3.1 *Collection of Accessions and Plantation*

Study of Growth, Morphological, Physiological and Flowering Parameters

The experiment was conducted at CSIR-North East Institute of Science and Technology (CSIR-NEIST), Jorhat which falls between 27.35–26.30˚N latitude and 93.45–94.30˚E longitude. The area enjoys moderate type of climate; with mean annual rainfall of 2244 mm. Stem or branch cuttings were collected from 24 sources from North-East states of India with considerable geographic isolation (Table 3.1). From each of the sources, cuttings were collected from a selected parent tree, located about 100 m apart from each other trees in order to avoid narrowing down the genetic base due to relatedness or inbreeding (Turnbull, 1975). The cuttings from each tree were collected and labeled to maintain their identities. In nursery, four replicates of each selected sources were maintained in poly-bags. Before planting in the poly bags the lower portion of the cuttings were treated with 0.8% of Rootex solution. The potting media consisted of soil, sand and farmyard manure in the ratio of 1:1:1 (by volume), treated with Aldrex to avoid any chance of insect attack. Thus the cuttings were maintained in poly-bags for 90 days for rooting. Three months old rooted seedlings from each accession selected for uniformity and seedling vigor, were planted in the field in a randomized complete block design with 3 replications and the spacing between plant was 2.5 x 2.5 m. Irrigation in first year and weeding were carried out regularly and when required. Observations were recorded on the trial periodically for plant height (cm), stem girth (cm), number of branches per plant and physiological parameters, *viz.* photosynthetic rate (µmol CO_2 m^{-2} s^{-1}) and stomatal conductance (cm s^{-1}). The first assessment was carried out after 12 months of field planting and subsequently after 24 months. Photosynthetic rate and stomatal conductance

were measured using Portable Photosynthesis System, TPS-2 (PP Systems). Leaf area was measured with a Leaf Area Meter 211 (Systronics) for five leaves chosen randomly from each tree and expressed as average leaf area per leaf. The leaf area was multiplied by the number of leaves occurring in the plant and was expressed as total leaf area per plant. Statistical analysis was done according to the standard procedure (Panse & Sukhatme, 1967). All the percentage data were suitably transformed and analysed in a completely randomized design (CRD).

3.2 *Seed yield and seed oil extraction*

The main objective of this part was to evaluate and compare the seed yield capacity and oil contents of all the *Jatropha* accessions studying under this experiment at NEIST, Jorhat.

The *Jatropha* trees begun to produce seeds in the second growing season, but only a part of accessions bear seeds in spring-summer period. However, all accessions gave seeds in autumn. So the seeds collected in this season from all the *Jatropha* accessions have been utilized for biochemical analyses.

Seeds were removed from ripe fruits, cleaned, dried in open air and weighted. Seeds, kernel and shell, were grounded and immediately subjected to oil extraction in Soxhlet apparatus using hexane as solvent. Three replicates for each seed lot has been extracted. Oil (raw oil), after total evaporation of hexane, were weighted for percentage in seeds and density and used for subsequent biochemical analyses.

Table 3.1. Accession sources of *Jatropha curcas* L. and their geographical locations:

Sl. No.	Accession Code	State	Locality	Latitude (N^0)	Longitude (^0E)	Altitude (m)	Av. R/F (mm)	Temp. (^OC)	
								Min.	Max.
1.	***Jc-1***	ASSAM	JORHAT	26.30	94.30	116.00	2244	9.0	39.0
2.	***Jc-2***	ASSAM	DERGAON	26.73	94.01	86.00	2052	9.3	35.8
3.	***Jc-3***	ASSAM	KARBI ANGLONG	26.04	93.67	86.00	2074	10.02	35.0
4.	***Jc-4***	ASSAM	BONGAIGAON	26.28	90.34	53.00	3500	12.9	31.7
5.	***Jc-5***	ASSAM	SONITPUR	26.60	92.78	86.00	1563	11.0	31.0
6.	***Jc-6***	ASSAM	DIBRUGARH	27.28	94.55	108.00	2758	10.0	31.0
7.	***Jc-7***	ASSAM	GOLAGHAT	26.75	94.25	86.00	2052	9.3	35.8
8.	***Jc-8***	ASSAM	LAKHIMPUR	27.65	96.25	87.00	2635	8.0	31.5
9.	***Jc-9***	ARUNACHAL PRADESH	NAHARLAGUN	27.00	93.42	200.00	2688	8.0	32.0
10.	***Jc-10***	ARUNACHAL PRADESH	ROING	28.05	95.89	300.00	2800	5.0	29.0
11.	***Jc-11***	ARUNACHAL PRADESH	ITANAGAR	27.06	93.41	146.00	3000	8.0	32.0
12.	***Jc-12***	NAGALAND	TULI	26.44	94.65	1325.00	2330	9.3	28.0
13.	***Jc-13***	NAGALAND	NAMSA	26.78	94.77	897.64	1644	13.3	25.5
14.	***Jc-14***	NAGALAND	KOHIMA	25.40	94.08	1433.00	2300	4.0	31.0
15.	***Jc-15***	NAGALAND	MOKOKCHANG	26.44	94.65	1325.00	2330	9.0	25.0
16.	***Jc-16***	MEGHALAYA	WEST GARO HILLS	25.30	90.13	870.00	2600	7.0	30.0
17.	***Jc-17***	MEGHALAYA	WEST KHASI HILLS	25.98	90.68	1496.00	3350	10.0	37.5
18.	***Jc-18***	MIZORAM	KOLASIB	24.13	92.40	660.54	2860	7.0	32.0
19.	***Jc-19***	MIZORAM	AIZAWL	23.36	93.00	1132.00	3000	11.0	30.0
20.	***Jc-20***	MANIPUR	LAMPHELPET	24.44	93.65	790.00	2027	8.2	34.5
21.	***Jc-21***	MANIPUR	IMPHAL	24.44	93.65	790.00	990	5.0	35.0
22.	***Jc-22***	MANIPUR	LOKTAK	24.30	93.55	768.00	1183	6.0	32.0
23.	***Jc-23***	TRIPURA	AGARTALA	23.50	91.25	12.80	2240	10.0	35.0
24.	***Jc-24***	TRIPURA	UDAIPUR	23.31	91.31	24.68	2100	12.0	35.0

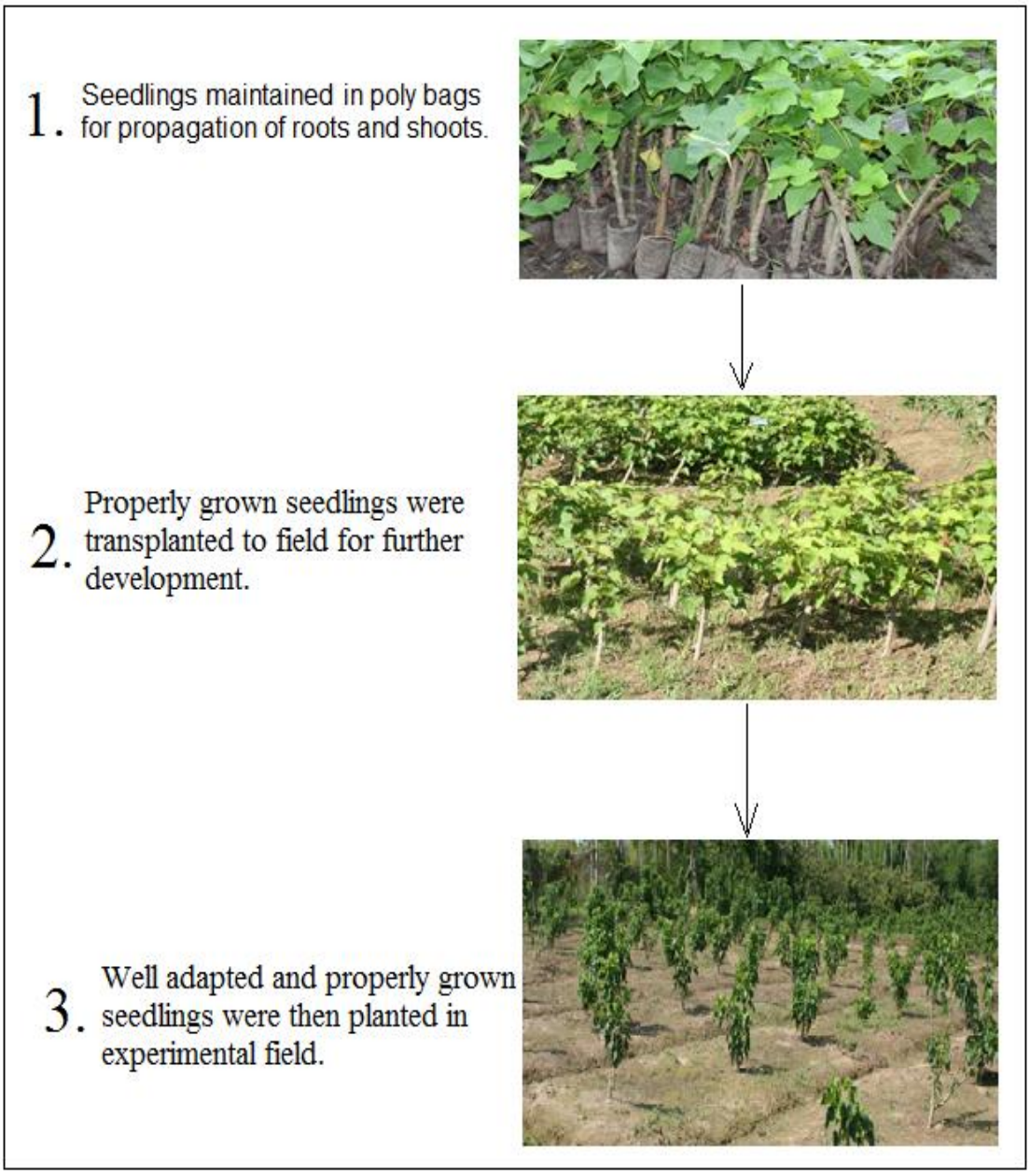

Plate 3.1: Pictures showing the stepwise plantation procedure of the collected *J. curcas* accessions from nursery to the experimental field.

3.3 *Biochemical Analysis of seed oil*

This part of the objective deals with the study and analysis of all the chemical constituents of *Jatropha* seed oil extracted from each and every accessions method.

3.3.1 *Quantification of Fatty acids, phorbol esters and tocopherols content*

The raw seed oil were extracted four times with equal volume of methanol to remove phorbol esters (PEs); the free fatty acids (FFAs) and tocopherols. The remaining triglyceride oil fraction was hydrolyzed to obtain fatty acids. Essentially, an aliquot of oil (20 μL) was hydrolyzed in 1 ml of 1 % NaOH in MeOH, at 80°C for 60 min. The solution dried under vacuum and the residue was dissolved in 2 ml of H_2O plus 0.3 ml of 1 N H_2SO_4, and shaken vigorously. The fatty acids, recovered from water sulfuric acid solution with 1 ml of hexane, were analyzed qualitatively and quantitatively on a HPLC system as described by Bravi *et al.* (2006) and Li *et al.* (2008) with some modifications. Briefly, an HPLC system pump and degasse PU2089 (Jasco, Tokyo, Japan) equipped with 3300 Evaporative Light Scattering Detector (ELSD) (Grace, Deerfield, USA) was used. The FAs were separated on Luna 5μm C8 column (150×4.6 mm) (Phenomenex, Bologna, Italy) thermostated at 18°C, eluted with acetonitrile:isopropanol:water (50:30:20, v/v/v) at a flow rate of 1 ml/min for 20 min. After each chromatographic run the column was washed for 10 min with acetonitrile:isopropanol:water (50:45:5, v/v/v, 1 ml/min) to check 176 the complete hydrolysis of oil triglycerides. Data from the detector were recorded, integrated and elaborated by the Borwin software program (JMBS Dev., Le Fontanil, France). The quantities of individual fatty acid were calculated in comparison to the respective FA standards (Sigma-Aldrich, St Louis, Mo, USA). In each sample composition was determined in triplicate and the quantities of individual fatty acid were recalculated

and expressed as a percentage of total FAs. The amount of free fatty acids (FFAs) was determined using methanol extract fractions. After evaporation of methanol the residue was diluted in hexane and analyzed according to the HPLC method described previously for FAs of hydrolyzed triglycerides. All analyses were conducted in triplicate and the quantities of FFAs were calculated and expressed as a percentage of total hexane extracted raw oil.

To detect phorbol esters, the methods described by Makkar *et al.* (2009) and Haas and Mittelbach (2000) have been adapted and used. Essentially, the methanol fraction extracted from raw oil was evaporated under nitrogen stream, the residue was dissolved/diluted in acetonitrile. In some samples the free fatty acid content was in very significant quantity as consequence samples were not dried under nitrogen stream, the volume were measured with micro-syringe and diluted to fixed volume with acetonitrile. The HPLC analyses were carried out using an HPLC Jasco Tritotar VI pump and Jasco MD910 Diode Array Detector (DAD). 20 µL of extract were loaded into a Phenomenex Kinetex 2.6u C18 column (100×4.6 mm), and eluted with 0.8 ml/min MeOH 93 %. This column and eluent system gave chromatograms resolution and phorbol esters separation similar to the Makkar *et al.* (2009) HPLC system, but in short time and with less solvent consume. The phorbol esters were identified by comparison of UV spectra reported by Makkar *et al.* (2009) and Haas *et al.* (2002) and quantification of phorbol esters were also done according to Makkar criteria using the phorbol myristate acetate (Sigma10 Aldrich, St Louis, USA) as reference (Makkar *et al*., 2009). The spectra 200 data and absorbance at 280 nm were acquired and elaborated by the Borwin software system.

Tocopherols were analysed in HPLC by direct injection of raw oil (Gimeno *et al.,* 2000 and Malvolti *et al.,* 2010). The tocopherol isomers were separated using an HPLC Jasco Tritotar III pump and Jasco MD910 Diode Array Detector (DAD). An amount of 5-10 µL of pure oil was loaded into a Kinetex 2.6 µm C18 100A (Phenomenex) column (100 × 4.6 mm), and eluted with 1.5 ml/min MeOH 95 %. The

DAD spectra gave information on the purity of tocopherols. Absorbance at 280 nm was elaborated by the Borwin software system to determine tocopherol amounts in comparison with standards (VWR, Darmstadt, Germany). Tocopherols can be also analyzed in methanol fraction extracted from raw oil giving results comparable to the raw oil analysis.

3.4 ***Analysis of variation in genetic composition:***

Assessment of genetic diversity is important for the study of biodiversity, population dynamics and ecological relationships. For that purpose, the molecular markers are now the widely used tool to assess the genetic diversity (Koebner *et al*., 2001 and Karp *et al*., 1998). Bardini *et al.* (2004), stated that most of the molecular markers that are used to assess the genetic diversity correspond to anonymous DNA sequences that, although efficient in providing a vast range of natural sources for DNA polymorphisms, almost exclusively associated within regions of unknown functions. In fact, most of the identified changes do not occur in functionally relevant regions of DNA and thus rarely lead to identification of variations occurring within the genes. They have also stated that the family of plant β-tubulin genes may be regarded as a valuable functional DNA target amenable for diversity assessment. Therefore a molecular level experiment was also carried out to verify the molecular basis of the phonetic diversity if present among the studied accessions of *Jatropha curcas* by using the TBP molecular marker in first level and secondly through studies performed on DNA methylation. Different versions of the Tubulin-Based Polymorphism (TBP) method (Bardini *et al.*, 2004; Breviario *et al.*, 2007; Galasso *et al.*, 2011) were performed on the genomic DNA extracted from all the 24 accessions analysed. The hTBP (Horse TBP) method was also used as it amplifies both introns present in the coding sequence of the beta-tubulin genes by combined effect of both the forward and reversed degenerated primers along with second exon present within, which may help in finding any polymorphisms more accurately than TBP-I (or simply TBP) and TBP-II (or cTBP) (Braglia *et al.*, 2010).

3.4.1 *DNA Extraction and PCR Conditions*

Total genomic DNA was extracted from younger leaves collected from each accession following the standard CTAB method with minor modifications (Doyle and Doyle, 1990). Five grams of leaves were ground in liquid nitrogen, then homogenized in 20 ml of extraction buffer (2% CTAB, 20 mM EDTA, 2% PVP, 1.4 M NaCl, 100 mM Tris–HCl pH 8.0 and 1% β-mercaptoethanol) and incubated at 65^{o}C for 1 h. The supernatant was treated with RNase A (100 µg/ml), incubated at 37^{o}C for 30 min and twice extracted with chloroform:isoamylalcohol (24:1 v/v). The DNA was precipitated with isopropanol and washed twice with 70% ethanol. The pelleted DNA was air dried and resuspended in 500 µl of sterile Millipore water and stored overnight at –20^{o}C. The quality and concentration of the purified DNA was determined both by UV absorbance and by comparison with a known quantity of lambda DNA (clind 1 ts857 Sam 7) following electrophoresis through a 1% w/v agarose gel. DNA samples were stored at –20°C. 30 ng of total DNA was used as the template for PCR amplification. TBP/cTBP analysis was performed according to Breviario *et al*. (2007). Hence, reaction conditions and primer combinations are like TBPfin2 (5′-GARAAYGCHGAYGARTGYATG-3′) / TBPrin2 (5′-CRAAVCCBACCATGAARAARTG-3′) for amplification of intron II (cTBP) were used, which were previously reported. For amplification of the whole *β*-tubulin gene family for each accession (*i.e.* to perform hTBP) primers used were TBPfex1 (5′-AACTGGGCBAARGGNCAYTAYAC-3′) and TBPrin2 (5′-CRAAVCCBACCATGAARAARTG-3′).

PCRs reactions (20 µL) were performed in 1X PCR buffer (10 mM Tris-HCl (pH 8.8), 50 mM KCl, and 0.1% v/v Triton X-100), 0.2 mM each dNTP, 2.5 mM $MgCl_2$, 50 ng of template DNA, 1 µM of each primer, and 1 U Taq polymerase (Sigma Aldrich Bangalore, India.) in a Eppendorf PCR thermal cylinder. The amplification procedure was programmed for 35 cycles of 94 °C for 30 s, 55 °C for 40 s, and 72 °C

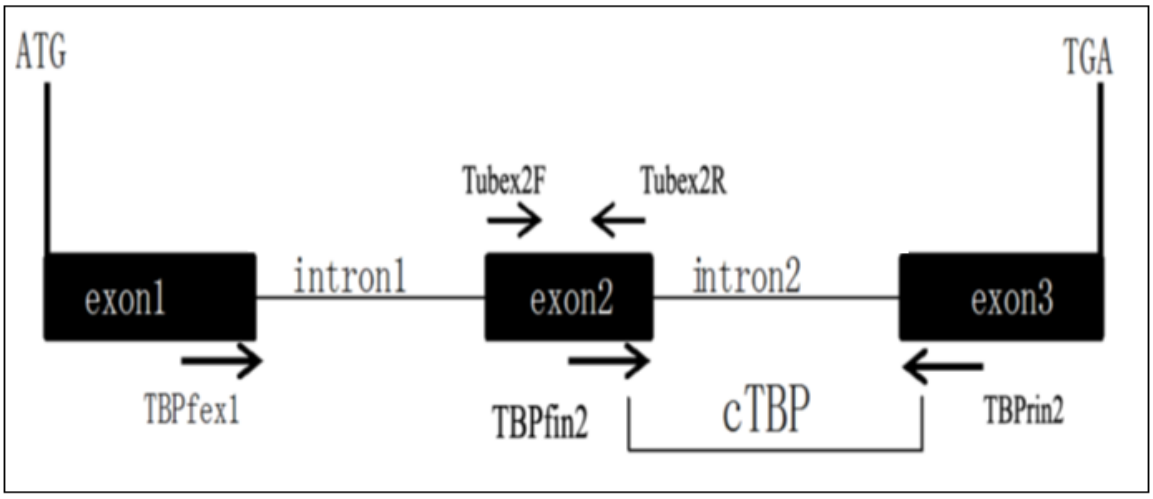

Fig. 3.1: Schematic representation of a typical plant β-tubulin genomic locus. Arrows indicate different primers in their respective position and orientation. ATG and TGA indicate the start and stop codon, respectively. The bracket encompasses the region amplified by the cTBP method.
(**Source:** *I. Galasso et al., 2015*)

for 90 s after the initial denaturation at 94^{o}C for 3 mins. Then the reactions were held at 15 °C after a final extension for 8 min at 72 °C. The amplified fragments were separated on a 2% agarose gel in 1 X TBE buffer (0.089 M Tris-Base, 0.089 M Boric acid and 0.002 M EDTA pH8) at 100 V for 1 h stained by ethidium bromide (1 µg mL-1) and photographed under UV light in a gel documentation system (UVP, UK). Moreover, 2 µl of each react Page I 26 on a sequencing sizes 6% w/v polyacrylamide native gel and run in 1X·TBE for four hours at a constant voltage 1,500 V. Amplicons were visualized by silver staining as described by Breviario *et al*. (2007). After staining, the banding patterns were scanned; data collected from reproducible and successful amplification were stored. Marker sizes were estimated by comparison to molecular mass standards included in each gel. All amplifications were repeated twice at the least, to ensure consistency of the TBP-PCR amplified products.

3.4.2 *Methylation studies*

The evidence of a large phenetic variety in *J. curcas* not sustained by a similar wide level of genetic diversity, led investigators to suggest that epigenetic modifications may play an important role in determining changes at morphological, physiological and developmental level. Up to now, this remains more a suggestion than a real demonstration. In fact few are the reports published on this matter and yet they do not convey a clearly straight forward message. This is the reason why we decided to investigate the status of DNA methylation in all our *J. curcas* accessions to verify the presence of some polymorphism. DNA methylation was studied at two levels: at the level of beta-tubulin genomic loci (Approach 1) and with a more randomized system that makes use of RAPD markers (Approach 2).

Approach 1

First, we ascertained the presence, in the TBP amplified products, of the recognition sites for those restriction enzymes whose activity is known to be influenced by DNA methylation such as *Hha*I, *Aat*II, *Stu*I and *Eco*RI. Among these enzymes we used the *Aat*II enzymes to perform experiments on the genomic DNA extracted from all the 24 *J. curcas* accessions. Once digested, the beta-tubulin loci were amplified with TBPI and pattern was compared with that of a straightforward TBP-I experiment (control) and the pattern obtained by cutting the TBP-I fragments with *Aat*II after their amplification, which is in the absence of methylation.

Approach 2

Because of the monomorphic data obtained when studying DNA methylation at beta-tubulin loci, an approach based on the use of the more versatile, wide spread, targeted to anonymous sequences, RAPD markers was enforced after restricting the genomic DNA with either the *Hpa*II or the *Msp*I enzyme. The RAPD marker used for this study is LBJ6= ACGCCAGAGG, which is a multi-locus primer with operon OPX-06.

3.5 *Statistical analysis*

Data obtained were subjected to descriptive statistical analyses, *viz.* Mean, Standard error of mean (SEM), Standard deviation (SD) & Coefficient of variation (CV). Analysis of correlation-coefficient between different parameters were carried out by performing the student's test. Data were elaborated with Microsoft Excel 2010 – Statistical Analysis and Graph-pad Prism 6 software.