Oryza australiensis*, only three families are responsible for doubling the genome size within the last three million years (Piegu et al., 2006) and nearly 10% of the barley genome is occupied by *BARE-1 copia*-like elements (Soleimani et al., 2006). Retrotransposons occupy most of the space in genome ranging from 5.5% in *Arabidopsis thaliana* to >50 % in *Zea mays* which in turn increase the C-value of the eukaryotic genome (Feschotte et al., 2002; Kumar & Bennetzen, 1999). Till date, Ty1-*copia* retrotransposons have been studied and characterized widely in plants. According to Flavell, there is an extreme heterogeneity observed in Ty1-*copia*-like retrotransposons of angiosperm due to high copy number and during evolution, it results in the accumulation of mutations which leads to genomic obesity (Flavell et al., 1992). *Copia* elements are well known for playing a vital role in genome diversification and evolution; most of them are found to be inactive during the plant development due to the accumulation of stop codons (Flavell et al., 1992; Hirochika & Hirochika, 1993). Nonetheless, due to the presence of a stress-responsive element in the promoter region of LTR, active elements have been reported which expresses during different stages of plant growth in response to biotic and abiotic stress (Kalendar et al., 2004). Furthermore, the activities have been observed during cell and tissue culture, pathogen infection and wounding (Hirochika, 1997). Use of retrotransposons is one of the potentially important tools for studying genetic diversity, genome evolution, gene mutation, gene cloning, gene tagging, and expression. Thus, characterization of Ty1-*copia* with respect to copy number could be helpful in understanding the genome organization and evolution.

The aim of this study was to verify whether the observed genome size variation within the species has any correlation with mobile elements specifically Ty1-*copia*.

4B.2 MATERIALS AND METHODS

4B.2.1 Plant material

Morphologically on the basis of growth and development among all the ecotypes, *P. pinnata* (NGPP46) collected from North Guwahati, Assam; *J. curcas* from Silchar, Assam (SILJC); *R. communis* from Tezpur, Assam (TEZRC) and *M. ferrea* from the part of North Guwahati, Assam (NGMF) were found to be better and considered in the present study for DNA isolation. Plants were maintained in the Greenhouse at Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati (IITG), Assam, India.

4B.2.2 Genomic DNA extraction

Genomic DNA was extracted from healthy and tender leaves according to the protocol described by Kesari (Kesari et al., 2009). To obtain a fine powder, about 5 g of young fresh leaves were collected and grounded in mortar and pestle using liquid nitrogen along with 2% PVP (Polyvinylpyrrolidone). The fine powder obtained from the leaves of the four plants under study were immediately transferred to 50 ml polypropylene centrifuge tube and suspended in two volumes of preheated extraction buffer at 65 °C for 30 mins in water-bath followed by gentle shaking at regular interval of 10 mins. Chloroform: isoamyl alcohol (24:1) was added in double volume and gently inverted for 15-20 times followed by centrifugation for 20 mins at 10,000 rpm at room temperature (RT). The upper aqueous was transferred carefully to a sterile 50 ml centrifuge tubes to avoid mechanical damage to DNA. To precipitate the DNA, two volumes of ice-cold isopropanol was added and shaken gently followed by 1 hr of incubation at - 20 °C. The precipitate was centrifuged at 12,000 rpm for 15 mins, and the supernatant was discarded. The obtained pellet was washed with 70% chilled ethanol by centrifugation at 12,000 rpm for 15 mins. The pellet was then air dried and suspended in 500 µl of TE buffer (pH- 8.0).

The extracted genomic DNA was purified by adding 3 µl RNaseA (10 mg/ml) and incubated at 37 °C for 30 mins. An equal volume of chloroform: isoamyl alcohol was then added and again centrifuged at 10,000 rpm for 5 mins. The aqueous phase was collected in a new vial followed by ethanol precipitation in the presence of 3 M sodium acetate (pH 5.2). The precipitated DNA was centrifuged and the pellet was washed in

70% ethanol followed by air or vacuum dried. The DNA extracted was then dissolved in 30-50 µl of TE buffer depending on the size of the pellet.

4B.2.3 Quantification and quality check of genomic DNA

The quality and quantity of the genomic DNA were assessed by agarose gel electrophoresis and spectrophotometry respectively. About 3 µl of the sample was run on 0.8% agarose gel to check its quality. The quantification was carried out by taking absorbance on Nanodrop (Eppendorf Biospectrometer, Germany) at 280 nm. Further, the purity of the extracted DNA was assessed by A260/280 ratio.

4B.2.4 PCR amplification of Ty1-*copia*

PCR amplification was carried out using isolated DNA from four plants using Mini Thermal Cycler (Applied Biosystems 9700, USA). The Reverse transcriptase (RT) and Reverse transcriptase-RNase H (RT-RH) domain of Ty1-*copia* was amplified from the genome of *P. pinnata, J. curcas, R. communis and M. ferrea* using degenerate primers (RT :- Forward : 5'ACNGCNTTYYTNCAYGG; Reverse: 5'ARCATRTCRTCNACRTA, RT-RH:- Forward: 5'-TATGTDGATGAYATGYTDATT-3'; Reverse: 5'-CCTCACATCWATRTGYTTBGW- 3') selected from close species (Flavell et al., 1992). PCR amplification was carried out in a final reaction volume of 25 µl containing 50 ng of genomic DNA, 1x assay buffer, 0.25 mM dNTPs each, 1.5 mM $MgCl_2$, 2.5 mM each primer and 1U of Taq DNA polymerase (B'LGenei, India). Thermal cycling conditions were: 95 °C for 5 mins; 35 cycles of 94 °C for 1 min; 47 °C for 1 min for RT and 44 °C for 1 min RT-RH; 72 °C for 1 min and the final extension at 72 °C for 5 mins. The amplification products were visualized on 1.5% agarose gel containing 0.5 µg/mL of EtBr in 1xTAE buffer and documented under BIO-RAD UV transilluminator. Amplified PCR products were purified and eluted using a Gel Extraction kit (Qiagen, Germany) according to the manufacturer's instruction.

4B.2.5 Cloning

The PCR eluted products were directly ligated into the high-quality ready-to-use TA cloning vector pTZ57R/T (Thermo Fisher Scientific, USA). The cloned product was sequenced by Macrogen sequencing service (South Korea). Before cloning competent

cells were prepared. The detailed procedure of competent cell preparation for transformation is described as follows.

4B.2.5.1 Competent cell preparation

First of all, a single colony of DH5-α strain was inoculated in 25 ml of Luria-Bertani (LB) in 250 ml bottle (incubation condition: 4-6 hrs at 37 °C at 250 rpm). Then, 1ml of saturated overnight culture was inoculated in 100 ml LB medium and kept for shaking at 37 °C. Once the OD at 600 increased to 0.4 (2-3 hrs), the culture was then transferred to two pre-chilled 50 ml falcon tubes. The tubes were centrifuged at 2700 x g for 10 mins at 4 °C. After the completion of centrifugation, the medium was removed and the cell pellet was suspended in 1.6 ml ice-cold 100 mM $CaCl_2$ and incubated on ice for 30 mins. The tubes were then centrifuged again at 2700x g for 10 mins at 4 °C, followed by the removal of the medium and the same step was repeated. The cell pellet thus obtained was then distributed in Eppendorf tubes with addition 0.5 ml of ice-cold 80% glycerol. Eppendorf tubes were then frozen in liquid nitrogen and stored at - 80 °C for future use.

4B.2.5.2 Transformation protocol

PCR product was added in the ligation mixture (10X ligation buffer, PCR product, TA vector 50 ng/µl, T_4 ligase 1U) and kept for 30 mins at 22 °C. DH5-α competent cells were thawed on ice for 15-30 mins and approximately 4 µl of ligation mixture was added to the competent cells. The competent cells mixed with ligation mixture were kept on ice for 20-30 mins together with mixing at every 5 mins of the interval. Heat shock treatment was then given to the competent cells in a water bath at 42 °C for 90 secs and was immediately kept on ice for 2 mins. LB broth (200 µl) was then added to the competent cells and was incubated at 37 °C on a shaker (180 rpm) for 1 hr. After the incubation of an hour, cells were centrifuged at 5000 rpm for 1 min, followed by the removal of approximately 600 µl of LB broth. The remaining broth was mixed well with the pellet and around 150 µl broth containing transformed cells were spread on LB agar (Ampicillin 100 µg/ml). The LB agar plates were incubated overnight at 37 °C for suitable growth.

4B.2.6 Colony PCR

Using M13/pUC primer: forward:- 5'- GTAAAACGACGGCCAGT-3' and reverse:- 3'- CA GTA TCG ACA AAG GAC-5' colony PCR was carried out with 6 µL of the reaction mixture which contained each transformed colony in separate PCR tube. PCR amplification was carried out with 2X Green GoTaqPCR master mix pH 8.5 (Promega, USA) containing 400 µM dNTP, 3 mM $MgCl_2$, 1U of Taq DNA and nuclease-free water (Promega, USA). Thermal cycling conditions were maintained at 95 °C for 5 mins; 35 cycles of 94 °C for 30 sec; 60 °C for 30 sec; 72 °C for 1 min and the final extension was for 72 ^{o}C for 5 mins. For colony PCR, from LB agar plate multiple transformed colonies were selected and the amplification products were visualized on 1% agarose gel containing 0.5 µg/ml of EtBr in 1xTAE buffer followed by visualization under BIO-RAD UV transilluminator.

4B.2.7 Plasmid isolation and sequencing

The transformed cell (positive colonies) were inoculated in 4 µl of LB broth and incubated overnight on a shaker at 37 °C. The overnight grown culture was then centrifuged at 12,000 rpm at 25 °C. The cell pellet obtained after centrifugation was dissolved in 300 µl of P1 buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 µg/ml RNaseA). P2 buffer (200 mM NaOH, 1% SDS) (300 µl) was then added in the same Eppendorf and incubated at 25 °C for 5 mins. After the 5 mins of incubation, chilled P3 buffer (3.0 M potassium acetate pH 5.5) (300 µl) was added and the reaction was incubated on ice for another 5 mins followed by centrifugation at 14,000 rpm for 10 mins at 25 °C. The supernatant was pipetted out with utmost care and mixed with 166 µl of 50% PEG 6000 and 118 µl of 5 M NaCl followed by centrifugation at 14,000 rpm for 10 mins at 25 °C. The supernatant was discarded and the Plasmid DNA pellet was then washed with 500 µl of 70% ethanol at 10,000 rpm for 10 mins. The pellet was air dried and dissolved in 30 µl of TE buffer. The dissolved plasmid was visualized on 1 % agarose gel containing 0.5 µg/ml of EtBr in 1xTAE buffer and documented under BIO-RAD UV transilluminator. Macrogen sequencing service (South Korea) then sequenced the cloned plasmid DNA.

4B.2.8 Sequence analysis

The sequences obtained from cloning were subjected to either BLASTN or BLASTX analysis (http://www.ncbi.nlm.nih.gov). Further, the edited sequences were analyzed through BLASTN against Repbase (http://www.girinst.org/censor/) and the Repeat Masker program (http://www.repeatmasker.org/). Using MUSCLE program the nucleotide sequence alignment was conducted (http://www.ebi.ac.uk/Tools/msa/muscle/) for the generating multiple sequence alignment followed by sequence annotation in Gene Doc V2.7 (http://genedoc.software.informer.com/2.7/). Using the Neighbor-Joining method, a phylogenetic tree was constructed using MEGA 6 software (http://www.megasoftware.net/).

4B.2.9 Dot blotting

Genomic DNA extracted from the four plants under study and heterogeneous 0.9 kb PCR product were denatured in 0.4 M NaOH for 30 mins followed by heating at 100 °C for 5 mins which were then quickly chilled. Denatured genomic DNA and PCR product were spotted on a positively charged nylon membrane (Hybond-N+ Amersham-Biosciences, England) in various amounts; genomic DNA: 25 ng, 50 ng, 100 ng, 200 ng, 400 ng; PCR product: 0.4 ng, 0.8 ng, 1.2 ng, 1.6 ng for *P. pinnata.* In case of *J. curcas* denatured genomic DNA and PCR product was spotted as genomic DNA: 50 ng, 100 ng, 200 ng, 400ng, and PCR product: 0.1 ng, 0.25 ng, 0.5 ng, 1 ng. For *R. communis*, the concentrations of spotted genomic DNA were 50 ng, 100 ng, 200 ng, 400 ng and the concentration of PCR product were 0.4 ng, 0.8 ng, 1.2 ng, 1.6 ng. The concentration of genomic DNA spotted for *M. ferrea* were 50 ng, 100 ng, 200 ng, 400 ng, 600 ng and PCR product were 0.1 ng, 0.25 ng, 0.5 ng, 0.75 ng, 1 ng, and 1.5 ng respectively. For the probe, ~900 bp product of (RT-RH) Ty1-*copia*-like retrotransposon was labelled by Biotin Deca Label DNA Labeling Kit (Fermentas, Germany). The hybridization was performed at 65 ^{o}C for 18-20 hrs (6x SSC, 5x Denhardt's solution, 0.5% SDS and 100 µg/ml salmon sperm DNA). After washing, the signal was visualized immunologically using Biotin chromogenic detection Kit (Fermentas, Germany) according to the manufacturer's protocol. Analysis of dot blot was performed using ImageJ 1.48v software (http://rsbweb.nih.gov/ij/). Copy number was calculated using the equation given by Ma

et al. (2008): Copy number = (the size of the haploid genome x average proportion of nuclear genomic DNA hybridizing to the probe) / size of the probe element.

4B.3 RESULTS AND DISCUSSIONS

The candidate plants: *P. pinnata, J. curcas, R. communis,* and *M. ferrea* collected from twelve different geographical locations of Assam were processed to estimate genome size (Chapter 3). Intraspecies genome size variations were observed in the candidate plants. The differences in nuclear DNA content among the ecotypes collected from different locations of Assam, do not seem to be caused due to polyploidization because the chromosome numbers in all the analyzed ecotypes for the candidate plants were identical as per the result obtained in chapter 3. The main reason for such a variation could be an increase or decrease in the copy number of repetitive DNA population especially retrotransposons which are known as a frequent source of genome size variation in angiosperms (Bennetzen et al., 2005).

Retrotransposons are important participant in genome diversification in plants as they are responsible for insertional mutagenesis and genome obesity (Zedek et al., 2010). To get the better insight, knowledge of retrotransposons and their copy number in the genome, the study was conducted for the isolation of partial Ty1-*copia* sequence from NGPP46, SILJC, TEZRC, and NGMF.

4B.3.1 Isolation and confirmation of Ty1-*copia* retrotransposons in *P. pinnata, J. curcas, R. communis,* and *M. ferrea* genome

In this study, partial *Pol* gene domains of retrotransposons were successfully amplified. RT domain of Ty1-*copia* has been amplified in *P. pinnata* and RT-RH domains were amplified in *J. curcas*, *R. communis* and *M. ferrea* genome using degenerate primers which belongs to Ty1-*copia*-like retrotransposons. An anticipated amplicon size of 260 bp of RT and 800 bp to 900 bp of RT-RH gene was amplified and eluted from the gel followed by quantification using Nanodrop for further experimental analysis **(Figure 4B.1).** The absorbance ratio at A260/280 was found between 1.7-1.9 which indicated the purity of the sample. In all the four plants, the size of PCR amplicons was the same, which is in line with the reported literature which suggests that the Ty1 *copia* motifs are conserved across all the plant kingdom. (Kumar & Bennetzen, 1999). Amplified RT and

RT-RH sequences were then used for cloning and the further RT-RH sequence was used in the dot blot hybridization.

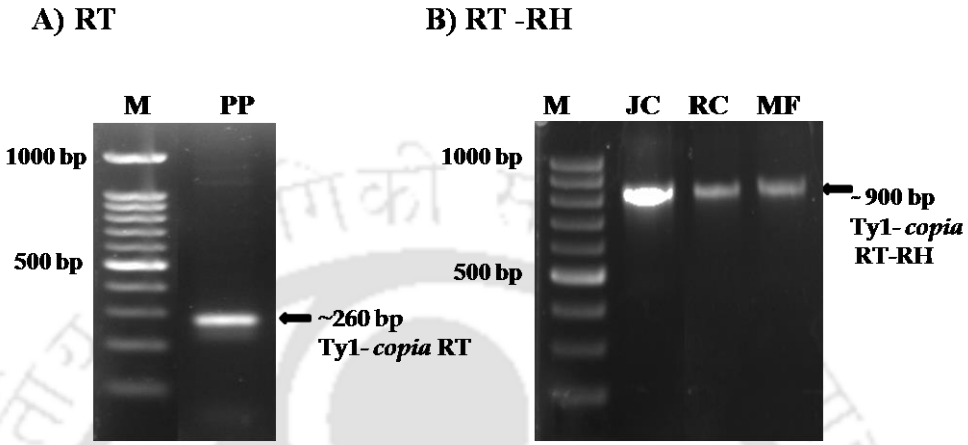


Figure 4B.1: PCR amplified product: A) RT and B) RT-RH of Ty1-*copia* retrotransposons. M: 100 bp DNA ladder, PP: *P. pinnata*, JC*: J. curcas*, RC: *R. communis and* MF*: M. ferrea* Ty1-*copia* PCR product

The PCR amplicons of RT and RT-RH from *copia* were cloned into the TA cloning vector using the methodology mentioned in section 4B.2.5. Multiple transformed colonies of RT and RT-RH opted for colony PCR. After cloning of PCR amplicons, plasmids were extracted from randomly selected colonies for Sanger sequencing. Homology-based search (BLASTN and BLASTX) for cloned sequences were carried out which revealed similarity (approximately 89-95% identity) to those of other plant species. Retrotransposons clones were then reconfirmed using *Gypsy* database 2.0, Repbase and Repeat Masker programs. The annotated sequences were deposited to NCBI-GenBank database under the accession number: *Pongamia* Ty1-*copia* RT (KP202847.1-KP202834.1, MH397570-MH397584), *J. curcas* Ty1-*copia* RT-RH (MK036332-MK036339), *R. communis* annotated Ty1-*copia* RT-RH sequences (MK036340-MK036345) and Mesua Ty1-*copia* RT-RH sequences were submitted to GenBank database under accession number KU507513-KU507530.

The Ty1-*copia* clones were found to be high in AT bases, the average AT/GC ratio was estimated to be 1.47, 1.55, 1.56 and 1.48 for *P. pinnata*, *J. curcas*, *R. communis*,

and *M. ferrea* respectively. In other species also the AT bases were reported to be higher in comparison to GC bases (Stergiou et al., 2002). This could be because of its lower thermal stability in comparison to GC base pair, which in turn helps in the mobilization of retrotransposons (Smarda & Bures, 2012). Translation of the PCR amplified sequences was performed using ORF finder of NCBI, which indicates that the Ty1-*copia* RT and RT-RH sequences contained in-frame stop codon. This also supports the finding that AT bases were higher than GC bases as most of the GC rich sequences are a coding region of genes (Smarda & Bures, 2012). Functional RT and RT-RH fragment were missing in the present sequences thus making them transcriptionally inactive. In higher plants the majority of the RT, sequences were mostly found to be inactive, containing stop codons and frame shifts (Stergiou et al., 2002). Mutations or mistakes made by the reverse transcriptase during reverse transcription could be the reason for defective retrotransposons.

4B.3.2 Multiple sequence alignment

To get a fair idea about *copia* partial sequences homology, we compared different clone sequences of *P. pinnata, J. curcas, R. communis* and *M. ferrea* using MUSCLE tool. The authentication of sequences was also confirmed using Repbase and Repeat masker programs. For conducting MSA, the nucleotide sequences were used. The nucleotide sequence of Ty1 *copia* of RT and RT-RH domain revealed the presence of an in-frame stop codon(s) suggesting the absence of a potential functional open reading frame (ORF), which could be one of the reasons for the repression of transcriptional activity of these sequences. Multiple sequence alignment of protein was not possible due to the presence of stop codons in nucleotide sequences. Nucleotide sequences of *P. pinnata, J. curcas, R. communis,* and *M. ferrea* were used for the construction of multiple sequence alignment having the range between 32.68% and 100%, 38.73% and 98.89%, 20% and 98.66%, 35.53 % and 96.65 % respectively. Representation of the conserved region has been shown in **Figure 4B.2-5.**

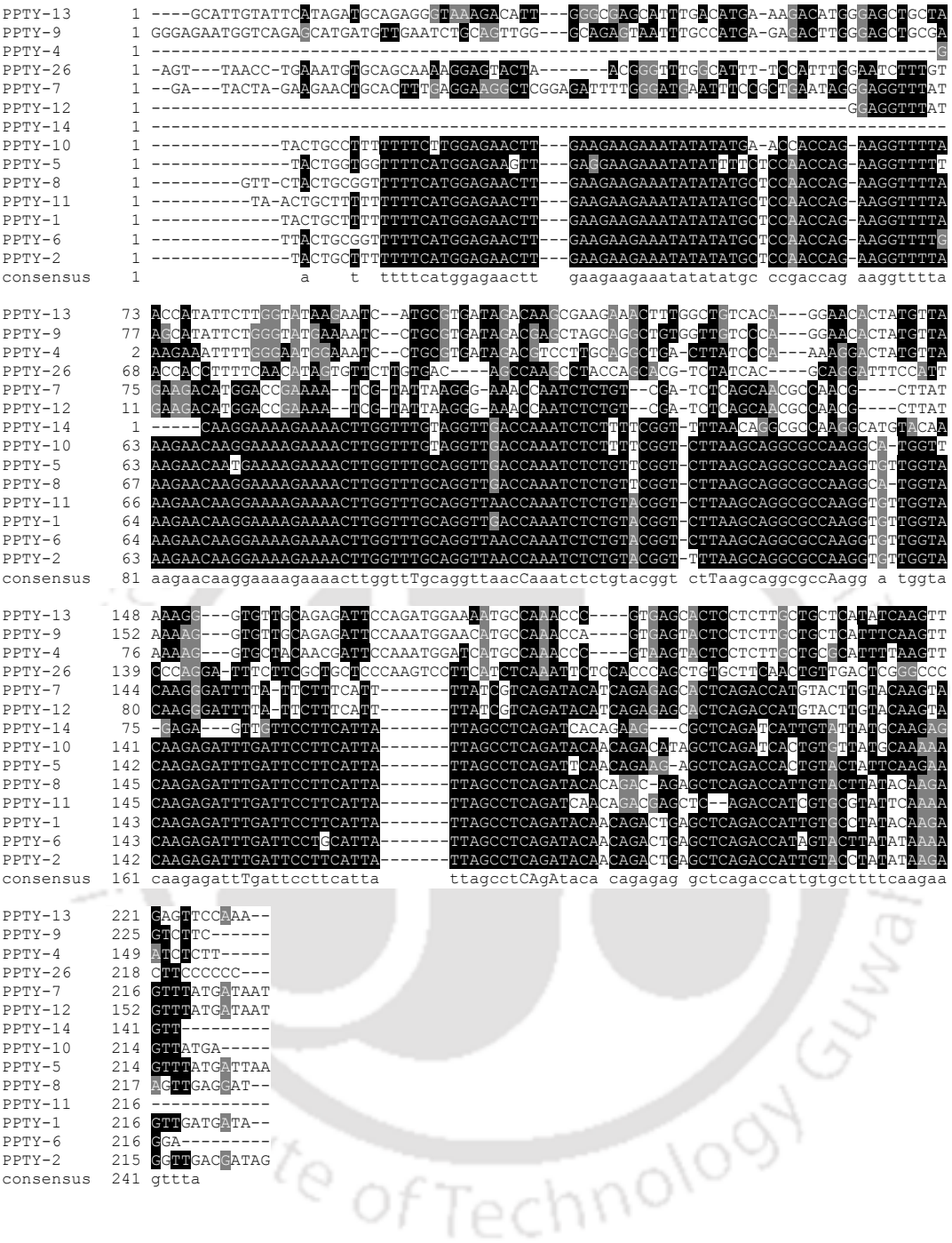
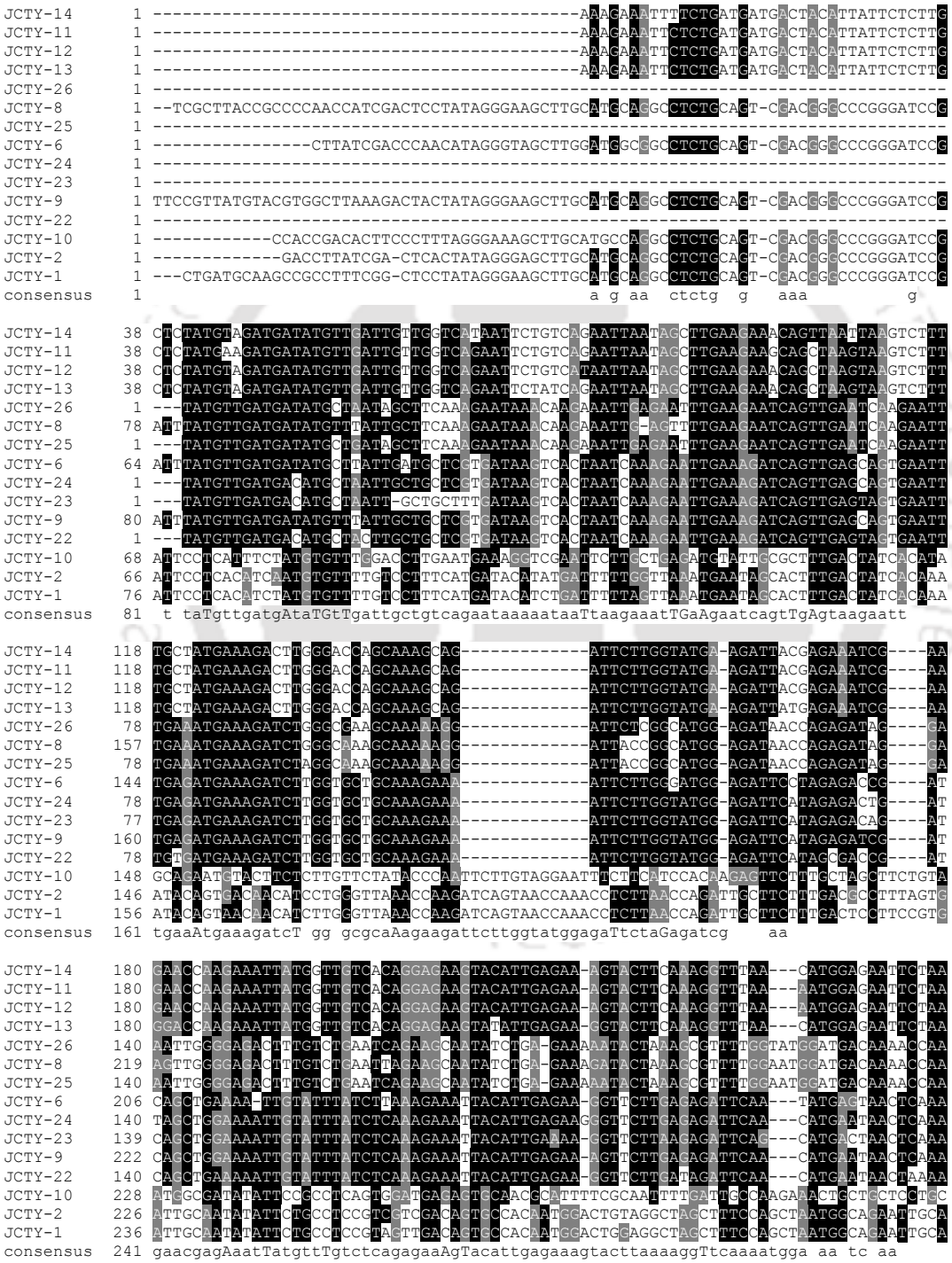
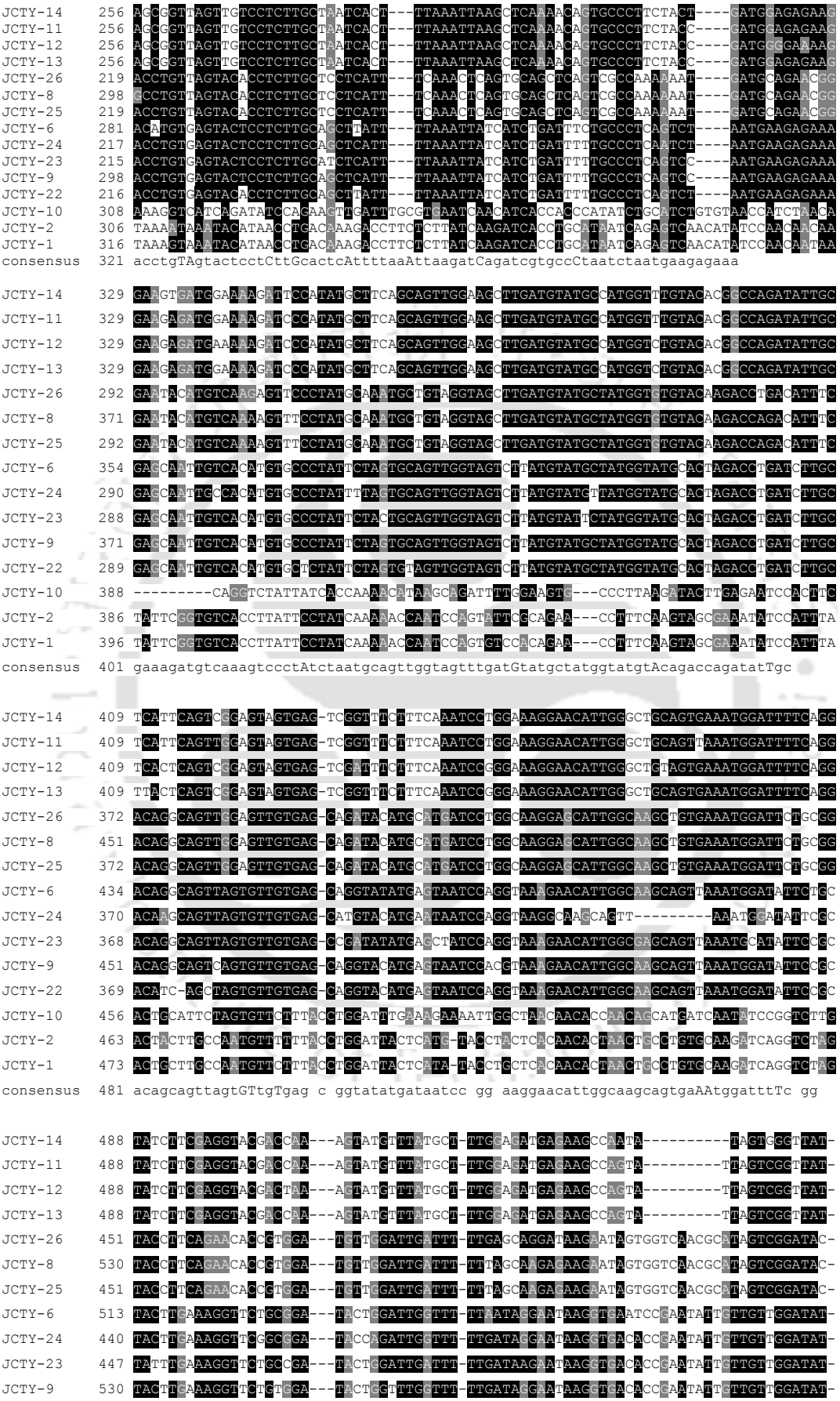


Figure 4B.2: Multiple sequence alignment of the nucleotide sequences corresponding to the partial reverse transcriptase (RT) domain of the Ty1-*copia* retrotransposons from *P. pinnata* transcriptome library. Shaded letters represent the conserved residues.

In *P. pinnata* the highest sequence homology (100%) existed between sequences PPTY7 (*Pongamia pinnata* Ty1- *copia*) and PPTY 29; lowest (32.68%) between PPTY 4 and PPTY 6. Some sequences showed conserved 'TTTTCATGGAGAACTT' nucleotide

sequences at 5′ end and 'GCTCAGACCATTGTGC' nucleotide sequences in the 3' end. In the middle, nucleotide sequence 'AAGAACAAGGAA' was found to be highly conserved in PPTY 14, 10, 5, 8, 11, 1, 6 and 2. Rest of the sequences was observed to have less conserved nucleotide sequences which indicate the presence of heterogeneity. Almost 50% of the sequences were found to be highly conserved at the nucleotide level.





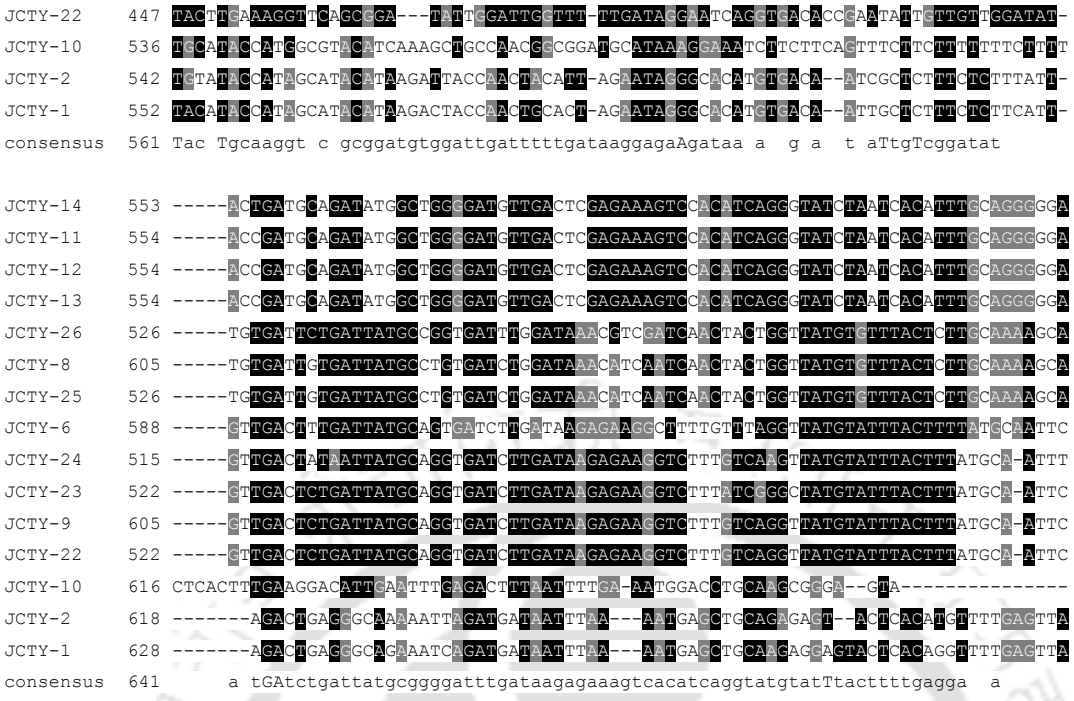
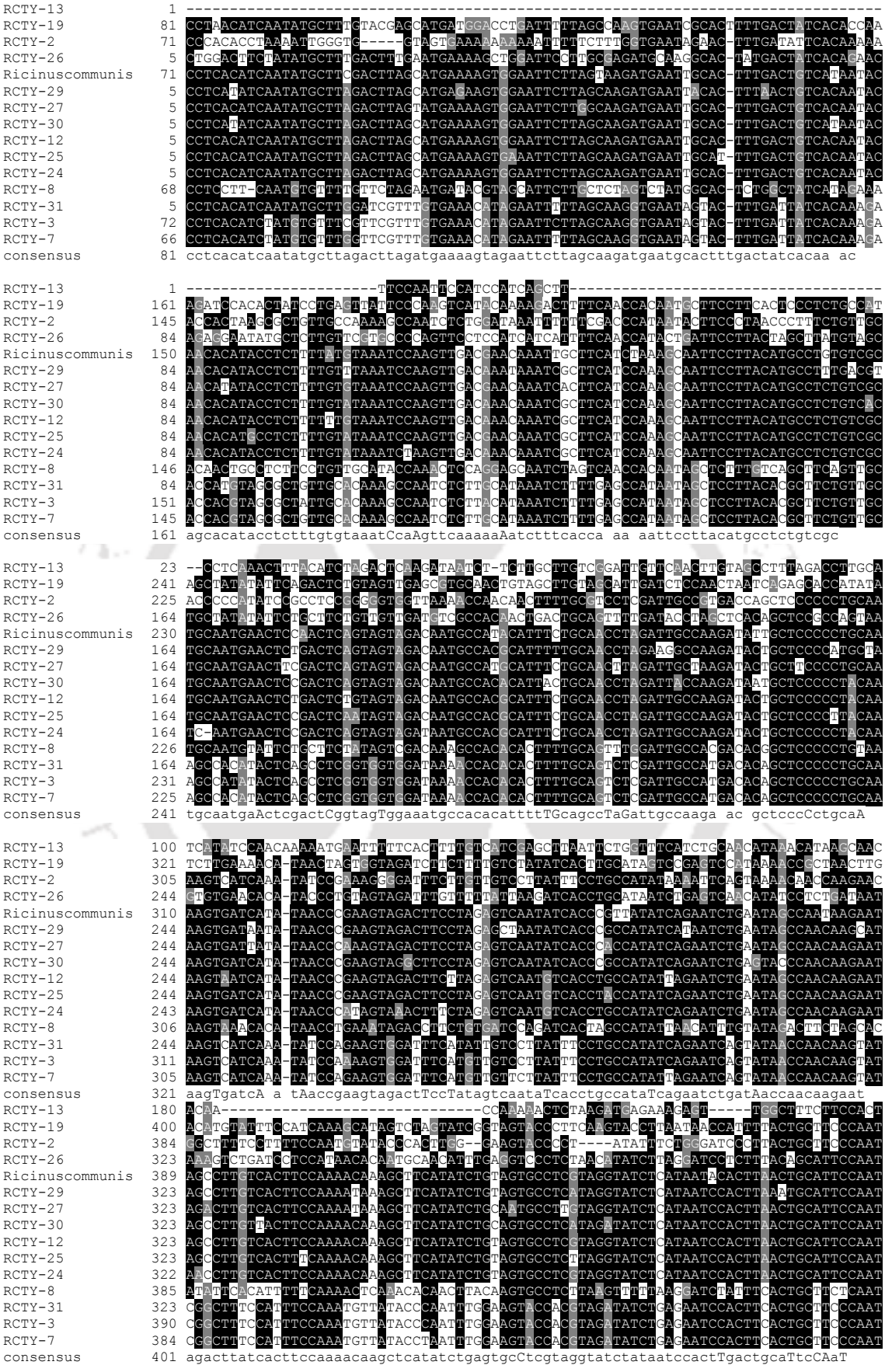
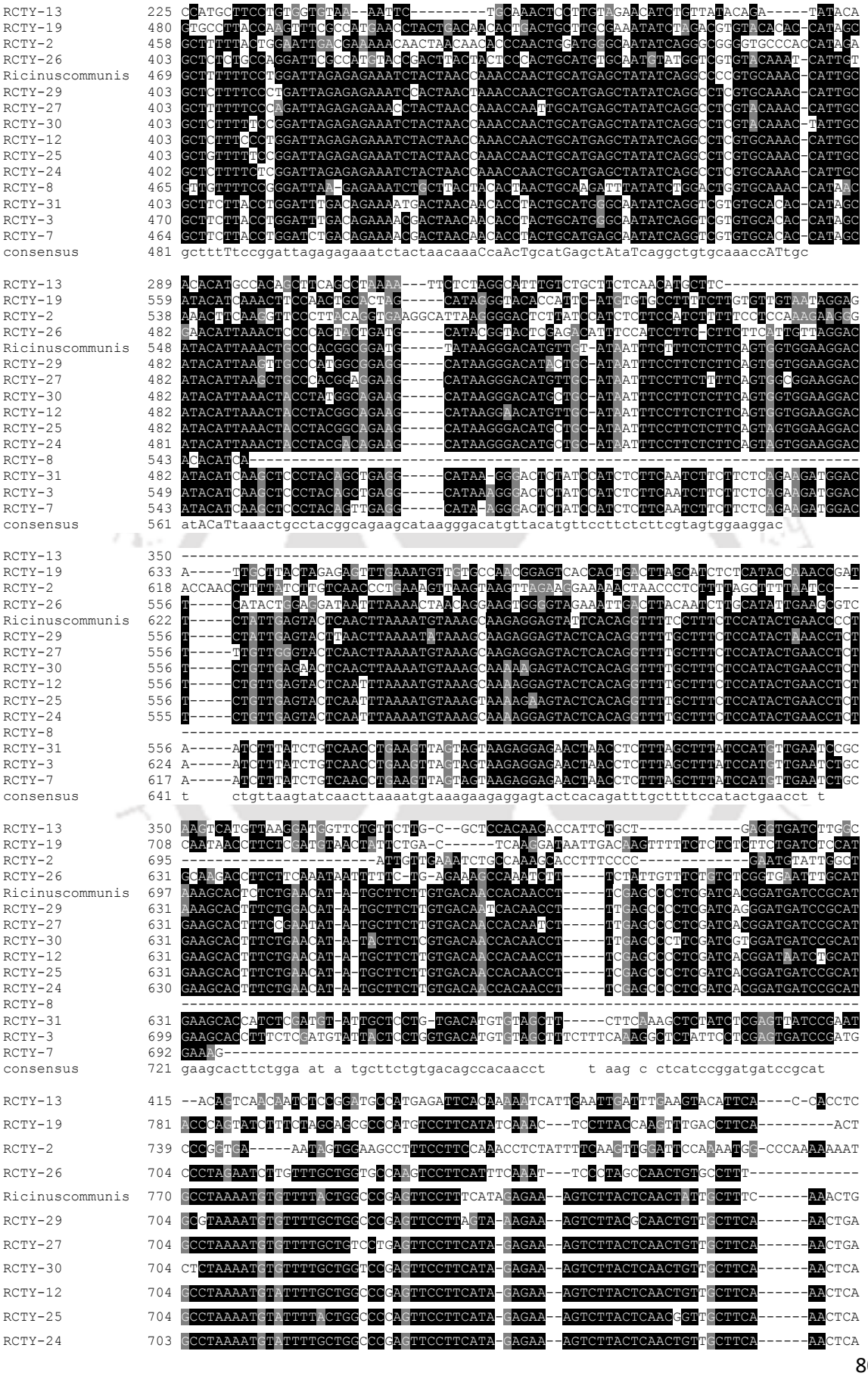


Figure 4B.3: Multiple sequence alignment of the nucleotide sequences corresponding to the RT-RH domain of the Ty1-*copia* element in *J. curcas.* Shaded letters represent the conserved residues

In the case of *J. curcas*, the highest sequence homology (98.89%) existed between sequences JCTY 5 (*Jatropha curcas* Ty1- *copia*) and JCTY 19; lowest similarity (38.73%) was found between JCTY 10 and JCTY 25. Some sequences showed conserved 'TATGTTGATGATATGTT' nucleotide sequences at 5′ end. Highly conserved nucleotide sequences were observed in the middle portion while in the 3′ end 'TGATTATGCG' and 'TTTACTTTTGAGGA' was found to be the conserved regions. Except the beginning of 3′ end rest of the sequences was observed to have conserved nucleotide sequences which indicate sequence homology whereas a detailed study of multiple sequence alignment of protein would have led to more heterogeneous sequences.





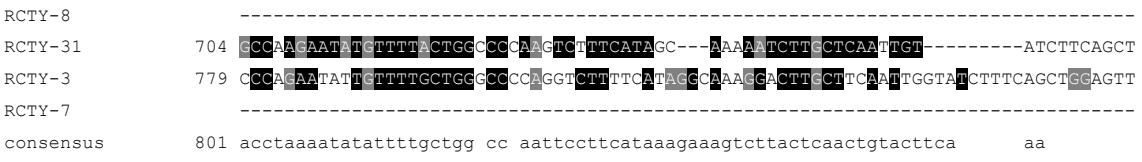
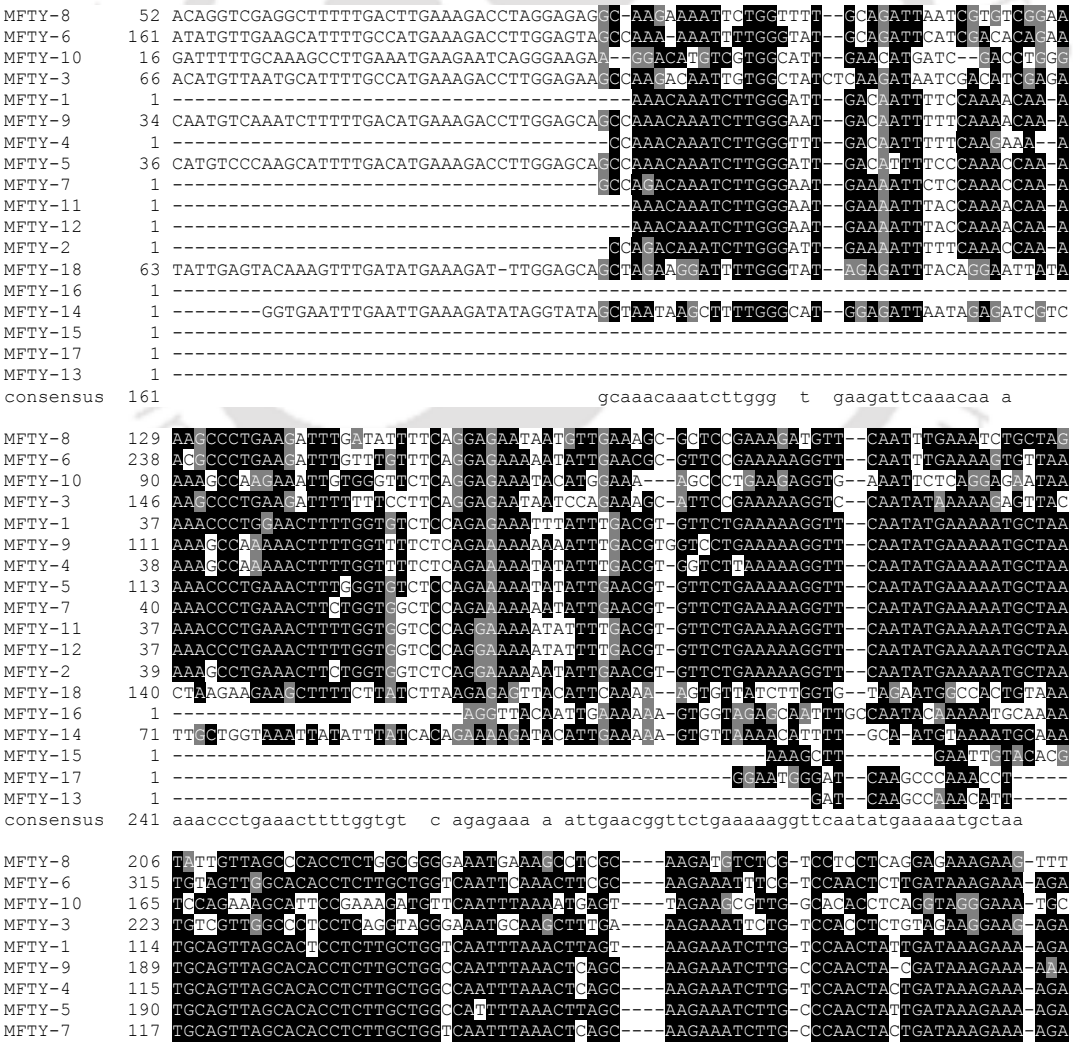
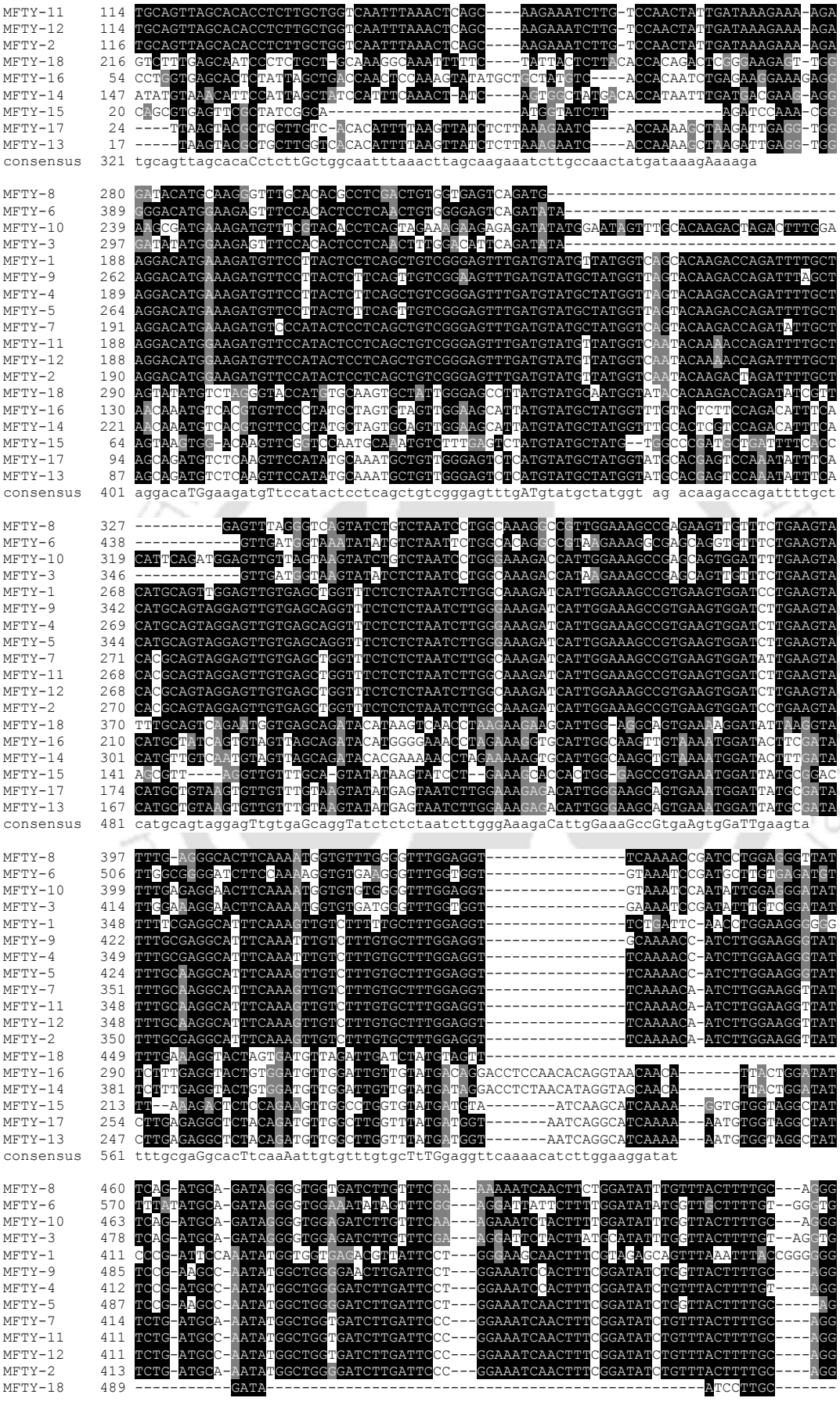


Figure 4B.4: Multiple sequence alignment of the nucleotide sequences corresponding to the RT-RH domain of the Ty1-*copia* element in *R. communis.* Shaded letters represent the conserved residues

Sequences RCTY 11 (*Ricinus communis* Ty1- *copia*) and RCTY 23 showed the highest similarity (98.66%) in case of *R. communis* whereas, sequences RCTY 14, RCTY 15 and RCTY 23 has the lowest similarity of 20%. Highly conserved sequences were observed throughout the nucleotide sequences.





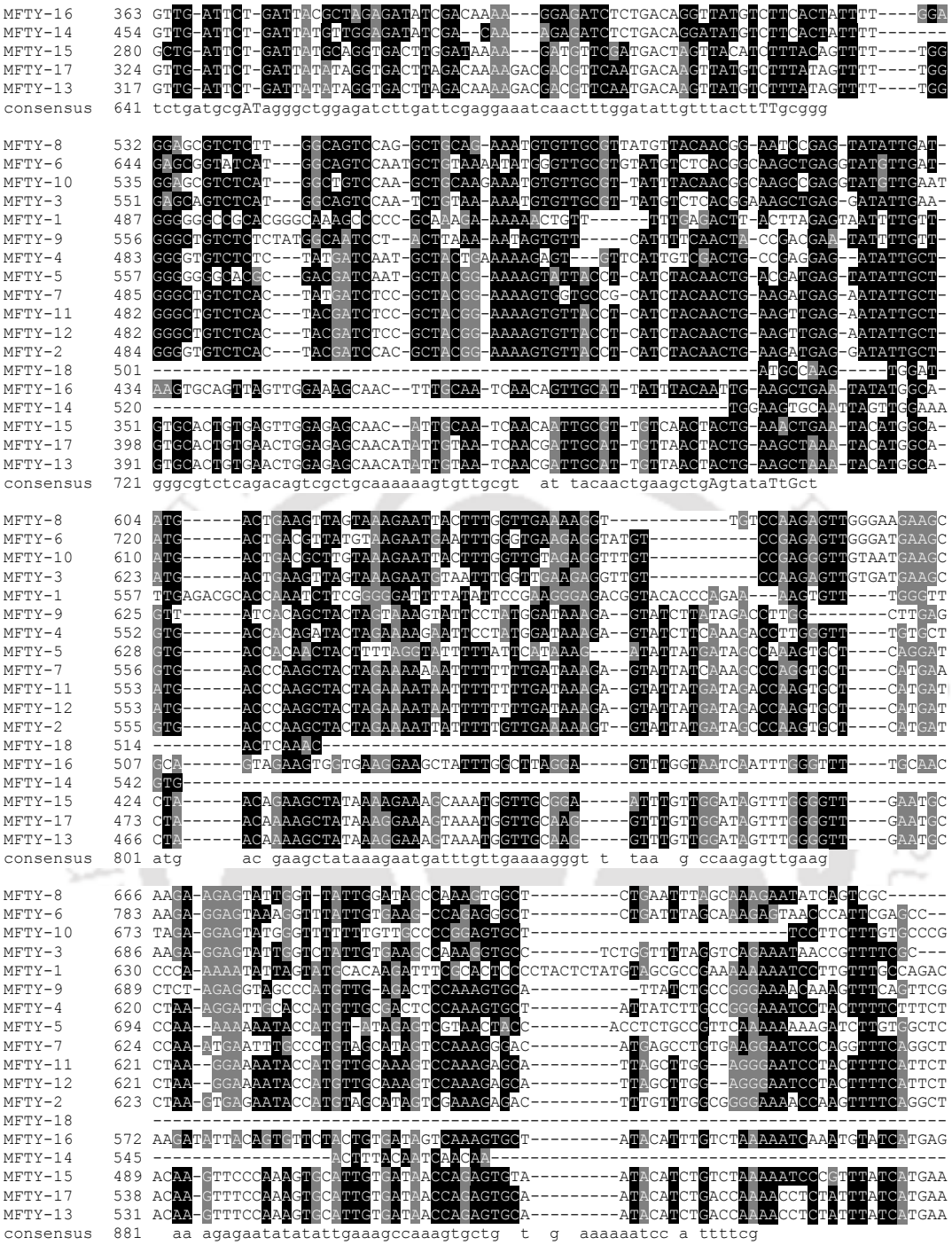


Figure 4B.5: Multiple sequence alignment of the nucleotide sequences corresponding to the RT-RH domain of the Ty1-*copia* element in *M. ferrea.* Shaded letters represent the conserved residues

In case of *M. ferrea*, the highest sequence homology (96.65%) existed between the sequences MFTY 13 (*Mesua ferrea*Ty1- *copia*) and MFTY 17 whereas; the lowest similarity (35.53%) was accounted between MFTY 8 and MFTY 15. Some sequences showed conserved 'GCAAACAAATCTTGGG' nucleotide sequences at 5′ end and 'CCAAAGTGCTG' nucleotide sequences in the 3' end. In the middle portion, the nucleotide sequences were found to be highly conserved in MFTY 8, 6, 10, 3, 1, 9, 4, 5, 7, 11, 12 and 2.

Overall the isolated sequences were highly heterogeneous as confirmed by multiple sequence alignment. High sequence heterogeneity was also observed in the previous studies, including chickpea, *J. curcas* etc. (Alipour, Tsuchimoto, Sakai, Ohmido, & Fukui, 2013; Rajput & Upadhyaya, 2010). Sequence heterogeneity is influenced by the copy number of these transposons. Presence of repetitive elements is a recognized reason for the variation in genome size of many plant species where an abundance of retrotransposon is quite high (Meyers et al., 2001). The extreme case of heterogeneity is observed in the potato genome (*Solanum tuberosum*) which contain hundreds of copies of transposons (Flavell et al., 1992). As the study was conducted with nucleotide sequences, most of the conserved sequences were absent which could be observed if multiple sequence alignment would have been carried out using protein sequences.

4B.3.3 Cluster analysis

To investigate the evolutionary relationship of Ty1-*copia* element clones were compared with another *copia* group of retrotransposons from different organisms obtained from the GenBank database. Phylogenetic analysis was conducted with 200 bootstrap replicates using NJ method with aid of MEGA 6 program. From various organisms, a total of 38 RT sequences of Ty1-*copia* for *P. pinnata,* RT-RH sequences of Ty1-*copia*: 35 for *J. curcas*, 35 for *R. communis* and 33 for *M. ferrea* were compared with 14 *P. pinnata,* 25 *J. curcas,* 27 *R. communis* and 18 *M. ferrea copia* clone sequences **(Figure 4B.6-9).** Phylogenetic analysis confirmed that Ty1-*copia* of above-mentioned plant sequences did not cluster into a single group; in fact, they were divided into more than one distinct group across the tree. According to GyDB database classification and sequences homogeneity of RT clones with previously identified sequences revealed that the sequences of *P. pinnata* probably belongs to the seven different groups based on *copia* lineages viz. "*Tork*",

"*Bianca*", "*Oryco*", "*PyRE1G1*", "*BARE*-1", "*Osser*" and "*Hopscotch*". The first and second group belongs to *Hopscotch* and *Osser* which consisted of 2 *Pongamia* Ty1-*copia* clones (PPTY) each. The third group belongs to *BARE1* containing 4 clones. The fourth group contained 1 PPTY clone which belonged to *PyRE1G*1 lineage followed by *Oryco* (fifth group) which contained 2 PPTY clones. *Bianca* which contained 1 PPTY clone was the sixth group in the cluster analysis and highest clones were found to be in group seven, containing 17 clones of PPTY. The RT-RH domain of Ty1-*copia* sequences of *J. curcas* belongs to "*Oryco*", "*Tork*", "*TOBAA*" lineages and also has been clustered in an unknown group. *Oryco* lineage consisted of 5 *Jatropha* Ty1-*copia* clones (JCTY). Four clones of JCTY were found to be clustered in both *Bianca* and *TOBAA* group. Highest JCTY clones were found to be clustered in an unknown group which did not have any characterized elements but nearly associated with *A. thaliana*. In case of *R. communis* groups identified were "*Oryco*", *Sire*", "*Bianca*", "*Martian*", "*Tork4*" and "*TOBAA*". Oryco consisted of 19 *Ricinus* Ty1-*copia* clones (RCTY), which was the highest among all the lineages. *Sire, Bianca, Martian,* and *Tork4* contained 1 RCTY clone each and 4 RCTY clones were clustered in *TOBAA* lineage. Lastly, sequences of *M. ferrea* have been observed belonging to the "*Osser*" and "*TOBAA*" type of retrotransposons group. *Osser* contained 2 *Mesua* Ty1-*copia* clones (MFTY) and TOBAA contained 12 MFTY clones. In addition, a group containing 5 MFTY clones did not cluster with any characterized retroelements but associated with *A. thaliana*. These results reflect that the existing Ty1-*copia* retrotransposons population in the genome is heterogeneous and harbor more than one lineage. The retrotransposons from related species, in general, made a cluster (Ahmed et al., 2011). The isolated sequences clustered into different groups suggesting that the existing population harbor more than one lineage of Ty1-*copia* which could have proliferated at different rates in different time intervals and occupied a significant proportion of the nuclear genome. Earlier studies have also shown the presence of different evolutionary Ty1-*copia* lineages in plants (Ahmed et al., 2011; Kolano et al., 2012). It is obvious that elements of a given category are clustered together and within a given category, elements show a close relationship with the elements from evolutionary distant taxonomic groups, which substantiates the widely accepted notion of horizontal transmission of retrotransposons to which Ty1-*copia* belongs (Woodrow et al., 2012). Till date, heterogenous Ty1-*copia* group retrotransposons were found in every plant species

tested so far. In the case of *A. thaliana*, 10 different Ty1-*copia* elements belonging to 2 subgroups were reported (Konieczny et al., 1991). A similar report was found for potato where 30 sequences were clustered in 6 sub groups (Flavell et al., 1992). As the current study was based on the limited number of RT and RT-RH sequences, it is our belief that extensive study of the whole genome could possibly end with more *copia* lineages.

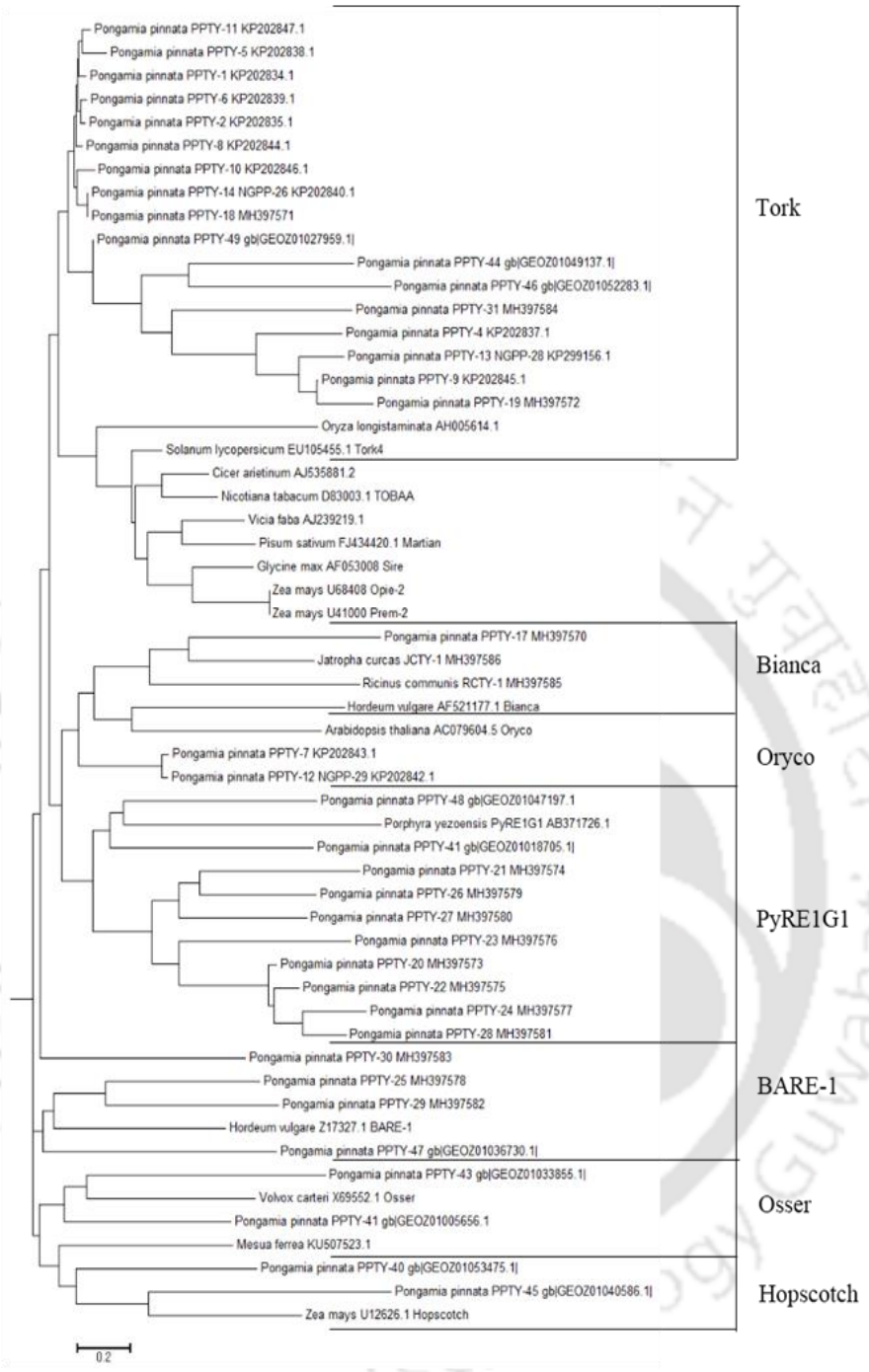


Figure 4B.6: Phylogenetic relationships among the nucleotide sequences of RT domain of Ty1-*copia* clones from *P. pinnata* and comparison with sequences from other species using the NJ method

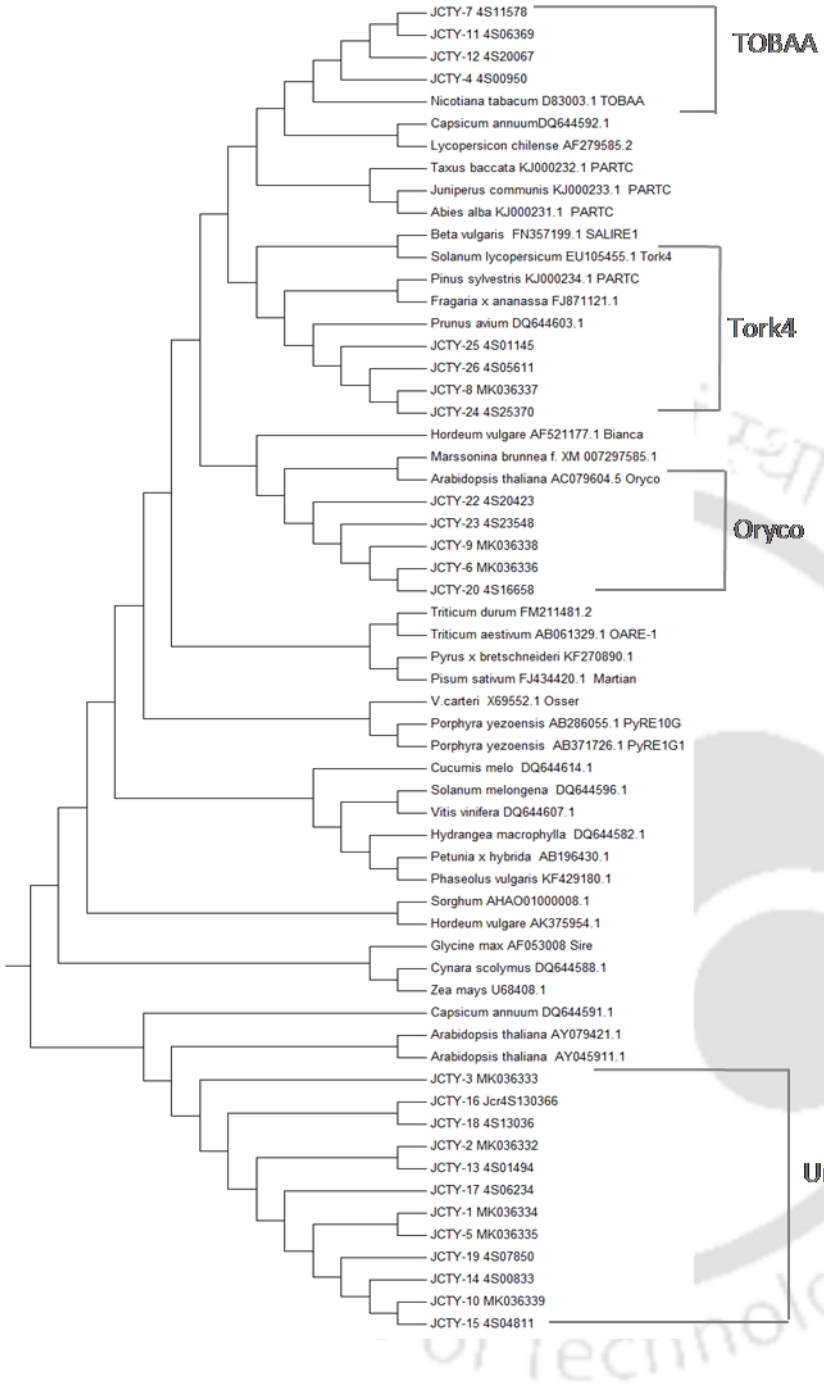


Figure 4B.7: Phylogenetic relationships among the nucleotide sequences of the RT-RH domain of Ty1-*copia* clones from *J. curcas* and comparison with sequences from other species using the NJ method

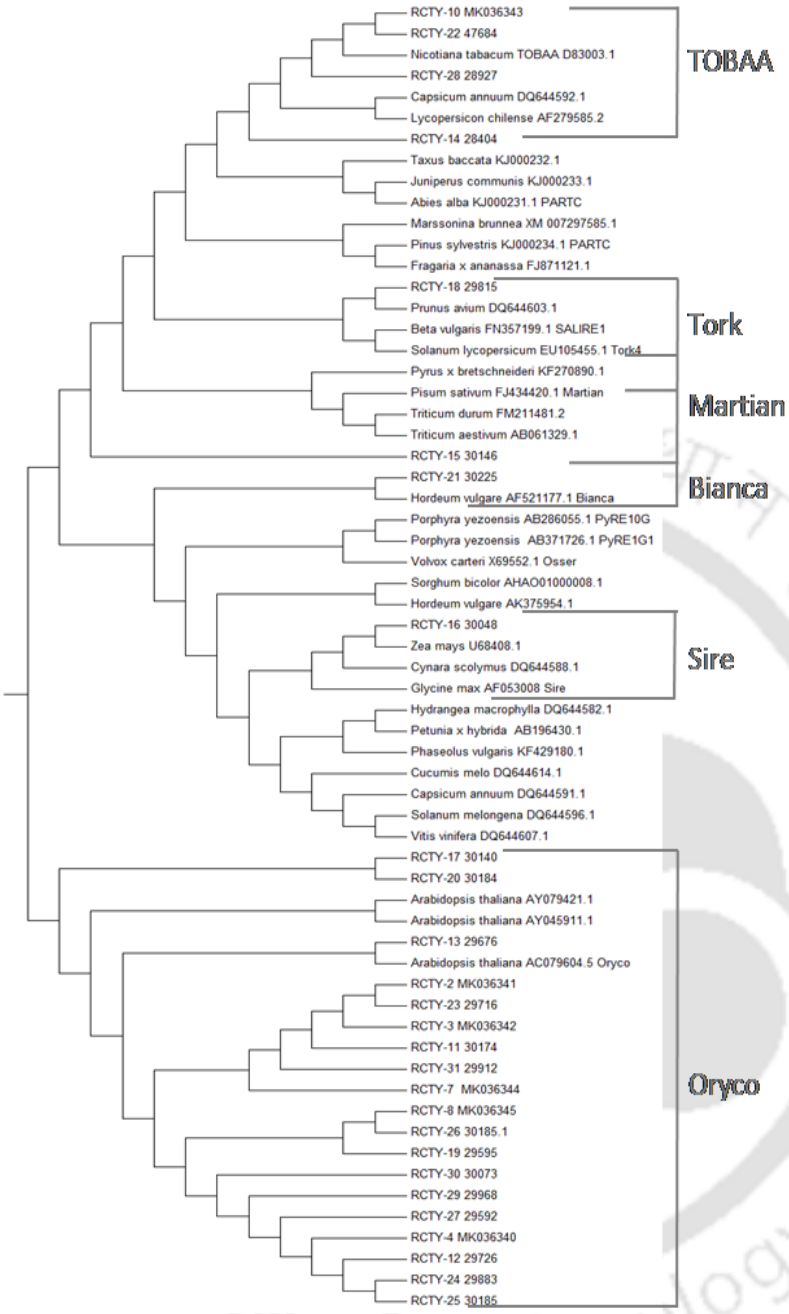


Figure 4B.8: Phylogenetic relationships among the nucleotide sequences of the RT-RH domain of Ty1-*copia* clones from *R. communis* and comparison with sequences from other species using the NJ method

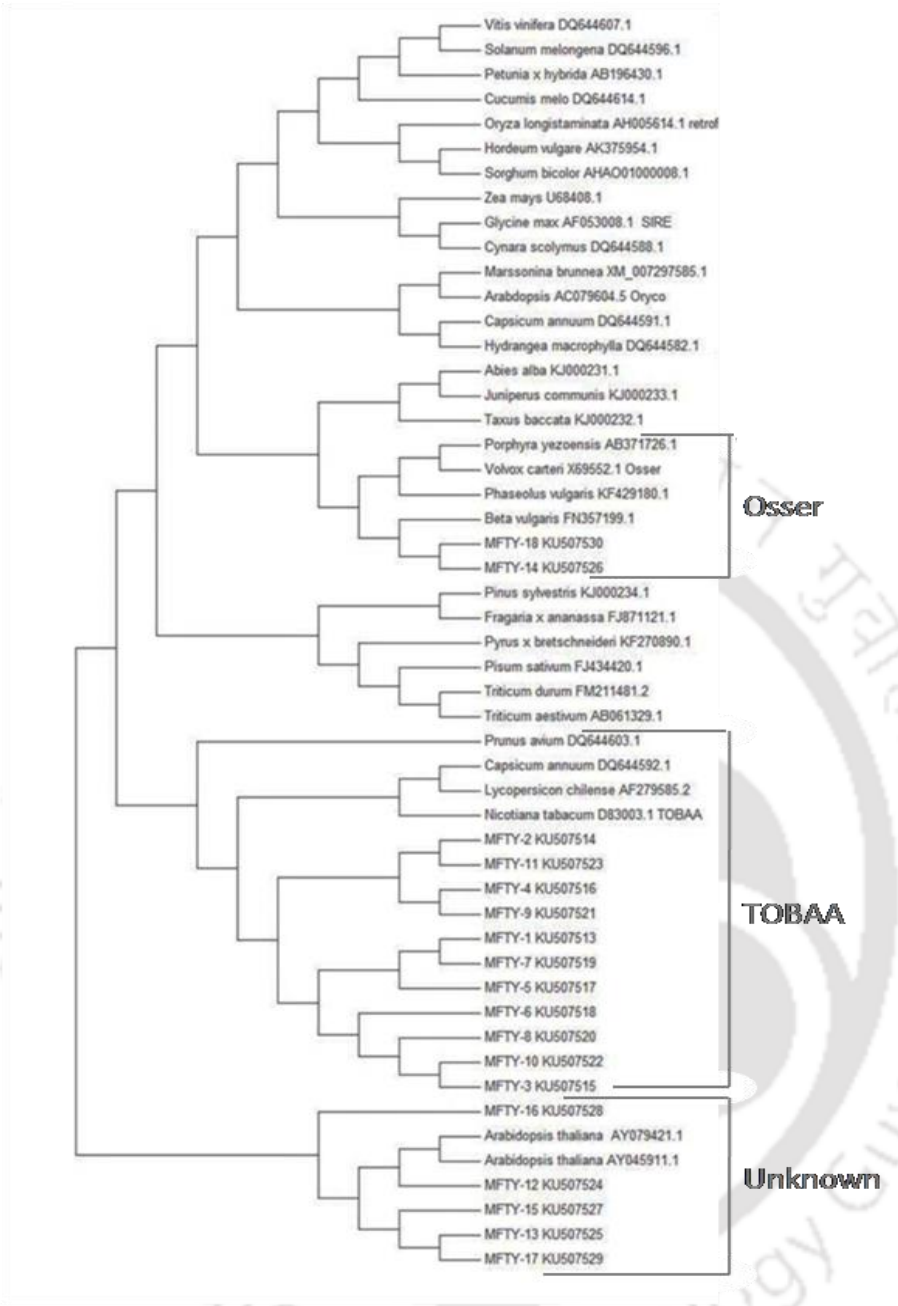


Figure 4B.9 Phylogenetic relationships among the nucleotide sequences of the RT-RH domain of Ty1-*copia* clones from *M. ferrea* and comparison with sequences from other species using the NJ method

4B.3.4 Estimation of copy number of Ty1-*copia* retrotransposon

The Ty1-*copia* studied so far in the candidate plants were found to be heterogeneous, which could be because of the following reasons stated by Flavell et al., (1992):

1. The distinctive strategy adopted by plants for germ-line determination,
2. Great tolerance of plant genome to chromosomal alterations and
3. The high copy number of retrotransposons, which is found to be the reason in most of the earlier reports (Khaliq et al., 2012).

In a population between any transposons, total genetic divergence is reported to be proportional to its copy number (Lee & Keem, 2014). Thus, dot blot hybridization was performed to determine the copy number of Ty1-*copia* element of *P. pinnata, J. curcas, R. communis,* and *M. ferrea* genome. Whole PCR amplified product (~900 bp) was used as a probe for dot blot hybridization for each species. Serially diluted PCR product was used as standards against total genomic DNA of *P. pinnata, J. curcas, R. communis* and *M. ferrea* which is approximately 1217 Mbp, 420 Mbp, 493 Mbp and 684 Mbp in length as recorded in the present study. For dot blot analysis, images were inverted using ImageJ tool for the hybridization signal intensity measurements. A standard graph was prepared based on the hybridization signal intensities of the PCR product and the genomic DNA **(Figure 4B. 10, 12, 14 and 16).** The selected values obtained from genomic DNA were thus compared with the standard graph. The values were also occupied to estimate the average proportion of nuclear genomic DNA hybridizing with the probe. Based on signal intensities, the copy number of RT-RH gene in Ty1-*copia* was estimated to be approximately 16967.574, 1212, 3726.04 and 2495 copies per haploid genome of *P. pinnata, J. curcas, R. communis,* and *M. ferrea* respectively **(Figure 4B. 11, 13, 15 and 17).** The present study focuses only on partial RT-RH domains, assuming average size for Ty1-*copia* (7 kb) (Hill et al., 2005). In the present study, it has been found that Ty1-*copia* retrotransposons constitute about 9.66% of *P. pinnata,* 2% of *J. curcas,* 6.21% of *R. communis* and 2.5 % of *M. ferrea* of the total genome and no significant variation in copy number is observed among the studied plants. Determination of copy number of copia elements in relation to genome size expansion and comparison with other species may be helpful for the understanding of genome evolution and organization (Lee & Kim, 2014).

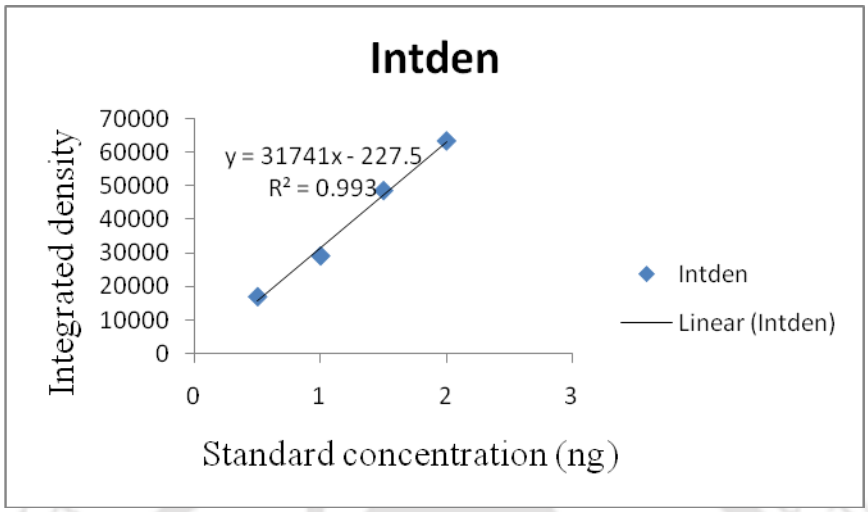


Figure 4B.10: Standard graph prepared for *P. pinnata* based upon the signal intensities of Tyl-*copia* (PCR product) dot blot hybridization

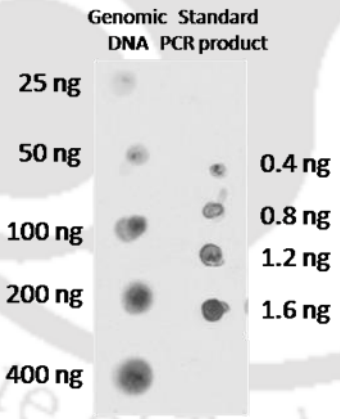


Figure 4B.11: Dot blot hybridization conducted for the determination of copy number of Ty1-*copia* like element in *P. pinnata*

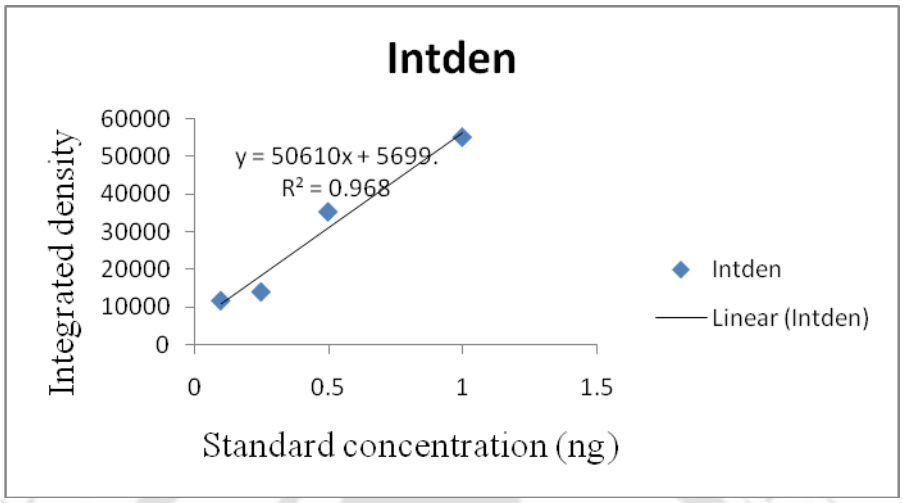


Figure 4B.12: Standard graph prepared for *J. curcas* based upon the signal intensities of Tyl-*copia* (PCR product) dot blot hybridization

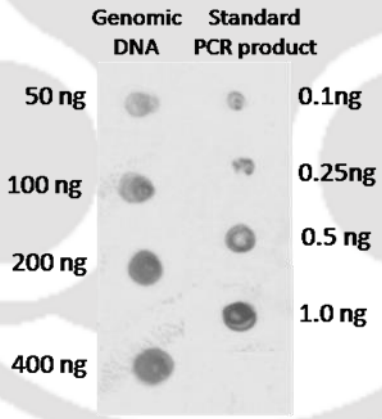


Figure 4B.13: Dot blot hybridization conducted for the determination of copy number of Ty1-*copia* like element in *J. curcas*

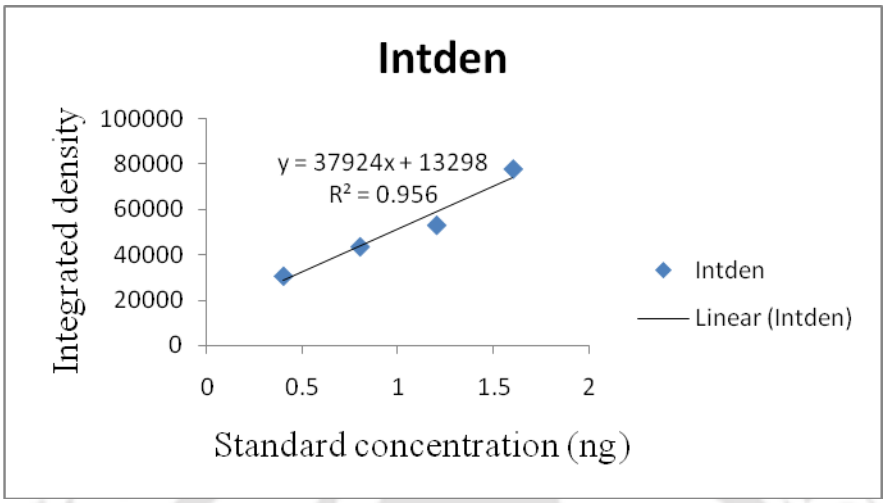


Figure 4B.14: Standard graph prepared for *R. communis* based upon the signal intensities of Tyl-*copia* (PCR product) dot blot hybridization

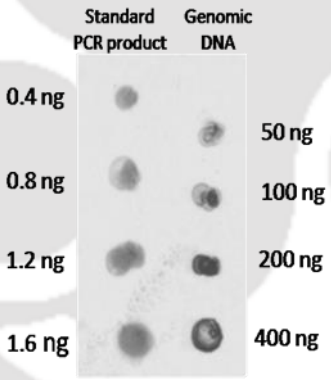


Figure 4B.15: Dot blot hybridization conducted for the determination of copy number of Ty1-*copia* like element in *R. communis*

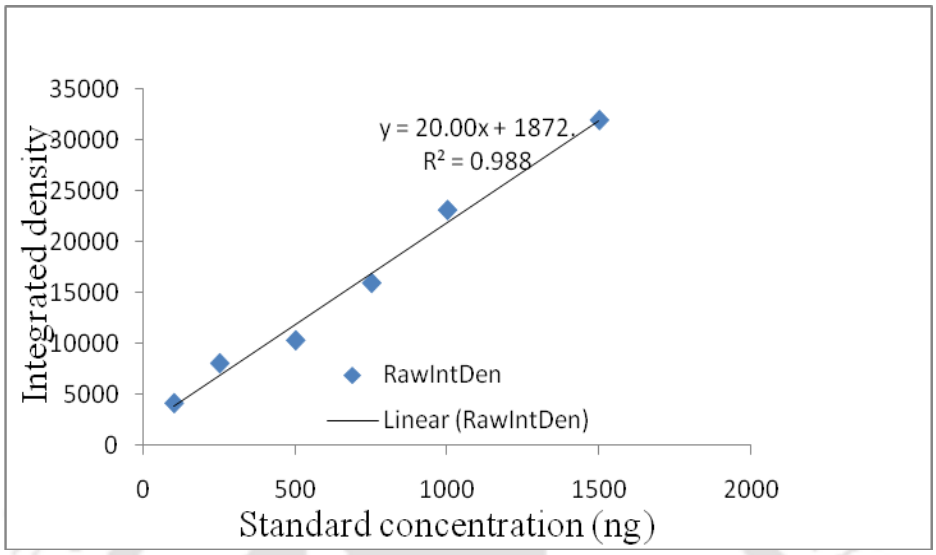


Figure 4B.16: Standard graph prepared for *M. ferrea* based upon the signal intensities of Tyl-*copia* (PCR product) dot blot hybridization

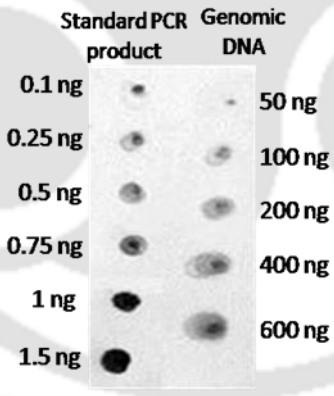


Figure 4B.17: Dot blot hybridization conducted for the determination of copy number of Ty1-*copia* like element in *M. ferrea*

In the previous report, *J. curcas* having a genome size of 370 Mbp, accounts for less than 10% of the genome to *copia* type retrotransposons (Alipour et al., 2013). This might be an indication of the importance of Ty1-*copia* retrotransposons in plant genome evolution and its size. No significant variation in copy number has been observed in *P. pinnata, J. curcas, R. communis* and *M. ferrea* belonging to Assam. Previous genome estimation

studies on *R. communis* showed some variation in genome size. Houben (2003) reported 510 Mbp haploid genome size in German *R. communis* accession which is 90 Mbp and 16 Mbp more compared to the reported genome size of 420 Mbp in USA accession (Chan et al., 2010) and the collected local accession from Assam. In the present study, we have estimated 6.21% of *copia* element in *R. communis* genome, whereas in the previous study Chan et al. (2010), reported 4.7%. This difference might be due to the use of whole PCR product as a probe for dot blot analysis, which results in non-specific hybridization. No such reports on the estimation of Ty1-*copia* copy number were found with respect to *P. pinnata* and *M. ferrea* till date. It is worth mentioning that, the population of retrotransposons is low in case of small genomes due to the pressure of rapid cell division (Cavalier-Smith, 1978), but it is opposite in case the genome size is moderate or large and retrotransposons selection is relaxed (Navarro-Quezada & Schoen, 2002) as the case here. All the sequences identified had either stop codons or frameshifts in the fragment of RT-RH reading frame suggesting that they are transpositionally defective. A similar case has been reported for the transposon group in *A. thaliana* and *S. tuberosum* (Flavell et al., 1992; Konieczny et al., 1991).

Copy number determination of Ty1-*copia* elements perhaps indicates the importance of retrotransposons in the candidate plants genome and also in their evolution. However, limited numbers of retrotransposon were employed for designing the degenerate primers. Thus, some lineages of Ty1-*copia* retrotransposons might have been missed out during the PCR amplification using degenerate primers (Park et al., 2007). Using the whole PCR product as a probe for the dot blot analysis could also be responsible for non-specific hybridization.

4B.4 CONCLUSION

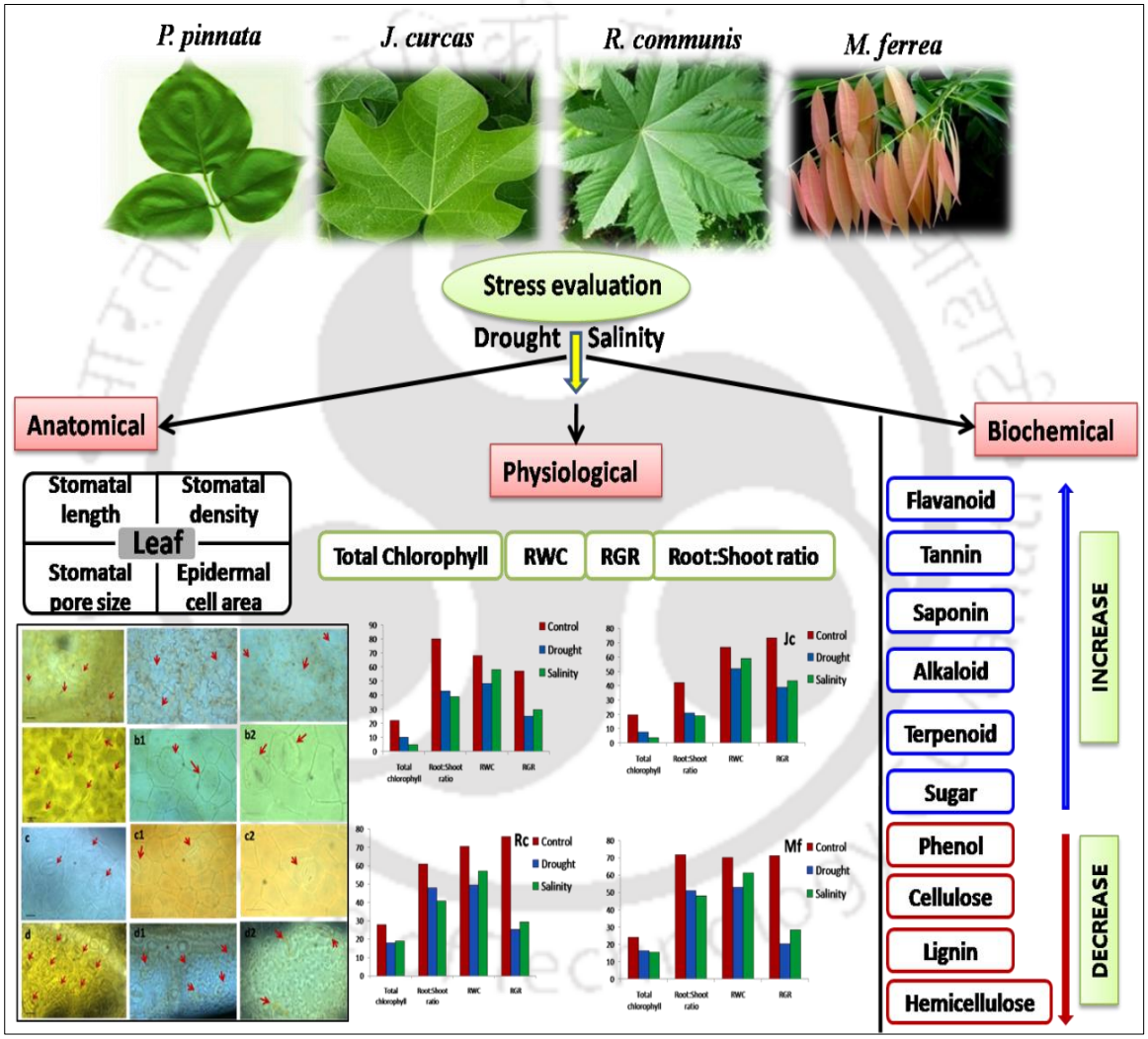
Retrotransposon is the major component of plant genome occupying large space. They play a significant role in all plant genomes and hence it is important to understand their relationships with the genome size. Among all the retrotransposons, Ty1-*copia* is considered as one of the cause for genome obesity. Thus, in the present chapter, a study has been conducted with a focus on Ty1-*copia* LTR-retrotransposons in *P. pinnata, J. curcas, R. communis and M. ferrea.* A significant heterogeneity was found among the

isolated conserved RT and RT-RH domain of Ty1-*copia* retrotransposons. Cluster analysis also revealed heterogeneity for RT and RT-RH sequences as they are dispersed all over the place in the phylogenetic tree. The sequence similarity between retrotransposons from different species indicates that Ty1-*copia* may belong to the group of horizontal transmission of retrotransposons. Dot blot analysis revealed no significant variation with respect to Ty1-*copia* copy number in the four biofuel crops under study. Thus, retrotransposons might not be responsible for the intraspecies variation in genome size of *P. pinnata, J. curcas, R. communis, and M. ferrea.* This study will help to understand plant genome evolution and organization. Additionally, this information will also give an idea about the genetic diversity in plants.

Moreover, since no significant variation in Ty1-*copia* copy number was observed, it was concluded that in the candidate plants the variation in genome size might be because of environmental variation.

Chapter 5

STRESS EVALUATION IN NON-EDIBLE OIL CROPS - ANATOMICAL, PHYSIOLOGICAL AND PHYTOCHEMICAL STUDIES



This chapter deals with the anatomical, physiological and phytochemical study conducted on *P. pinnata, J. curcas, R. communis* and *M. ferrea* under abiotic stress (drought and salinity).

STRESS EVALUATION IN NON-EDIBLE OIL CROPS - ANATOMICAL, PHYSIOLOGICAL AND PHYTOCHEMICAL STUDIES

5.1 INTRODUCTION

Plants have been used from the time immemorial in various sectors by mankind. Starting from agricultural, medicinal, industrial, biofuel production etc, plants have contributed immensely. Socio-economic plants are one of the major contributors to the economy in developing countries like India. Thus, in order to maximize the yield, plants having proper growth are required to be cultivated in large scale. The productive potential and yield of plants are strongly affected by the environment. Harsh environmental stresses adversely affect the plant growth, their metabolism, and yield. Among all the environmental stresses, salinity and drought are known to have the most adverse effect on plants (Anjum et al., 2011; Golldack et al., 2014; Negrao et al., 2017), that can directly influence the growth and yield (Buchbauer & Baser, 2010; Stanev, 2010). The immediate effects of salinity on plants include decreased osmotic potential, nutritional imbalance (due to high ionic concentration), and the toxic effects of certain ions, especially chlorine and sodium (Flowers, 2004; Willadino & Camara, 2010). Soil salinity results in reduced biomass accumulation because of the expense of huge metabolic energy in adapting to salt stress conditions. The increase in osmotic pressure of the soil solution and the reduction in the water infiltration rate results in water limitation for plants (Taiz & Zeiger, 2009), which, in turn, has a negative effect on the growth and development of most plant species. Drought stress in plants reduces the plant cells water potential and turgor, which elevates the solutes' concentrations in the cytosol and extracellular matrices (Amin & Davood, 2016). As a result, cell enlargement decreases leading to growth inhibition and reproductive failure. Drought not only affects plant-water relations through the reduction of water content, turgor, and total water but also affects stomatal closure, limits gaseous exchange, reduces transpiration and arrests photosynthesis rate. Effects in physiological and anatomical parameters due to stress conditions often lower the growth, development and productivity of the plants.

P. pinnata, J. curcas, R. communis, and *M. ferrea* are socio-economic plants well-known for their medicinal and biofuel properties. The seeds contain oil that can be processed to produce a high-quality biodiesel that is usable in a standard diesel engine (Mardhiah et al., 2017; Openshaw, 2000). As the four crops have the potential to produce high oil-yielding seeds, they are considered as pioneers of second-generation sustainable biofuel crop. Different parts of these plants are well known for their traditional use and pharmacological properties (Asif et al., 2017; Khan et al., 2017; Pulipati et al., 2018; Rampadarath et al., 2016). Number of reports are available on the oil aspect of these four biodiesel crops (Achten et al., 2007; Aydin et al., 2010; Bora et al., 2013; Kesari et al., 2008) but no information is available on the anatomical, physiological and biochemical changes in the plant during abiotic stress.

For large-scale biofuel production from these plants, they ought to be planted over a wide range of geographical and climatic conditions. Thus, the aim of the present work was to examine the effect of salinity and drought on several anatomical, physiological and phytochemical parameters required for the commercial production of plant products. This study is essential for a clear perception of plant resistance to stress conditions.

5.2 MATERIALS AND METHODS

5.2.1 Growth of the plants and stress application

Healthy seeds of *P. pinnata, J. curcas, R. communis,* and *M. ferrea* seeds were grown for 30 days in the plastic pots containing soil and peat (4:1), in a greenhouse maintained at a temperature 25 °C and relative humidity of 60 ± 5%. Plants were further grown for approximately 30 days more to obtain individuals of comparable sizes. Well grown plants were exposed to five levels of salt stress by adding 50, 100, 150, 200 and 250 mM of NaCl to the irrigation water (Ragagnin et al., 2014). Healthy plants without any stress were chosen as control. Another set of plants were exposed to drought stress, out of which, one group of plants were provided with 50% less water than ideal irrigation water given to controls (moderate drought) and another group was subjected to drought stress by withholding irrigation water i.e., 100% (high drought stress) for 30 days (Makbul et al., 2011).

5.2.2 Plant anatomical study

5.2.2.1 Stomatal parameters and epidermal cell area measurement

Estimation of stomatal parameters and epidermal cell area was done as reported in the previous chapter (section 4.2.2.1).

5.2.3 Plant physiological study

The experiments for determination of all the physiological parameters were performed for both the control and stressed (drought and salinity) plants. For all the control and stressed (drought and salinity treated) plants the below-mentioned methodology was used.

5.2.3.1 Chlorophyll content

Total chlorophyll content was determined using the Arnon method (Arnon, 1949). Fresh leaf samples were selected and washed 2-3 times with distilled water. The leaf samples were then dried and homogenized using mortar pestle in 80% acetone, followed by centrifugation at 5000 rpm for 5 mins. The supernatant was separated and the absorbance of the supernatant was recorded at 663 and 645 nm by UV-vis spectrophotometer (AnTech, India)

5.2.3.2 Determination of root: shoot ratio

Shoots from both control and stressed plants were collected, washed and weighed immediately after their excision. The potted plants were plucked to get roots followed by cleaning thoroughly with tap water. Cleaned roots were then blotted with blotting paper before weighing. Dry weights of both root and shoot were determined after drying at 70-80 °C for 48 hrs using dry heat.

5.2.3.3 Determination of relative growth rate

The relative growth rate (RGR) signifies the temporal increase in dry weight and was calculated by the following equation:

$$RGR = \frac{\log W2 - \log W1}{d2 - d1} \quad (1)$$

Where, *W2* and *W1* are the dry weights of plants at 30 days of exposure (*d2*) and 15 days of exposure (*d1*), respectively.

5.2.3.4 The relative water content of leaves

Three leaf discs of known diameter from both the control and stressed plants were taken randomly, washed thoroughly and dried followed by weighing to determine the fresh weight. Same discs of leaves were then placed in water-soaked filter paper in Petri dishes for 10 hrs in the dark in a biological oxygen demand (BOD) incubator (at 25 °C and 80% relative humidity). Subsequently, the discs were weighed to determine the turgid weight and dried using dry heat at 70 °C. The relative water content (RWC) was quantified by the following formula and expressed in percentage:

$$RWC = \frac{Wf - Wd}{Wt - Wd}.100 \quad (2)$$

where, *Wf*, *Wd*, and *Wt* are the fresh, dry, and turgid weights, respectively.

The values of total chlorophyll content, root: shoot ratio, relative growth rate, and relative water content were estimated in percentage.

5.2.4 Phytochemical study of leaves

Leaves from the four plant species were collected and cleaned using running water. Samples from both control and stressed plants were shade dried for 6 - 7 days to make it moisture free. The dried samples were powdered using a mechanical grinder to obtain fine sized leaf powder. Finely powdered leaf (5g each) for *Pongamia, Jatropha, Ricinus,* and *Mesua* were extracted with aqueous: methanol (1:1), using water bath treatment. The extraction period for *P. pinnata* was optimized at 30 mins, for *J. curcas* and *R. communis* at 15 mins and for *M. ferrea* the extraction time exceeded to 45 mins. The resulting crude extract was filtered by Whatman no. 1 filter paper. These extracts were concentrated using flash evaporator and were stored in sealed glass vials for further studies.

5.2.4.1 Quantitive analysis of phytochemicals

The leaf extracts of the four plants under study were subjected to qualitative phytochemical analysis. Presence of various phytochemicals such as phenols, flavonoids, saponins, tannins, terpenoids, alkaloid, and carbohydrate was determined by various phytochemical tests (Mir et al., 2016). For quantitative analysis estimation of both primary and secondary metabolites were carried out in the four plants under study.

Primary metabolites like cellulose, hemicellulose, lignin, and sugar content were estimated whereas, for secondary metabolites, estimation of total phenol, flavonoids, tannin, saponin, alkaloid, and terpenoids were carried out.

5.2.4.1.1 Estimation of total flavonoid content

Total flavonoid content was examined using Quercetin as standard. Methanolic leaf extracts (0.1 ml) were taken in test tubes and the volume was made up to 5 ml with distilled water followed by the addition of 0.3 ml of 5% $NaNO_2$. After 5 mins of incubation, 3 ml of 10% $AlCl_3$ was added and were mixed thoroughly. After 6 mins, 2 ml of 1M NaOH solution was added and the absorbance was recorded at 510 nm. The results were expressed as mg quercetin/g of the plant tissue.

5.2.4.1.2 Estimation of total phenolic content

Around 20 µL of leaf extracts were taken from the control and stressed samples and volume was made up to 1 ml with distilled water. About 0.5 ml of freshly prepared Folin ciocalteu phenol reagent, followed by 2.5 ml of 20% sodium carbonate, was added to each extract. The contents were agitated and left in dark for 40 mins. The absorbance of the samples was taken at 725 nm using a UV-vis spectrophotometer (Varian, Cary 100). Gallic acid was used as a standard. Amount of phenolic content was expressed as mg of Gallic acid per gram of the plant tissue (mg GA/g).

5.2.4.1.3 Estimation of tannin content

Leaves of the four plants were dried, powdered using a mechanical grinder and 500 mg of each sample was weighed into a 250 ml conical flask. To the powdered samples, 50 ml of distilled water was added and kept in mechanical shaker incubator for 1 hour. After an hour of incubation, the mixture was filtered into a 50 ml volumetric flask and made up to the mark. Then 5 ml of the filtered was pipette out into a test tube and mixed with 2 ml of 0.1 M FeCl3 in 0.1 N HCl and 0.008 M $K_4Fe(CN)_6 \cdot 3H_2O$. Total tannin content was determined using Gallic acid equivalent (50 to 1000 mg/ml) as the standard and was expressed as mg Gallic acid equivalent per gram of dry sample. The samples were incubated for 10 mins and absorbance was measured at 120 nm immediately using a UV-vis spectrophotometer (Varian, Cary 100).

5.2.4.1.4 Determination of saponin

Around 20g of plant leaves powder from the four plants under study was taken in 4 different 500 ml conical flask and to those 200 ml of 20% ethanol was added. The suspensions of leaves powder were then heated over a hot water bath for 4 hours with continuous stirring at about 55 ºC. The mixtures were filtered and the residues obtained were then re-extracted with another 200 ml of 20% ethanol. The combined extracts were then boiled in a water bath at about 90 ºC and reduced to 40 ml. The concentrate was transferred into a 250 ml separating funnel followed by addition of 20 ml of diethyl ether with constant shaking. The aqueous layer was recovered while the organic layer was discarded. The purification process was repeated twice and 60 ml of n-butanol was added to it followed by washing with 10 ml of 5% (w/v) aqueous sodium chloride. The remaining solution was heated in a water bath and the concentrated samples were then dried in the oven into a constant weight. The saponin content was calculated in percentage Shanmugapriya (2017).

5.2.4.1.5 Determination of alkaloid

Determination of alkaloid was carried out using the Harborne method (Mir et al., 2016). Around 5 g of the leaf powder from each plant were taken into four 250 ml beaker and 200 ml of 10% (v/v) acetic acid in ethanol was added to each beaker. The mixtures were covered and allowed to stand for 4 hrs at room temperature followed by filtration. The filtered extract was then concentrated on a water bath to one-quarter of the original volume. Concentrated ammonium hydroxide was added dropwise to the extract until precipitation was obtained. The solution was then allowed to settle for an hour and the precipitate was collected by slowly pipetting out the supernatant with utmost care. The precipitate was then washed with dilute ammonium hydroxide followed by filtration. The residue obtained was an alkaloid, which was then dried and weighed. All tests were carried out in triplicate.

5.2.4.1.6 Determination of terpenoid

Around 2 g (wi) of all the four-leaf powder was soaked in 180 ml of 99.9% ethanol for 24 hrs in four different test tubes. For *P. pinnata* and *M. ferrea* the mixture was slightly heated 3-4 times before incubating for 24 hrs. The mixtures were then filtered using

Whatman no. 1 filter paper. The filtrate was then again extracted using petroleum ether using separating funnel. The ether extract was separated in pre-weighed glass vials and incubated for complete drying (wf). Ether was evaporated and the yield (%) of total terpenoids contents was measured by the below-mentioned formula (Malik et al. 2017).

$$\left(\frac{W_i - W_f}{W_i}\right) \times 100 \quad (3)$$

5.2.4.1.7 Total cellulose estimation

Cellulose content was estimated using standard Updegraff method (Bauer & Ibanez, 2014). Powder leaves (0.1 g) were mixed with 5 ml of acetic reagent. Thorough mixing was followed by incubation at 100 °C for 30 mins and centrifugation at 5000 rpm for 20 mins. For *P. pinnata* and *M. ferrea* the centrifugation time was extended to 30 mins. The residue was washed with double distilled water and then dissolved in 10 ml of 67 % (v/v) H_2SO_4 followed by 1 hr incubation. From the solution, 1 ml was taken and diluted to 100 ml. Anthrone reagent (10 ml) was added to 1 ml of diluted solution. The mixture was boiled for 10 mins using a boiling water bath and was cooled. Absorbance was recorded at 630 nm using a spectrophotometer (Varian, Cary 100). A calibration curve was drawn using carboxymethyl cellulose and the amount of cellulose in the sample was calculated.

5.2.4.1.8 Determination of lignin content

Lignin content was calculated using a standard protocol given by TAPPI (Dence, 1992). About 1 g of raw biomass was mixed with 10 ml of 72% sulphuric acid. The final volume of the mixture was made up to 300 ml with distilled water. The solution was refluxed for 3 hrs and was then transferred to a pre-weighed sintered glass crucible. The biomass was washed with hot Mili Q water and the residue was dried at 105 °C. The residue obtained was considered the lignin present in the weight percentage of raw biomass.

5.2.4.1.9 Determination of hemicellulose content

The hemicellulose contents were measured using the method described by Van Soest (Van Soest, 1967). A sample of 1 g leaf powder was analyzed. All of the reagents used in this study were of analytical grade. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were estimated separately to determine hemicellulose content.

The neutral detergent solution was prepared by dissolving 6.81 g of sodium borate dehydrate and 18.61 g of disodium ethylene diamine tetra acetate dehydrate (EDTA) in 800 ml of distilled water. For better dissolving, the mixture was heated for 2-3 min. The solution was then cooled down and to this, 30 g sodium lauryl sulfate, 4.5 g disodium hydrogen phosphate and 10 ml of 2 ethoxy ethanol were added. The volume of the solution was made up to 1 L by adjusting the pH to 7.0. On the other hand, the acid detergent solution was prepared by dissolving 20 g of cetyltrimethyl ammonium bromide (CTAB) in 1 L of 1N H_2SO_4. For estimating Neutral detergent fiber (NDF), the powder leaves (1 g) of the four plants were individually taken in a refluxing flask and 100 ml of cold neutral detergent solution was added to it. To this solution, 2 ml of decahydronaphthalene and 0.5 g of sodium sulfite was added. The solution was then refluxed for 1 hr and was transferred to a pre-weighed sintered glass crucible. Filtration was done by applying suction. The residue was then washed with hot water followed by acetone and dried at 100 °C till constant weight was achieved.

For estimating Acid detergent fiber (ADF), 1 g of powdered leaves of the four plants were transferred to a refluxing flask and 100 ml of cold acid detergent solution was added to it. The solution was refluxed and processed similarly as described in NDF. The deduction of ADF from NDF determines the hemicellulose content of the biomass.

5.2.4.1.10 Total sugar estimation

For acid hydrolysis dried leaf biomass was pretreated as per the methodology described by Singh (Singh et al., 2014). The optimized conditions for acid hydrolysis have been determined as follows: For all the four plants, 1% (v/v) H_2SO_4 (equivalent to 0.36 N) was added to 10% w/v powdered leaves followed by autoclaving at 121 °C and 15 psi for 30 min with the rapid steam release. Dried biomasses of all four plants were pretreated under these conditions. The biomass from the reaction mixture was then separated by filtration using double-layered muslin cloth followed by a successive water wash to remove the residual chemicals left on the biomass surface after acid pretreatment. To maintain the neutral conditions, the washing procedure was continued till the pH of wash water became 7. Biomass residues were then dried in a hot air oven for 24 hrs at 60 °C. The dried biomass that contains cellulose and traces of lignin was used for further processing. Acid pretreatment causes hydrolysis of hemicellulose in biomass resulting in the release

of pentose sugars. The filtrate of the acid pretreatment or the acid hydrolyzate was analyzed for the total sugar content. The hydrolysate was then detoxified to remove any inhibitory compounds. Hydrolyzates were subjected to centrifugation for 10 mins at 10,000 rpm at 4 °C. Total reducing sugar in the hydrolyzate was estimated using the method of Nelson (1944) and (Somogyi, 1945).

5.2.5 Statistical analysis

All the above experiments were repeated three times independently. Approximately similar results were obtained and the data shown were expressed as a mean ± standard deviation. Data analysis was based on a one-way analysis of variance. All statistical analysis was performed using Microsoft Excel Version 2007.

5.3 RESULTS AND DISCUSSION

5.3.1 Plant anatomical study

Anatomical changes induced by stress conditions in higher plants are considered to be good indicators of plant growth. Anatomical features of the stressed and control plants were analyzed based on transverse sections of the leaf using microscopic analysis. Under abiotic stress conditions, stomata play a controlling role for optimizing the use of water and hence, are considered as a major physiological factor. Here, stomatal movements are considered to be dynamic, involving complex regulation by several environmental factors. The entire surface of the leaf was studied for stomatal size, stomatal pore size, and stomatal density along with epidermal cell area **(Figure 5.1)**. Isolateral leaf of *Pongamia, Jatropha*, and *Ricinus* had paracytic stomata on the abaxial surfaces, whereas, *Mesua* having bifacial leaf was observed to have paracytic stomata on both the surfaces. Stoma index was found to be higher in upper surface as compared to the lower surface in all the four plants. No significant difference with respect to the control was observed at salinity down to 250 mM concentration of NaCl, and below 100% high drought stress in all the studied parameters, thus, the conditions (250 mM concentration of NaCl and 100% drought) were chosen for the rest of the study to analyze the tolerance limit of the selected plants.

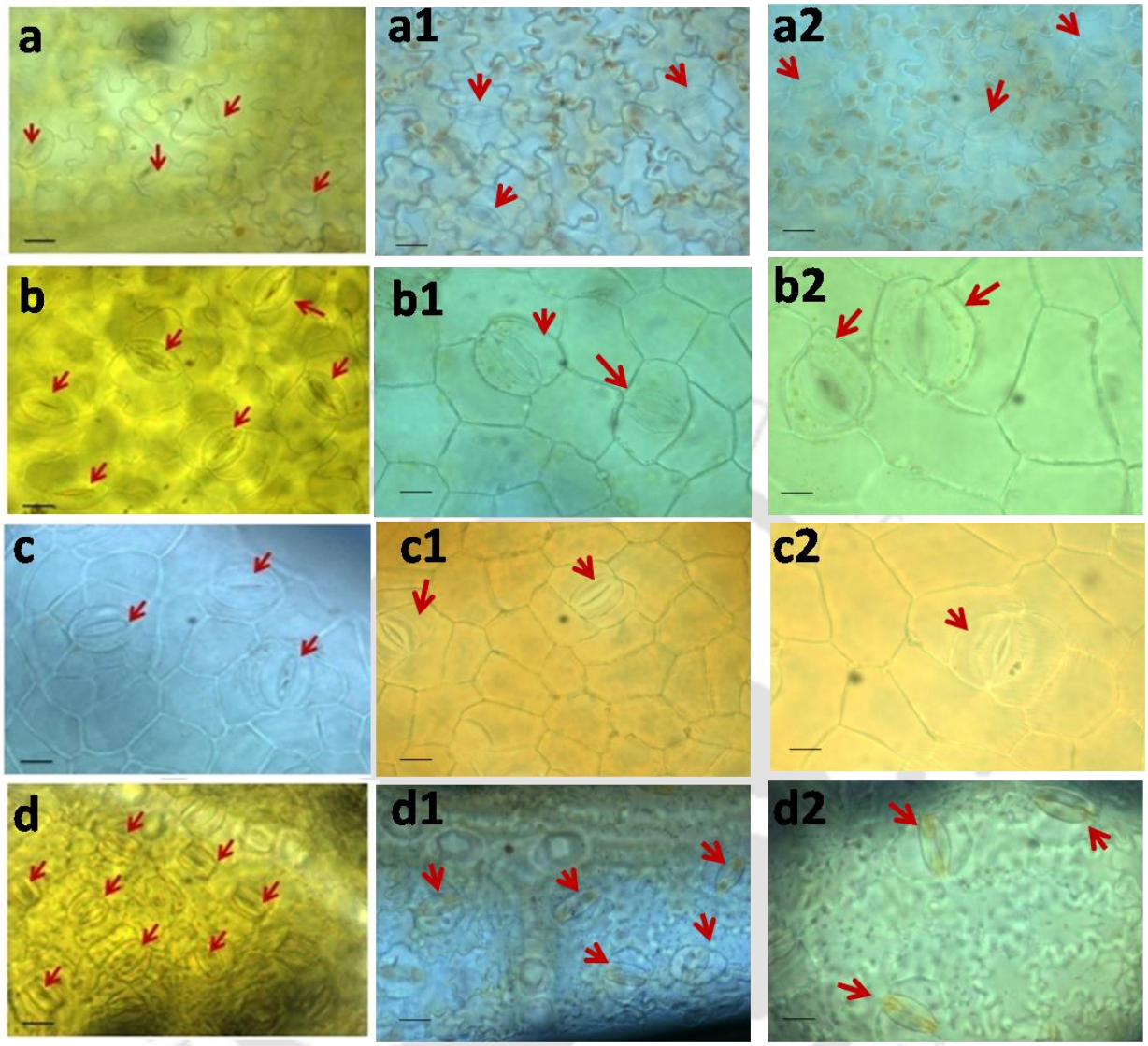


Figure 5.1: Detail of a microscopic observation of abaxial leaf epidermis showing epidermal cell, guard cell length, stomatal density, and pore size. The a, a1 and a2 represents control, drought and salinity affected *Pongamia* plants. Similarly, b, c and d refer to *Jatropha, Ricinus,* and *Mesua* respectively. Images were taken at 40X magnification. Bar 6 µm

The average length and the pore size of stomata were found to be reduced in drought and salinity stressed plants as compared to control **(Figure 5.2 and 5.3).** Stomatal length in *P. pinnata*, *R. communis* and *M. ferrea* was found to be less in the stressed plants (250 mM NaCl) in comparison to drought-stressed plants (100% drought), which indicates that the effect of salinity is more pronounced in these plants whereas in *J. curcas* the result was found to be opposite. In case of *P. pinnata* and *M. ferrea* the estimated stomatal pore size was higher in salinity stressed plants (250 mM NaCl) in comparison to drought-stressed plants (100% drought), whereas the reverse was observed in case of *J. curcas* and *R. communis*. It may be speculated that salinity affects shrubs more than tree species, which might be because of the low resistance of the roots of shrubs which can sense the osmotic stress easily, unlike the hard roots of trees. Stress induces root to leaf signaling, which is promoted by soil drying through transpiration stream, resulting in stomatal closure (Makbul et al., 2011). Among the four plants, *J. curcas* had least stomatal density and *M. ferrea* had the highest but with stress, a decrease in stomatal density with respect to all the four plants were observed as compared to control **(Figure 5.4).** Larcher in his study had reported that leaves under well-maintained conditions usually had larger and number of stomata than leaves during stress (Larcher, 1995). This is because, under short-term water stress, plants increase their water utility efficiency by reducing stomatal opening followed by transpiration rate; however, under prolonged drought stress, plants produce leaves with reduced stomatal conductance due to a reduction in stomatal parameters (Li et al., 2017). Thus the results in the present study are in accordance to the previous reports as the length, pore size and density of stoma cells decreased in the stressed plants (250mM concentration of NaCl and 100% drought) in comparison to control.

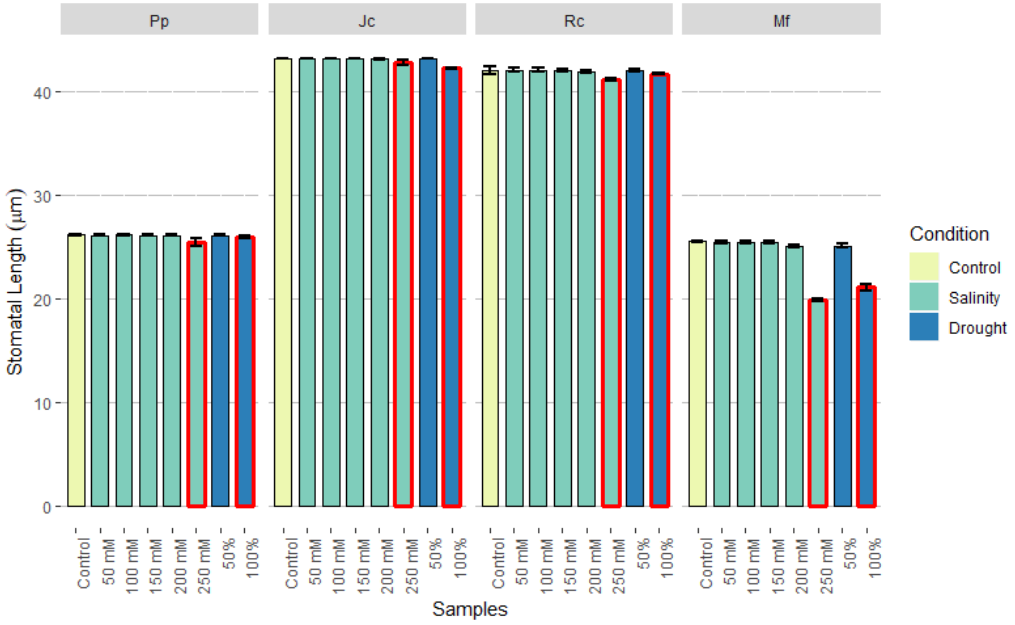


Figure 5.2: Variation in stomatal length with respect to the different concentration of salinity and drought stress in *P. pinnata*, *J. curcas*, *R. communis*, and *M. ferrea*. No significant difference was observed in salinity (50 mM, 100 mM, 150 mM, and 200 mM NaCl) and drought (50%) in comparison to control. Plants with salinity stress (250 mM NaCl and drought (100%) showed a significant decrease compared to control

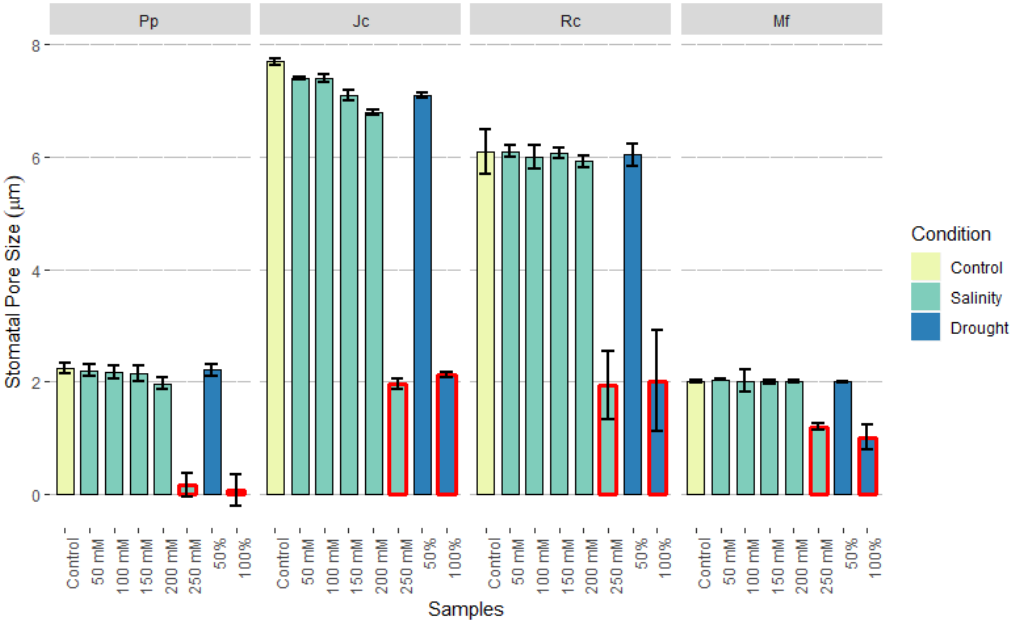


Figure 5.3: Variation in stomatal pore size with respect to the different concentration of salinity and drought stress in *P. pinnata*, *J. curcas*, *R. communis*, and *M. ferrea*

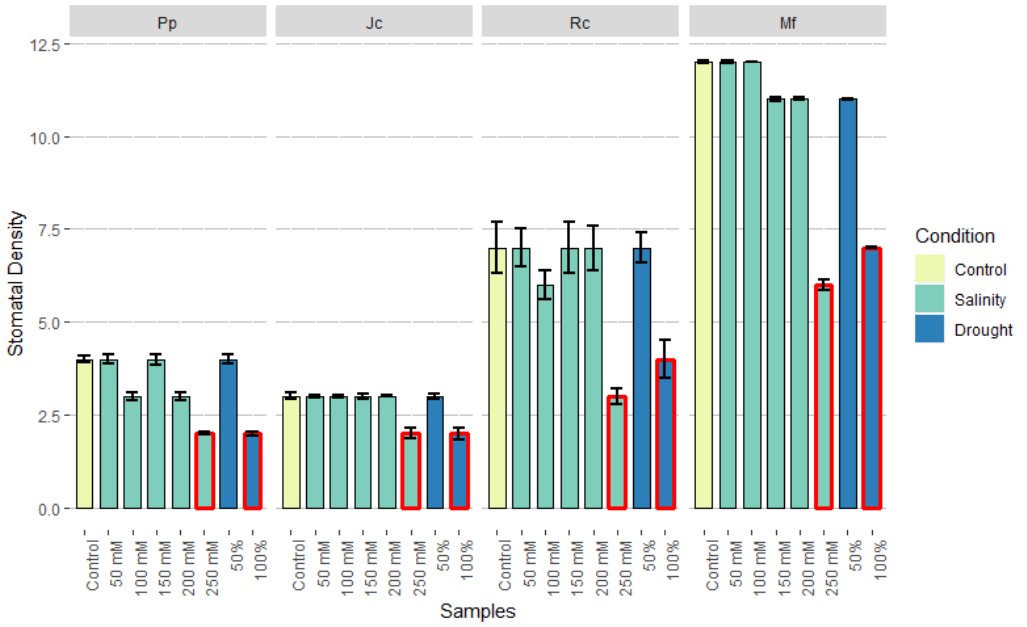


Figure 5.4: Variation in stomatal density with respect to the different concentration of salinity and drought stress in *P. pinnata*, *J. curcas*, *R. communis*, and *M. ferrea*

The epidermal cell area estimated for all the four plants revealed a gradual decrease in stressed plants (250 mM concentration of NaCl and 100% drought) as compared to control **(Figure 5.5).** The reduction of water uptake by plants creates an osmotic which leads to a decrease in epidermal thickness. Increase in epidermal cell area in cases of salinity (250 mM) was observed in comparison to drought stress (100%) except in *R. communis*, this can be explained based on the fact that there is extensibility of epidermal cell wall under salt stress as has been reported by Zorb et al., (2015), in maize. This extensibility is caused due to a shift in apoplastic pH of epidermal cells, which results in the cell growth even under acidic conditions. Apart from *R. communis*, the other three plants showed more effect in epidermal cells due to drought stress as compared to salinity stress. It has been reported that the tissues exposed to environmental stress have in general reduction in the cell size and stomatal parameters (Guerfel et al., 2009). As per the statistical analysis, the differences in anatomical values of control and the test plants (250 mM concentration of NaCl, and 100% high drought stress) were considered to be statistically significant; the two-tailed P value was less than 0.05. Whereas, below the 250 mM concentration of NaCl, and 50% moderate drought stress the differences in anatomical values of control and the test plants were statistically insignificant ($P > 0.05$).

Thus, it can be concluded that the plants subjected to a treatment of 250 mM NaCl, and 100% drought experience the major stress. These anatomical traits are considered within the environmentally influenced characters and may vary among species (Makbul et al., 2011).

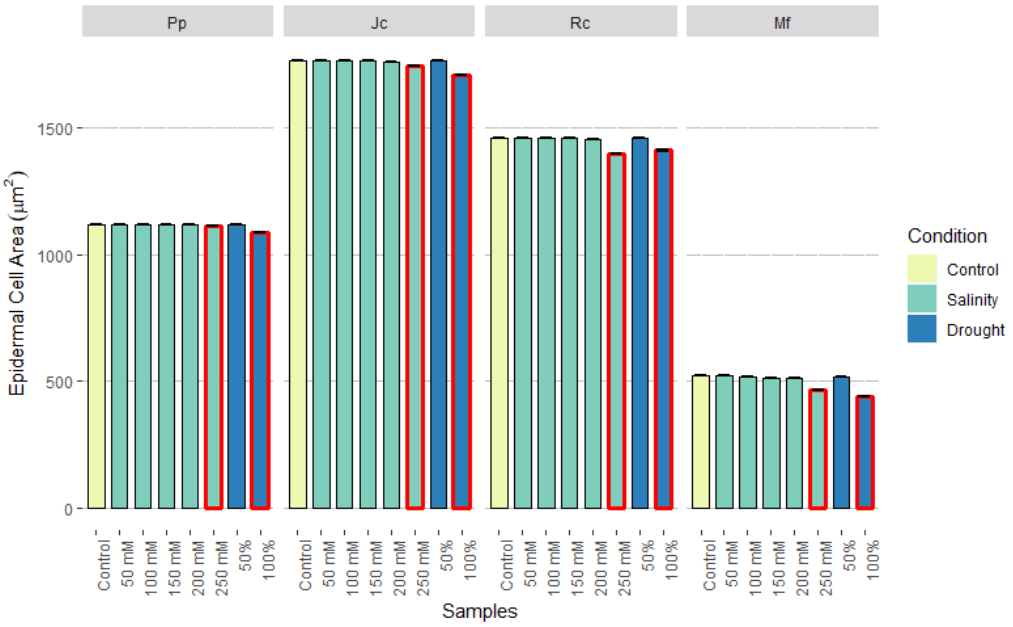


Figure 5.5: Variation in epidermal cell area with respect to the different concentration of salinity and drought stress in *P. pinnata*, *J. curcas*, *R. communis*, and *M. ferrea*

5.3.2 Plant physiological study

Drought and salinity affect total chlorophyll content, relative water content, relative growth rate, and root: shoot ratio, which are useful means of determining the physiological status of plants (Makbul et al., 2011). The estimated value for the studied physiological parameters decreased during the stressed period in leaves **(Figure 5.6 and 5.7)** except the salinity concentration down to 250 mM NaCl and below 100% high drought stress. No significant difference with respect to the control was observed in plants having moderate drought and with salinity concentration 50 mM, 100 mM, 150 mM, and 200 mM NaCl. Exposure to stress resulted in the decrease in total chlorophyll content in drought and salinity stressed leaves as compared to the control leaves. In *Ricinus*, the effect was visualized more in drought stress as compared to salinity. The percentage of the decrease in chlorophyll content was calculated and found to be 53% and 77%; 61%

and 81%; 35% and 32%; 32% and 36% respectively for *Pongamia*, *Jatropha*, *Ricinus*, and *Mesua*, respectively under drought and salt stress. A similar phenomenon was reported by Taiz and Zeiger in other species highlighting the sensitivity of chlorophyll to increasing environmental stress (Taiz & Zeiger, 2010). The decrease in total chlorophyll content can be explained by the decrease in leaf water status i.e. decrease in RWC. Root: shoot ratio, on the other hand, showed a significant increase in the stressed plants. This is in accordance with the previous reports where root: shoot ratio was found to increase under drought stress (Makbul et al., 2011). When plant experience limited availability of water, there is an increase in root: shoot ratio because roots are less sensitive than shoots to grow inhibition by low water potential (Wu & Cosgrove, 2000). In contrast to root: shoot ratio, though the estimated value for relative water content and relative growth rate decreased as compared to control, it was observed to be higher in salt-stressed plants as compared to the drought-stressed ones. Thus, drought stress effect was more in comparison to salt stress in this respect. Drought stress was applied to the plants under study to their threshold limit. Thus the reason of survival of plants can be explained by the fact that with osmotic adjustment mechanism there is decreasing the osmotic potential of the cells which involves in maintaining of full turgor of tissue under water stress conductions (Ranney et al., 1991). Osmotic adjustment is a reason for accumulation of solutes within the plant in response to a decrease in soil water potential, thus reducing the detrimental effects of water deficit. Also, under stressed conditions cell membranes are subject to changes often associated with the increase in the cell permeability (Blokhina et al., 2003; Iqbal, 2009).

The difference in the physiological values of the test plant with respect to control is considered to be statistically significant ($P < 0.05$) for salinity (250 mM NaCl), and 100% high drought stress. As physiological changes are in accordance with the anatomical changes it was clear that the plants were experiencing abiotic stress. Since, the environment has a direct effect on plant productive potential and yield, the study of the phytoconstituents in both control and stressed plants will help to understand the productive potential of the plants.

Total Chlorophyll Content (%)

	Salinity							Drought			
	Control	50 mM	100 mM	150 mM	200 mM	250 mM	Correlation	Control	50%	100%	Correlation
Pp	▲21.8	▲21.8	▲21.11	▲21.1	▲21	▼4.8		▲21.8	▲20.4	▼10.1	
Jc	▲19.6	▲19.74	▲19.5	▲19.21	▲18.7	▼3.7		▲19.6	▲17.62	▼7.5	
Rc	▲28	▲28.33	▲28	▲27.91	▲27.28	▼19.1		▲28	▲27.33	▼18.1	
Mf	▲24.1	▲24	▲24	▲23.87	▲23.11	▼15.4		▲24.1	▲23.1	▼16.3	

Relative Water Content (%)

	Salinity							Drought			
	Control	50 mM	100 mM	150 mM	200 mM	250 mM	Correlation	Control	50%	100%	Correlation
Pp	▲67.97	▲67.9	▲67.91	▲67.5	▲67.02	▼58.18		▲67.97	▲65.12	▼48.29	
Jc	▲66.78	▲66.8	▲66.67	▲66.21	▲66	▼59.12		▲66.78	▲64.97	▼52.01	
Rc	▲70.62	▲70.5	▲70.5	▲70	▲70	▼57.23		▲70.62	▲69.11	▼49.62	
Mf	▲70.21	▲70.11	▲70.1	▲70.1	▲69.82	▼61.38		▲70.21	▲68.2	▼53.04	

Relative Growth Rate (%)

	Salinity							Drought			
	Control	50 mM	100 mM	150 mM	200 mM	250 mM	Correlation	Control	50%	100%	Correlation
Pp	▲ 57	▲ 57	▲ 56.8	▲ 56.5	▲ 56	▼ 29.5		▲ 57	▲ 52	▼ 24.83	
Jc	▲ 73.31	▲ 73.09	▲ 73	▲ 73	▲ 71	▼ 43.39		▲ 73.31	▲ 70.5	▼ 38.92	
Rc	▲ 75.94	▲ 75.9	▲ 75.5	▲ 75	▲ 73	▼ 29.5		▲ 75.94	▲ 73	▼ 25.52	
Mf	▲ 71.3	▲ 71	▲ 71	▲ 70	▲ 69.8	▼ 28.35		▲ 71.3	▲ 69.5	▼ 20.17	

Figure 5.6: Abiotic stress (salinity and drought) induced physiological changes in Pp: *P. pinnata*, Jc: *J. curcas*, Rc: *R. communis* and Mf: *M. ferrea*. All the values were estimated in percentage

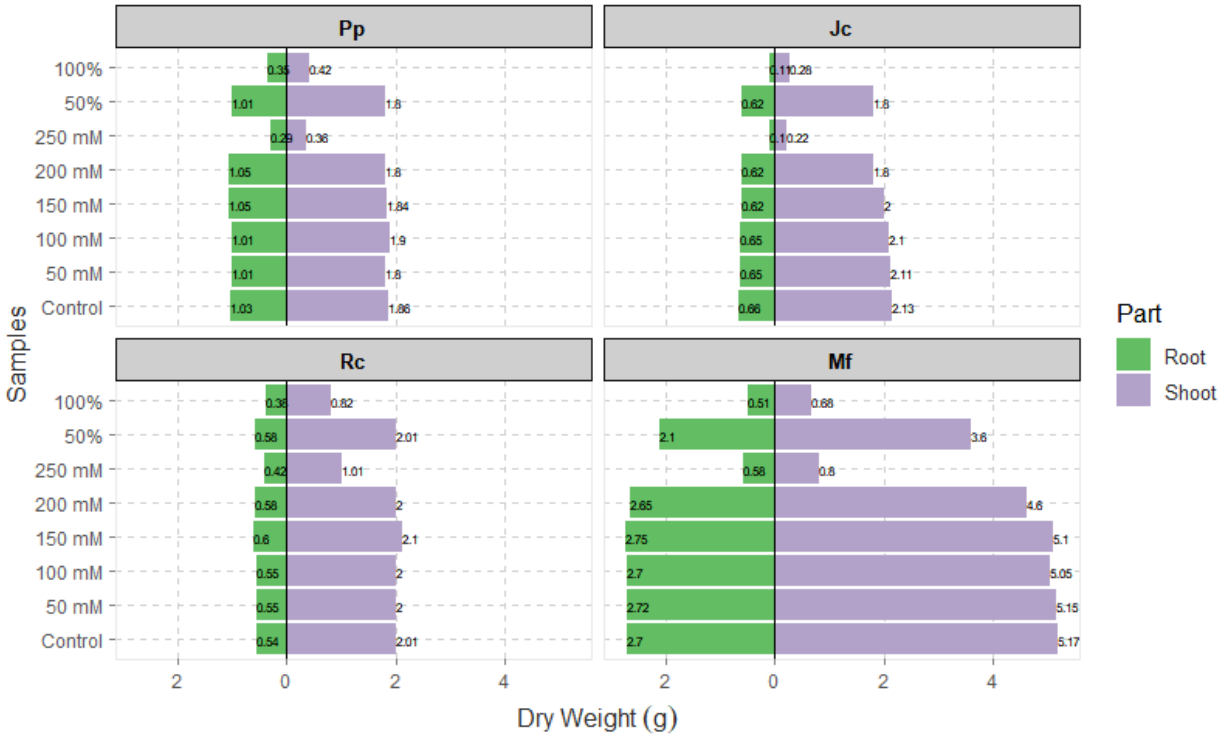


Figure 5.7: Abiotic stress (Salinity: 50 mM, 100 mM, 150 mM, 200 mM and 250 mM NaCl concentration; drought: 50% and 100%) induced increase in root: shoot ratio in Pp: *P. pinnata*, Jc: *J. curcas*, Rc: *R. communis* and Mf: *M. ferrea*

5.3.3 Abiotic stress induced phytochemical study in leaves

5.3.3.1 Qualitative analysis of phytochemicals in leaves

Phytochemicals are crucial for plant defenses against stress. Plant-derived phytochemicals could be used as a therapeutic agent and also for commercial production of bioproducts. In the present study, preliminary phytochemical screening of the methanolic extract was carried out by chemical testing with different chemical reagents. The experiment was carried out for both the control and plants under stressed conditions. The response for the chemical test was the same in case of both control and plants under stressed conditions. Results were found to be positive for alkaloid, tannin, terpenoid, phenol, flavanoid, carbohydrate, and saponin. The most important of these bioactive constituents of plants are terpenoids, alkaloid, tannin, flavonoid, and phenolic compounds. The presence of phytoconstituents such as flavonoids, phenolic compounds, and tannins in considerable amount may be responsible for wound healing property and antimicrobial property. Other

phytochemicals like steroids, alkaloid, terpenoids, and saponin were also screened. Leaves of *Pongamia* showed the absence of terpenoid, while, *Jatropha* leaves lack in alkaloid. In the present study, leaves of *Ricinus* showed positive results for all the screened phytochemicals whereas, in an earlier study, *Ricinus* roots were reported to be deficient in alkaloid, flavanoid, and saponin (Mittal & Ali, 2013). *Mesua* like *Jatropha* was found to be deficient in alkaloid. The result will serve in the development of pharmacological standards for future studies.

5.3.3.2 Quantitative analysis of phytoconstituents

Anatomical and physiological parameters indicated that drought and salinity stress had an impact on the stressed plants, which may have an influence on various industrially important phytochemicals produced by the plants. For protection and repair of the biological processes, plants produce phytochemicals. The diverse uses of plants in the treatment of various diseases are attributable to the presence of the phytoconstituents (Lekhak & Yadav, 2012). The phytochemicals estimated in the present study has much industrial use. Flavanoid, tannin, phenol, saponin, terpenoid, cellulose, hemicellulose, etc are having varied industrial use thus are economically valuable (Leavell et al., 2016; Martin & Appel, 2009; Oleszek & Hamed, 2010; Prior et al., 2005; Philippe, 2012; Scalbert et al., 2005; Reddy & Yang, 2005; Williams & Spencer, 2012). The quantitative estimation of these phytochemicals from the plants under study (control and stressed) will help to know whether the economic value of these plants reduces under stress environment. The estimation of phytoconstituents of the selected plant showed that leaves of all the four plants were rich in both secondary and primary metabolites which are industrially much in use. The present study was carried forward taking both control and plants under stressed conditions (Salinity: 250 mM NaCl concentration and Drought: 100%). The estimated total phenolic content, flavanoid, tannin, saponin, alkaloid, terpenoids, cellulose, hemicellulose, lignin and reducing sugar are tabulated in **Table 5.1**. The results indicate that the stressed plants had a significant decrease in the value of cellulose, hemicellulose, lignin and reducing sugar as compared to control. The decrease in percentage yield was calculated to be less than 10%. In contrast, the total phenol, flavanoid, tannin, saponin, alkaloid, and terpenoid were found to increase during stress condition by around 11% except in case of *Pongamia* where the terpenoid value

decreased with stress. The differences in the values of phytoconstituents obtained from the methanolic leaf extract of *Pongamia, Jatropha, Ricinus,* and *Mesua* of both treated and control were significant as the P value obtained was found to be less than 0.05 for each plant. Effect of drought was seen to be more in comparison to salinity stress for most of the phytoconstituents, whereas in *Pongamia* the value of flavanoid and saponin was found to be more with the effect of drought stress. In *Ricinus* also the estimated value for Terpenoid, phenol, and hemicellulose was higher in drought-stressed plants. Also, it was found that in *Mesua* the total reducing sugar obtained from acid hydrolysis was estimated to be more in drought-stressed plants. This indicates that the salinity effect was more in the production of these metabolites with respect to these plants. Throughout all the experiments, *J. curcas* and *M. ferrea* were found to be affected more by drought stress in comparison to salinity stress; thus can be considered more salinity tolerant plants among the four plants under study. The estimation of phytoconstituents showed a minimal increase in secondary metabolites since more phytochemicals are produced by plants for protection and repair the biological processes during stress; whereas, a decrease in primary metabolites was observed, which they use as a source of energy for survival. Also, soil stress results in reduced biomass accumulation, because of the expense of huge metabolic energy in adapting to stress conditions. Thus, the significance of the study carried out with the leaves in terms of differences in phytoconstituents content was comparable to previous reports from other plants (Durai et al., 2016; Santhi & Sengottuvel, 2016; Siddhuraju & Becker, 2003). Although phytoconstituents are responsible for antioxidant activities, antioxidant activity exhibited by plant parts do not always signify the presence of large quantities of all phytoconstituents (Badarinath et al., 2010). Thus, the study was not carried out with respect to the estimation of antioxidant. The results above indicate minimal differences in all the industrially important phytoconstituents during abiotic stress in comparison to control which indicates the plant's resistance to abiotic stress with respect to their yield. Thus, these plants can be grown in both salinity and drought region without any economically important productive loss. Though these plants are rich in oil content, in the future study has to be conducted with the aspect of oil production.

Table 5.1 Quantitative analysis of phytochemicals in crops during abiotic stress

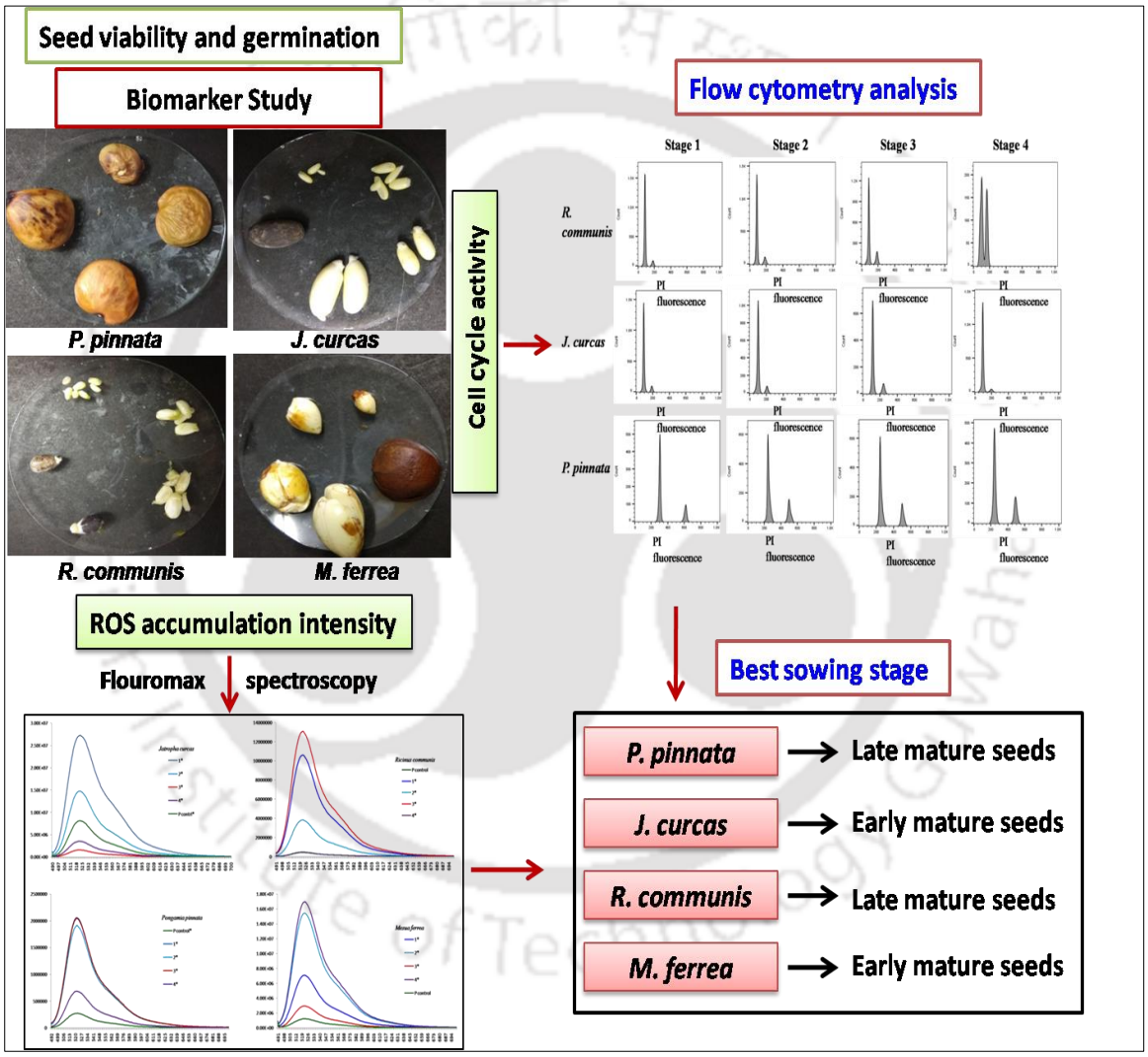
Plant	Environmental condition	Flavanoid (g/g)	Total phenol (%) w/v	Tanin g/g	Saponin g/g	Alkaloid g/g	Terpenoid g/g	Cellulose*(wt % raw biomass) w/v	Hemi-cellulose (wt% raw biomass) w/v	Lignin(wt% raw biomass) w/v	Total reducing sugar (g/g) by AH
P. pinnata	Control	0.016	9.97	0.0027	0.08	0.61	0.006	56.0	19.04	16.23	0.09
	Drought	0.018	10.23	0.0029	0.092	0.77	0.002	55.7	17.91	14.92	0.086
	Salinity	0.016	11.01	0.0029	0.087	0.79	0.003	55.4	18.02	15.11	0.086
J. curcas	Control	0.015	10	0.0037	0.02	0.69	0.02	48.0	25.36	9.7	0.214
	Drought	0.015	11.53	0.0038	0.037	0.75	0.04	46.2	24.01	8.01	0.209
	Salinity	0.017	11.97	0.0038	0.042	0.79	0.48	47.06	24.81	8.66	0.212
R. communis	Control	0.010	26.25	0.0046	0.2	0.45	0.028	61.2	23.32	29.2	0.186
	Drought	0.011	27.86	0.0047	0.31	0.53	0.037	60.11	22.21	28.78	0.182
	Salinity	0.011	27.04	0.0048	0.34	0.6	0.033	60.33	22.17	28.34	0.184
M. ferrea	Control	0.015	22.95	0.0028	0.05	0.47	0.025	34.8	27.12	18.1	0.125
	Drought	0.017	24.51	0.0029	0.07	0.54	0.034	34.01	25.86	17.01	0.123
	Salinity	0.017	24.66	0.003	0.077	0.58	0.041	33.64	26.45	17.66	0.121

5.4 CONCLUSION

Abiotic stress study is an important area with respect to an increase in plant productivity. Based on the data obtained regarding anatomical, physiological and biochemical parameters, it is clear that abiotic stress has an effect on the plants. There was a gradual decrease in most of the anatomical and physiological parameters with an increase in the stress. Though the plant experience abiotic stress, minimal differences were observed in the estimated phytoconstituents. This signifies that the plants can be grown under stress conditions without much productive loss. In conclusion, the studied plants are resistant to harsh environmental conditions and can be considered as abiotic stress tolerant plants. Moreover, salinity and drought are the most severe threats to the crops in many parts of the world which can be helpful in the selection of a suitable habitat for industrially important crops and in addition, can be a key molecular factor for genetic engineering of abiotic-tolerant plants.

Chapter 6

STUDY OF BIOMARKER FOR SEED GERMINATION IN NON-EDIBLE OIL CROPS



This chapter summarizes the successful identification of biomarkers for the seed germination. Both fluorescence intensities with respect to ROS accumulation and cell cycle activity were studied for identifying the best sowing stage.

STUDY OF BIOMARKER FOR SEED GERMINATION IN NON-EDIBLE OIL CROPS

6.1 INTRODUCTION

Nowadays, much research are being conducted with respect to biofuel; crops having high oil content are used as feedstock for the production of biofuel (Ho et al., 2014). Here, focus has been restricted to the biofuel second generation non edible oil crops such as *P. pinnata*, *J. curcas*, *R. communis* and *M. ferrea*. These plants contain high oil yielding seeds, which can be processed to produce biofuel (Bobade & Khyade, 2012; Bora et al., 2014; Lavanya et al., 2012; Pandey et al., 2012). Additionally, these four crops are considered as multipurpose plants with considerable potential in the field of medicinal, environmental and industrial aspects (Muqarrabun et al., 2013; Asif et al., 2017; Madalena et al., 2017; Sabandar et al., 2013). Since, these socio-economic plants are highly valuable, large scale cultivation will maximize its yield. Generally, the productive potential and yield of the plants are strongly affected by the environment. On contrary to this, our results from Chapter 5 infer that these plants can be grown in different geographical locations including abiotically stressed regions without any significant loss in their productivity.

Along with environmental factors, seeds are the vital source for growing crops and germplasm preservation. Seeds provide the required nutrition and suitable environment for the proper germination, which protects the species from various stresses (McDonald, 1998). Additionally, they help the tiny species adapt the environment. Quality of seeds is crucial for the successful growth of seedlings (Kumar et al., 2016). Seeds can be considered to be of good quality; if it consists of vigour and genetic purity along with the ability for fast germination (McDonald, 1998). Quality seeds show complete germination at a faster rate with physical strength and good health of plantlet; thus, enable the plantlet to adapt the climatic changes and a broad range of environmental conditions (Corbineau, 2012). To the best of our knowledge, for the germination of plant at an industrial scale, mature seeds have been used, which demands the visual expertise of the farmers. Since, high oil containing seeds are used as feedstock for biofuel production; seed losses in case of candidate plants are not affordable. Hence, it is essential to explore markers that may assist in identifying an ideal harvest time of seed. Biomarkers can be developed by understanding the physiology and

molecular mechanism of seed germination. Several biomarkers such as antioxidants, membrane integrity, ethylene as indicator, raffinose family oligosaccharides and cell cycle markers have been reported as remarkable markers for seed quality and germination (Abts et al., 2013; Souza et al., 2016; Rewers & Sliwinska, 2012; Ventura et al., 2012). Along with the identification of seedling stages, biomarkers also facilitate a better understanding of seed priming treatments. Membrane integrity biomarker and ethylene as indicator biomarker are not suitable for seeds containing high sugar content and high phenolic content (Hampton & Tekrony, 1995). Also, these markers cannot serve for the high oil yielding seeds. Therefore, these markers are not suitable for the candidate plants as well.

Synthesis of reactive oxygen species (ROS) occurs during the metabolic process of seeds; hence seed quality maintenance is an important factor (Kumar et al., 2016). ROS includes peroxides, superoxides, hydrogen peroxide, hydroxyl radical and singlet oxygen. It plays important role in cell signaling and homeostasis. The effects can be seen on DNA, RNA, and proteins, which eventually direct to cell death. It damages the cell membranes integrity, affecting the permeability and fluidity through lipid peroxidation (Avila et al., 2007). More deteriorative conditions have been reported for the high oil yielding seeds. (Ishibashi et al., 2012). Thus, it has been hypothesized that the decrease in ROS concentration acts as a messenger in seed germination (Jeevan Kumar et al., 2015).

In developing and germinating seeds, the cell cycle activity is more pronounced. It has been reported that an increased percentage of 4C nuclei usually are considered as analytic for higher mitotic activity (Rewers & Sliwinska, 2012). The low quality seed would require a longer period for DNA repair and consequently suffers from integrity in the course of imbibitions (Sliwinska, 2009). Given the fact that the active cells conclude its cell cycle within a short span, the 4C/2C ratio is possibly a good marker for the seed germination in addition to its quality assessment (Chamberlin et al., 1993a).

This chapter highlights the study of ROS intensity and cell cycle activity, which can be used as a good marker for seed germination.

6.2 MATERIALS AND METHODS

6.2.1 Sample collection

Seed samples from *P. pinnata* (NGPP46), *J. curcas* (SILJC), *R. communis* (TEZRC) and *M. ferrea* (NGMF) were collected freshly. Seeds at different stages of development were

collected and among all, four stages of seeds were considered and used for further study **(Figure 6.1).** Stage 1 (early immature), stage 2 (late immature), stage 3 (early mature) and stage 4 (late mature) were collected at different time intervals: 90, 180, 225 and 315 days after flowering (DAF) for *P. pinnata*; 35, 55, 75 and 95 DAF for *J. curcas*; 20, 30, 40 and 60 DAF for *R. communis* based on their morphology, oil content and protein storage (Chen et al., 2011; Kermode et al., 1989; Kesari & Rangan, 2011). In case of the *M. ferrea*, the seed collection was done completely based on morphological changes on 30, 50, 70 and 90 DAF. Collected seeds were washed thoroughly with distilled water (3-4 times) and were used freshly for the experiments.

Figure 6.1: Different developmental stages of seeds extracted from A) *P. pinnata*, B) *J. curcas*, C) *R. communis* and D) *M. ferrea*

6.2.2 Determination of ROS accumulation intensity

Seeds of different stages of the candidate plants were isolated from their pods and were crushed in 10 ml of 10 mM Potassium phosphate buffer in an ice cold mortar. The samples were then filtered using Whattman no. 1 filter paper (0.45 µ) and the filtrate was collected. Around 1ml of the aliquot was taken out from each filtrate and 20 µl of 10 µM Dichloro-dihydro-fluorescein diacetate (DCFH-DA) was added to all the aliquots. The samples were then incubated in dark for 1 hour followed by sonication for 5 mins. For positive control, 1 ml of 30% H_2O_2 was taken and was stained similarly. Fluorescence of the samples and the positive control were measured using spectrofluorometer (Fluoromax-4, Horiba scientific) at

excitation 485 nm and emission 519 nm wavelengths. Fluorescence intensity at 519 nm was corrected against blank (unstained sample).

6.2.3 Cell cycle activity

6.2.3.1 Sample preparation and isolation of nuclei

All the four stages of seeds belonging to the candidate plants were used for the preparation of suspension of intact nuclei. Seeds were extracted from their respective pods and embryos were dissected into embryo axis and cotyledons. For the preliminary study, Propidium iodide/Hypotonic citrate buffer (Krishan, 1975) was used for the extraction and staining of the cells. With little modification it gave clear histograms with minimum nuclear disruption and background noise. The embryo axis was chopped using surgical scissor dipped in 1ml of propidium iodide (PI) hypotonic citrate buffer following the protocol described by Ramesh et al., (2014). The propidium iodide (PI) hypotonic citrate buffer modified for seed sample contains 0.5% v/v detergent IGEPAL CA-630 (Sigma–Aldrich) and 0.1% w/v sodium citrate (Sigma-Aldrich), 2% w/v PVP40 (Polyvinylpyrrolidone) and 2% v/v β-mercaptoethanol. Staining was done using PI (50 µg/ml; Sigma–Aldrich) in a solution containing DNase–free RNase (2 mg/mL; Sigma–Aldrich). The incubation time for staining was optimized for each of the seed stages. For obtaining clear nuclei clusters the incubation period for *P. pinnata* was optimized at 10 mins. Whereas, for *J. curcas* and *R. communis* the incubation time optimized for staining was 20 mins and 30 mins respectively. Nylon mesh of 50 µm (Swedesboro, NJ) was used to filter the suspension. The filtrate obtained was then subjected to flow cytometric analysis.

6.2.3.2 Flow cytometric analyses

Number of nuclei in the G0/G1 phase (2C) and G2/M phase (4C) was estimated with BD FACSCalibur flow cytometer (BD Biosciences, New Delhi, India). PI stained samples were excited with 15 mW argon ion laser at 488 nm. Fluorochrome fluorescence was collected through a 645 nm dichroic long-pass filter and a 620 nm band-pass filter. Throughout the experiment, the voltage and gain were kept constant and three dot plots; *viz* FSC vs. SSC (for understanding size and granularity of the nucleus), SSC vs. FL2A (to spot the signals due to intact fluorescence nuclei) and FL2A vs. FL2W (for discriminating singlets and doublets) were plotted. For each sample, 10,000 nuclei were analyzed at the rate of 20-50 nuclei per

seconds. Histograms obtained were analyzed using FlowJo v. 7.6.5 (FlowJo, TreeStarInc, Ashland, OR) software for the estimation of mean fluorescent intensity (MFI) and co-efficient of variance (CV). The ratio 4C/2C was calculated, which is the ratio between the numbers of nuclei with 4C DNA content to the numbers of nuclei with 2C DNA content.

6.2.3.3 Statistical analysis

All the experiments were performed in triplicates and the statistical analysis was done using the one-way analysis of variance and Duncan's test ($P < 0.05$).

6.3 RESULTS AND DISCUSSION

The cell activity and the ROS concentration in different stages of seed development gives an idea about the quality of seeds and also helps to predict the optimum development stage for sowing seeds. Thus, in the present study, four different stages of seed i.e. early immature, late immature, early mature and late mature were selected based on their cotyledonary reserves distribution and morphological appearances.

6.3.1 Reactive oxygen species concentration

ROS accumulation at lower concentration helps in breaking seed dormancy and protects against pathogens, whereas, at higher concentration it is detrimental (Kumar et al., 2016). Reports have shown that ROS along with antioxidants, plant hormones and other reactive species are a part of the signaling networks, which are important for plant stress responses, cell division, and cell death (Bailly et al., 2008). As a result, they play significant roles in the regulation of seed development, maturation, germination, seedling establishment and seed ageing (Kumar et al., 2015). Notably, although ROS are required at low concentrations for seed development, it must be under the rigid control of antioxidants. Oxidative stress occurs due to the imbalance between the pro- and antioxidative processes, which can promote seed death. Seed deterioration during prolonged storage is generally attributed to the ROS (Bailly et al., 2008). However, reports also suggest that the ROS accumulation play vital roles in signaling linked with germination (Bailly et al., 2008). ROS are reported to be present at all the stages of seed germination (Kibinza et al., 2006); thus, studying ROS concentration will

give a better insight into the seed physiology, thereby aiding in the selection of proper germination stage of seeds.

For studying the intensity of ROS accumulation, seeds of all the four plants with different developmental stages were pre-incubated with fluorescence probe DCFH-DA. DCFH-DA is a cell permeable dye, which undergoes enzymatic cleavage of the diacetate groups by cytoplasmic esterase inside the cell, followed by the further oxidation to produce highly fluorescent product dichlorofluorescein (Kalyanaraman et al., 2012). For positive control, H_2O_2 was used as it is the long living form of ROS (Bailly et al., 2008). Fluorescence intensity with respect to ROS concentration was found to be present in all the stages of seeds **(Figure 6.2-6.5)**. Area under the curve of the intensity spectra was calculated to compare the stages.

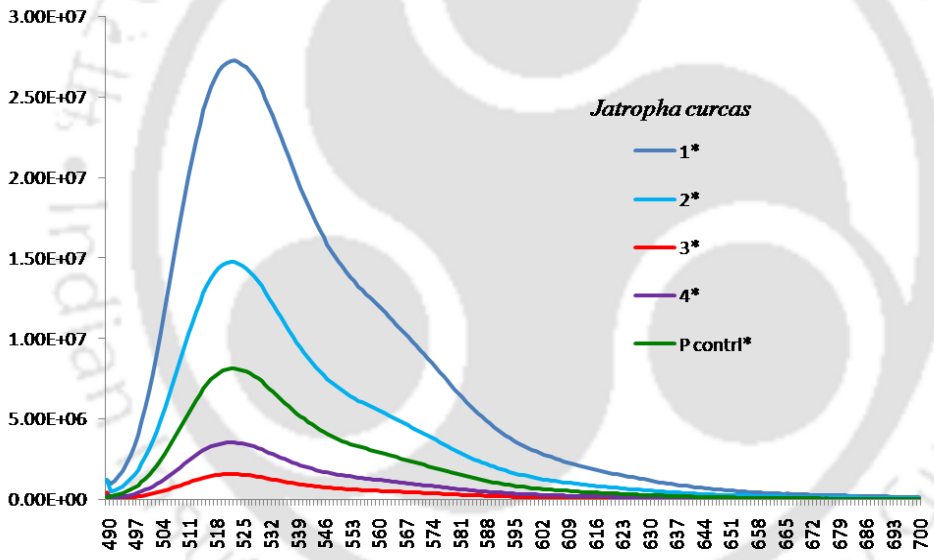


Figure 6.2: Quantification of reactive oxygen species treated with DCFH-DA at different stages of seed development of *J. curcas*. Fluorescence, 485 nm (excitation) and 519 nm (emission) was finally quantified after 1-hr incubation. ROS accumulation intensity of 1*) Early immature stage, 2*) Late immature stage, 3*) Early mature stage, 4*) Late mature stage and P*) Positive control was acquired. According to the intensity measured at stage 3 (early mature) is having the lowest ROS intensity than the rest of the stages

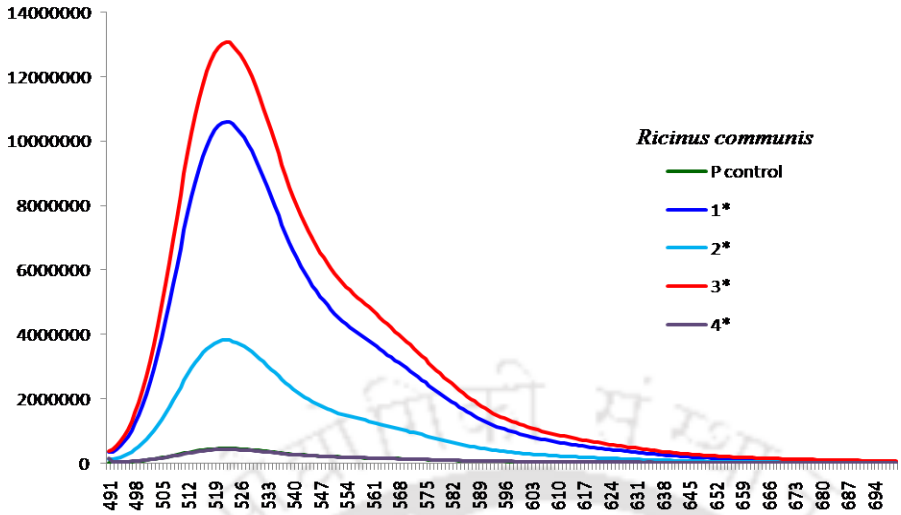


Figure 6.3: Quantification of reactive oxygen species treated with DCFH-DA at different stages of seed development of *R. communis*. ROS accumulation intensity of the four stages along with the positive control was acquired. According to the intensity measured stage 4 (late mature) is having the lowest ROS intensity than rest of the stages

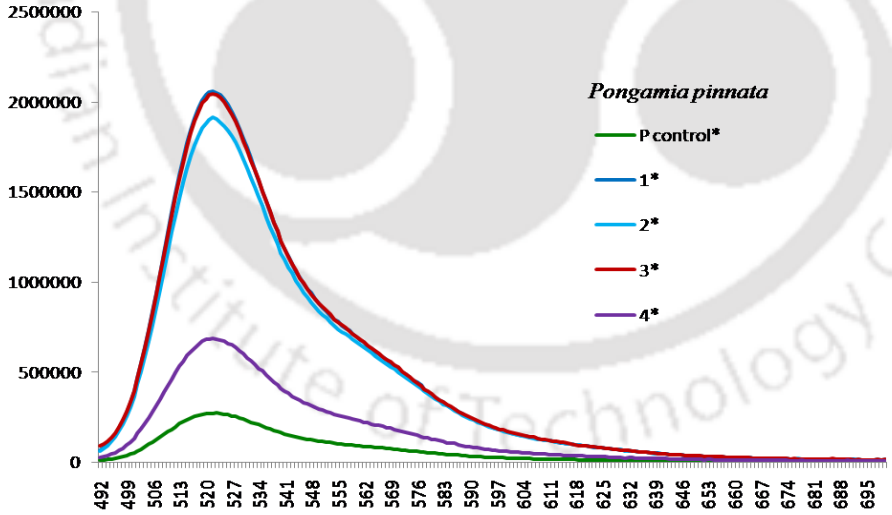


Figure 6.4: Quantification of reactive oxygen species treated with DCFH-DA at different stages of seed development of *P. pinnata*. ROS accumulation intensity of the four stages along with the positive control was acquired. According to the intensity measured stage 4 (late mature) is having the lowest ROS intensity than rest of the stages

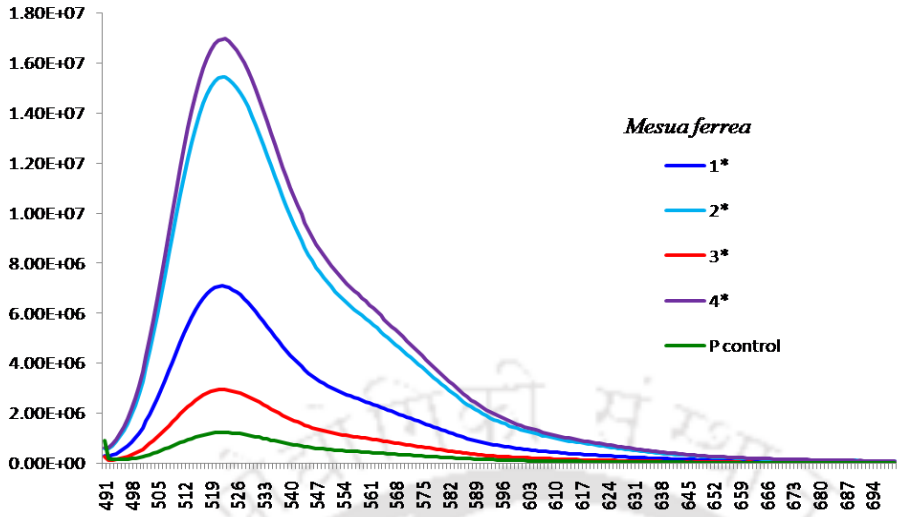


Figure 6.5: Quantification of reactive oxygen species treated with DCFH-DA at different stages of seed development of *M. ferrea*. ROS accumulation intensity of the four stages along with the positive control was acquired. According to the intensity measured stage 3 (early mature) is having the lowest ROS intensity than rest of the stages

In the early immature and late immature seeds, ROS concentration was observed comparatively higher than the early and late mature seeds, which is indicative of the undergoing stress. This result complements with the previous study on sunflower seeds where ROS content was found to be higher in the initial stages of seed development (Kibinza et al., 2006). One of the reasons could be the presence of high moisture in the preliminary stages of seeds, which leads to high metabolic activities. However, as the seed matures, there is a decrease in the metabolic activity due to seed desiccation (Kibinza et al., 2006). From the start of embryogenesis to the end of germination, ROS concentration fluctuates significantly. Such variation in ROS production occurs due to the molecular mobility and cytoplasmic viscosity, which are responsible for governing the occurrence and rate of the metabolic reactions (Bailly et al., 2008). The presence of free water in hydrated seeds allows the mobility of ROS within the seed that aids ROS to reach to its targets site far away from its production site. ROS which are produced in the dry state, are effective only when the seeds are hydrated during germination and act as cellular messenger. The intensity of ROS accumulation was observed to be the lowest in early mature seeds for *J. curcas* and *M. ferrea* whereas, in *P. pinnata* and *R. communis*, the intensity was lowest in the late mature seeds. According to literature, reduction in the concentration of ROS act as a good marker for seed

germination (Kumar et al., 2015; Sano et al., 2015). Hence, the best seed harvesting stage for *J. curcas* and *M. ferrea* is the early mature seed stage (stage 3) and for *P. pinnata* and *R. communis* is the late mature stage (stage 4).

6.3.2 Cell cycle activity

Along with ROS accumulation, another seed germination marker was also studied. Cell cycle activity is considered as one of the superlative biomarker to determine the quality of seed (Kumar et al., 2016). In different developmental stages of seeds, mitotic/endoreduplication is observed which indicates the physiological state of a seed. In the embryo, proliferative cells undergo replication in S phase via mitosis where 2 n is divided into 4 n conditions. DNA content is thus divided into two daughter nuclei (4 n to 2 n) and 4C nuclei indicate the mitotic activity. Hence 4C/2C ratio is used to monitor germination rate and seed development (Chamberlin et al., 1993b). 4C/2C ratio do not consider the occurrence of endopolyploid cells thus, in polysomatic seeds (∑>2C)/2C ratio is recommended as a biomarker for seed germination. In some of the Fabaceae species, endoreduplication intensity was reported as a biomarker for developing seeds (Rewers & Sliwinska, 2012). Four stages of seeds were exercised for examining cell cycle activity using Flow cytometer (BD FACS caliber). Flow cytometry is an easy and less time consuming method to detect cell cycle activity in developing and germinating seeds. PVP40 and β-mercaptoethanol was used in higher concentration to remove the inhibitors from nucleus. Staining period was optimized for all the four plants where it was observed that the seeds (*J. curcas* and R. communis) with the capability to yield high oil content take longer time of incubation to stain the nuclei. Flow cytometric analysis revealed the absence of endopolyploid nuclei in the candidate plant seeds. Since the plants are true diploids, the embryonic tissues of *Jatropha*, *Ricinus* and *Pongamia* seeds contained 2C and 4C nuclei which were recorded in histogram plot **(Fig 6.6-6.8)**. To determine the percentage of nuclei in the histogram, background signal was subtracted from the peak height. Number of nuclei in both G0/G1 phase and G2/M phase was estimated using Flowjo software. Thus, 4C/2C ratio was determined for all the seed stages of the four plants and the values were tabulated in the **Table 6.1**. In stage 1 and 2 the rate of cell division was found to be higher than the rate in stage 4 of *J. curcas* and in Stage 3 of *R. communis* and *P. pinnata*. As per previous reports, in early developmental stage of embryo the 4C nuclei content in G2 phase of the cell cycle is comparatively higher than the mature embryos of

orthodox seeds, which contains high 2C nuclei content with small portion of 4C nuclei content (Sliwinska, 2009; Sliwinska, 2000). It happens due to the occurrence of cell cycle arrest at the G0/G1 phase. Additionally, it has been reported that the proportion of the 4C nuclei increases again during the germination stage (Rewers & Sliwinska, 2012). This is in accordance with the result obtained in the present study where the rate of cell division increases in stage 3 for *J. curcas* and in stage 4 for *R. communis* and *P. pinnata*. This indicates that the rate of cell division is faster in the germinating stage of seed. Therefore, the 4C/2C ratio has been projected as a biomarker for seed quality assessment for proper germination. In the present study, the 4C/2C ratio was found to be highest in the late mature seeds of *Ricinus* and *Pongamia* whereas, in case of *Jatropha* the 4C/2C ratio was more in early mature seeds. It was difficult to extract the nuclei from *Mesua* seeds because of their recalcitrant nature, due to which cell cycle activity marker study was not carried out successfully.

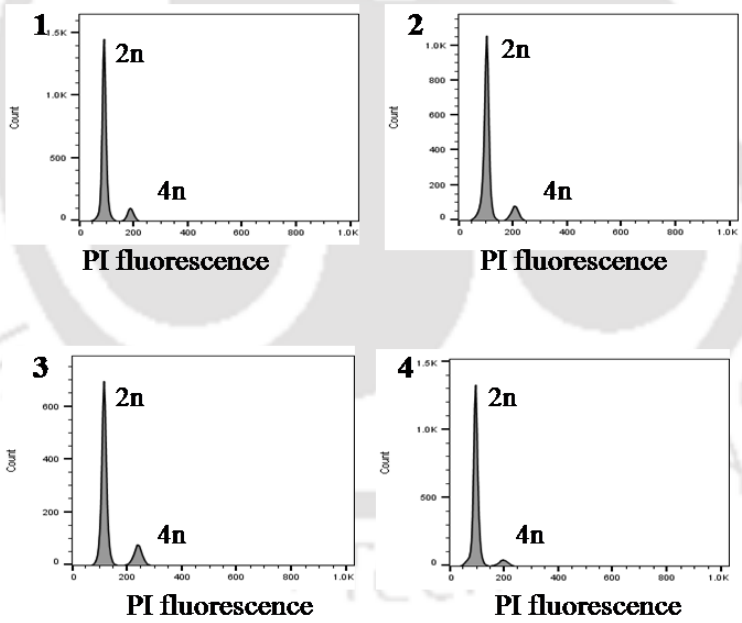


Figure 6.6: Selected histograms of PI fluorescence in the embryo axis of *J. curcas*. G0/G1 and G2/M phase of cell cycle were arrested in 1) early immature, 2) late immature, 3) early mature and 4) late mature stages of seeds. 4C/2C ratio was estimated to obtain the higher cell division stage. According to the number of dividing cells, stage 3 was found to be best for harvesting among the four stages

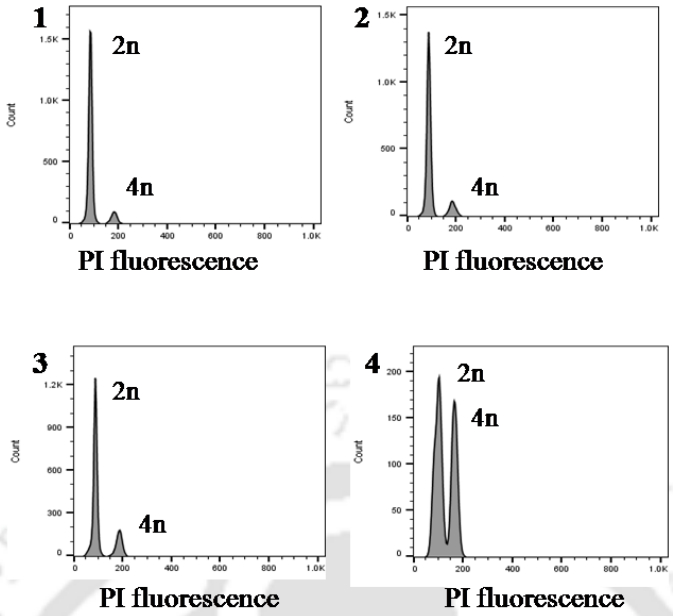


Figure 6.7: Selected histograms of PI fluorescence in the embryo axis of *R. communis*. G0/G1 and G2/M phase of cell cycle were arrested in 1) early immature, 2) late immature, 3) early mature and 4) late mature stages of seeds. According to the 4C/2C ratio, stage 4 was found to be best for harvesting among the four stages

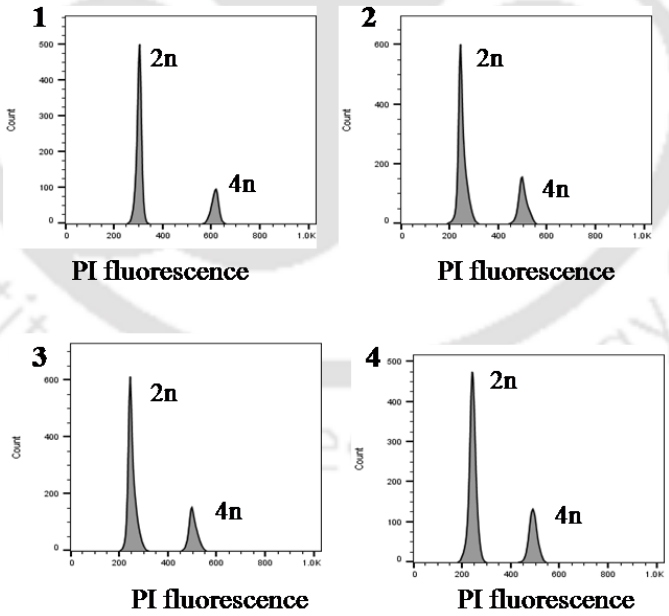


Figure 6.8: Selected histograms of PI fluorescence in the embryo axis of *P. pinnata*. G0/G1 and G2/M phase of cell cycle were arrested in 1) early immature, 2) late immature, 3) early mature and 4) late mature stages of seeds. According to the 4C/2C ratio, stage 4 was found to be best for harvesting among the four stages

Table 6.1: Determination of biomarker (4C/2C ratio) in the different developmental stages of seeds

Plants	4C/2C ratio			
	Early immature	**Late immature**	**Early mature**	**Late mature**
J. curcas	0.13 ± 0.11	0.14 ± 0.21	0.07 ± 0.07	0.2 ± 0.11
R. communis	0.17 ± 0.06	0.2 ± 0.11	0.37 ± 0.12	0.7 ± 0.06
P. pinnata	0.34 ± 0.1	0.35 ± 0.04	0.29 ± 0.2	0.42 ± 0.07

The results of cell cycle activity upheld the results obtained for ROS. The present study revealed two robust, time and cost effective techniques for the identification of the suitable seed stage for the plantation of the studied plants. In addition to the identification of best stage for seed harvesting, biomarkers also facilitate a better understanding of seed priming treatments. Seed priming treatments are known to have many beneficial effects on biochemical, metabolic, cellular and molecular events. Its positive effect has also been reported in proteins and RNA synthesis, mobilization of reserves and induction of cell-cycle processes. A positive correlation was reported between the 4C/2C ratio and the efficacy of the priming treatment in tomato, sugar-beet and pepper seeds (Elwira et al., 1999; Lanteri et al., 1994; Ozbingol et al., 1999). In future, a correlation study between the biomarker and the efficacy of priming treatment will be suitable for optimizing the best priming treatment. The genome size was found to have a negative correlation with the markers of seed developmental stage (Rewers & Sliwinska, 2012). The cell cycle activity marker can also be correlated with the genome size of the plants for the better understanding of seed proliferation at molecular level, in *P. pinnata, J. curcas and R. communis*. The present study will be helpful in gaining the relation between the ROS as well as the cell cycle with the seed quality, which may be applied to other commercially important plants and crops as well, for the scale up production of those species.

6.4 CONCLUSION

This chapter summarizes the successful identification of biomarkers for the determination of the best stage for seed germination. Both fluorescence intensities of ROS accumulation and cell cycle activity strongly signify the sowing ability of early and late mature seeds. The seed embryo was found to be a suitable model for following seed development; hence the obtained results can be used to study *in vivo* as well as *in vitro* cell/tissue differentiation. Flow cytometry was proved to be an easy and fast technique for detecting cell cycle activity. This study will be helpful for the research community in scrutinizing the seed quality, and its maturation stage ideal for germination.

Chapter 7

SUMMARY AND FUTURE PROSPECTS

This chapter reveals the overall summery of the doctoral thesis and the future directions.

SUMMARY AND FUTURE PROSPECTS

7.1 SUMMARY

The escalating demand for biofuel has inspired the exploration and identification of many plants as potential biofuel crops. Some plants that appear to have the potential to produce biofuel efficiently and economically with minimal impact on food crops of tropical importance are *Pongamia pinnata*, *Jatropha curcas*, *Mesua ferrea*, and *Ricinus communis*. Genome size estimation has been done on these four biofuel crops, which can be helpful to search a potent trait in this field. This will also help the researchers in their attempt to exploit and improve the traits of significance. Unlike traditional methods, flow cytometry has made it possible to measure the nuclear DNA content in a cost and time-effective way. In this study, with the estimation of genome size, intraspecies variations have been reported. The repetitive DNA content occupies a large portion of the nuclear genome leading to the variation of genome size in different species. To date, Ty1-*copia* retrotransposons have been studied and characterized widely in plants. Thus, calculating the copy number of Ty1-*copia* and understanding its probable role in the genetic diversity will serve as an important background for further understanding of retrotransposons in the genomic study. Environmental factors also affect genome size, thus, the knowledge of genome content and cell phenotypic characters makes it possible to infer the relationship between them and may lead to the genome evolution. Environmental stresses, salinity, and drought have the most adverse effect on plant productivity. Thus, a study was conducted to examine the effects of the abiotic stresses on several anatomical, physiological and biochemical parameters. This study is essential for a clear perception of plant resistance mechanism to stress conditions. Since the mentioned crops are having versatile importance, it should be grown in a large scale for higher yield. Several promising markers have been reported so far for monitoring seed quality process. Thus, identifying a biomarker for seed germination will be beneficial to decrease seed loss during sowing.

Nuclear DNA content was estimated in the four plants under study. The genome size was in the order of *J. curcas* (0.86 pg/2C DNA), *R. communis* (1.01 pg/2C DNA), *M. ferrea* (1.4 pg/2C DNA) and *P. pinnata* (2.49 pg/2C DNA). Intraspecific variation was observed in

the genome size of all the studied plants collected from different geographical locations. However, no variation was detected when chromosome number was estimated. Ecotypes of the non-edible oil crops collected from different locations of Assam revealed chromosome number 2n = 2x = 22, 22, 20, 30 respectively without any polyploidy and were true diploid plants. The environmental factors affect phenotypic characters and thus, influence genome size. When analyses were partitioned across growth forms, it was found that the relationships within shrubs and trees were not significant for 2C DNA content and phenotypic characters. Moreover, no significant variation in Ty1- *copia* copy number was observed, thus, it is concluded that the variation in genome size is mainly because of environmental variation. Study was also conducted to understand the plant resistant capacity. Though abiotic stress had effect on plants, there was a gradual increase in most of the biochemical parameters with an increase in stress. Though the plants experience abiotic stress, minimal difference in the value of phytoconstituents was observed. This signifies that the plants can be grown in a stressed environment without any productive loss. In conclusion, the studied plants are resistant to harsh environmental conditions and can be considered as abiotic tolerant plants. Also, research was conducted for the successful identification of biomarkers for the seed germination. Both fluorescence intensities with respect to ROS accumulation and cell cycle activity strongly signify the sowing ability of early and late mature seeds. This study will be helpful for the research community in scrutinizing the seed quality, and its ideal maturation stage for germination.

7.2 FUTURE PROSPECTS

- Genome size estimation will help in the study of genome sequencing, which is important for better understanding of the genomic study.
- Flow cytometry based new protocol can be developed for the extraction of nuclei from the seeds of *Mesua ferrea.*
- Sometimes genome analysis is hampered by sequence redundancy due to the presence of repetitive DNA. This can be solved by flow cytometry based chromosome sorting, which enables purification of chromosomes. Study of chromosome sorting along with next generation sequencing will provide a cost effective way to tackle the complexity in genomes.
- Quality and quantity of oil yield can be examined under abiotic stress, which can be further rectified in the future by genetic manipulation.
- Study of biomarkers for seed germination offers a powerful approach for real-time monitoring and understanding the quality stage for seed sowing. This study will also help in understanding the seed priming treatments.
- Thorough understanding of the effects of ROS will provide great scope for the improvement and maintenance of seed vigor and quality.

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RESEARCH OUTPUT

RESEARCH PAPER

1. **R Das**, RG Shelke, L Rangan, S Mitra (2018). Estimation of nuclear genome size and characterization of Ty1-*copia* like LTR retrotransposon in *Mesua ferrea* L. ***Plant Biochemistry and Biotechnology*** (doi.org/10.1007/s13562-018-0457-7).

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1. **R Das**, G Juerges, P Nick, L Rangan* (2014) Genome size of four biofuel crops. 4th International Science Congress, Udaipur, 8th - 9th Dec 2014, pp 67.
2. **R Das**, G Juerges, P Nick, L Rangan* (2015) Flow cytometry based correlation of nuclear DNA content with cell phenotypic characters. International Conference on New Horizons in Biotechnology: 12th Annual Convention of The Biotech Research Society, Trivandrum 22nd - 25th Nov 2015, pp 341.
3. RG Shelke, **R Das**, L Rangan* (2015) Evolution and distribution of LTR-retrotransposons in *Pongamia pinnata* and its correlation with genome size. 2nd International Conference on Biotechnology and Bioinformatics (ICBB-2015), Pune 6th -8th Feb 2015, pp 27 - 28.
4. L Rangan*, A Singh, RG Shelke, **R Das**, AM Ramesh, V Kesari, P Scott, P Gresshoff (2015), New positives of biotech research of renewable energy resources- Success story of *Pongamia*. Proceedings National Seminar on Biofuel A Search for New Fire SIBB R&D, Cochin 17th - 18th Dec 2015, pp 22-25.
5. **R Das**, L Rangan* (2016) Estimation of nuclear genome size, chromosome number determination and cell phenotypic investigation in *M. ferrea* L., a valuable biodiesel plant. Proceedings of 9th TCS annual event and Flow cytometry workshop on Flow application in basic, applied and clinical biology, Guwahati 3rd – 5th Nov 2016.
6. **R Das**, L Rangan* (2017). Variation in leaf anatomical characters and total chlorophyll content in *Mesua ferrea* during abiotic stresses. Proceedings of the International Symposium on Plant Biotechnology for Crop Improvement, IIT Guwahati, India 20th - 21st Jan 2017, pp 65.

7. **R Das**, L Rangan* (2017). Assessment of genome content, chromosome number and cell phenotypic features in *Mesua ferrea* L. Research Conclave, IIT Guwahati, India 16th - 19th March 2017, pp 64.
8. **R Das**, L Rangan* (2018). Genome size and Ty1-*copia* retroelements in biofuel crops. 24th ISCB Frontier Research in Chemistry & Biology Interface, Jaipur, India 11th - 13th January, pp 176.

WORKSHOP ATTENDED

1. Workshop on Basic and Clinical Flow cytometry course (2014). Organized by Cachar Cancer Hospital and Research centre, Silchar and IIT Guwahati, Assam 10th - 11th Nov 2014.
2. 9th TCS annual event and Flow cytometry workshop on Flow application in basic, applied and clinical biology (2016), Organised by TCS and IIT Guwahati, Guwahati 3rd - 5th Nov 2016.
3. Indo-Japan Workshop on Translational Agriculture Avenues for International Cooperation (2017). Organized by DBT- program support centre, IIT Guwahati and Gifu University, Japan 29th March 2017.
4. Research conclave workshop on intellectual property rights (2017). Organized by Indian Institute of Technology Guwahati, Guwahati 16th - 19th March 2017.
5. Workshop on advanced microscopy and imaging techniques (2017). Organized by DSS Imagetech Pvt Ltd, Olympus Medical Systems India Pvt Ltd and IIT Guwahati, Guwahati 18th - 20th April 2017.
6. Sensitization Workshop on Technological Empowerment of Women (2017). Organised by IIT Guwahati and The National Academy of Sciences, Allahabad 3rd – 4th Nov 2017.

NCBI GENBANK SUBMISSION

1. *Pongamia pinnata* Ty1-*copia* RT (KP202847.1- KP202834.1, MH397570-MH397584)
2. *Jatropha. curcas* Ty1-*copia* RT-RH (MK036332-MK036339)
3. *Ricinus. communis* annotated Ty1-*copia* RT-RH sequences (MK036340-MK036345)
4. *Mesua ferrea* Ty1-*copia* RT-RH sequences (KU507513-KU507530).