5.1 Collection, plantation and survivability test of the accessions

Utilization of *Jatropha curcas* oil as a new source for diesel engine has tremendous scope in contributing to the growing needs of energy resources in the country. Owing to its importance, the species has gained popularity and is being scaled up in different parts of India on a large scale. In the present study, a total of 24 sources of *J. curcas* collected from different zones of NE India, were screened and evaluated. The objective of this study was to understand the magnitude of genetic variation in growth behavior and adaptability under Jorhat, Assam, environment to identify the best sources to be utilized for reforestation and future genetic improvement work. Soil of the experimental field of Jorhat, Assam, was classified as wet acid and rich in available phosphorous and nitrogen (Singh *et al.*, 2013). The sources used in this study had mean annual rainfall range from 129 to 3500 mm. The survivability rate was different for different accessions. The percentage of survival recorded was 100% in 06 out of 24 sources (*Jc-01, 03, 07, 14, 18 & 20*) and was 50% in 07 out of 24 sources (*Jc-06, 08, 09, 10, 19, 23,& 24*). The corresponding mean performance values are presented in Tables 4.1 to 4.4. After 36 months of field planting, significant differences were noticed among accession sources in height, stem girth, number of branches, leaf area, photosynthesis rate, stomatal conductance and survival per cent. The apparent variability in growth performance indicates that economic benefits may be obtained. The results of the present study will be valuable for the conservation of genetic variation, prospects of improvement and assessment of the potential of the locally adapted accession source.

5.2 Study of morpho-physiological parameters

A clear-cut distinction in the performance of the accession sources was observed at three years of observations for all the morphological growth parameters studied. The highest plant height found in the accession *Jc-10* which was of 157 cm and shortest was only of 50cm (*Jc-03*) in the first year. But till the end of third year scenario became changed and maximum height showed by accession *Jc-16* (203.67 cm) which was followed by *Jc-14* with 198.67cm of height (Fig. 4.1). At the third year end, Jc-14 also showed maximum measure for stem girth which was of 23cm, whereas the lowest was of 15.38cm found in *Jc-02*. Regarding the primary branch number there was no significant variation found though the number is maximum in third year comparison to the first year data (Table 4.1). In this case also *Jc-14* showed the better performance along with *Jc-02* which was of around 12 nos. (Fig. 4.2). *Jc-09* being the follower of *Jc-14* & *Jc-02* in case of primary branch no. showed largest canopy size with 187cm in third year. The CV% for these four parameters was found in the range from 11%-16%.

Regarding the flowering data, the total numbers of inflorescences, male flowers and female flowers per accession for all three years have been calculated. Also the ratio between male and female flowers per inflorescences from the average for every accessions was calculated. It was found that with the increase in age of the plant, along with other morphological parameters the flowering parameters were also significantly increased (Table 4.2). From first year to 3rd year there was a vast difference in the total no. of inflorescences. In the 1st year the highest no. of total inflorescences was 19 in *Jc-20* & *Jc-04*, which gone up to 144 in the 3rd year in accession no. *Jc-14* followed by *Jc-03* with 112 no. of total inflorescences (Fig. 4.3).

The CV% found for it in third year is 25.67. The highest number of male flowers per accession raised from 983 (*Jc-16*) in 1st year to 7482 (*Jc-19*) during 3rd year with CV% 35.68. Similarly the highest total number of female flowers per accession was calculated was 18 in three accessions *viz*. *Jc-10, Jc-12* & *Jc-17* where CV% was 23.09, while in the 3rd year this figure has been raised to 179 (*Jc-14*) which was followed by *Jc-05* & *Jc-17*with 177 (Fig. 4.4). Here too the CV% calculated was around 23. The ratio between the male flower and female flower was ranged from 22.94:1 to 100.87:1 with CV% 37.18 in the first year of observation. But in the third year this ratio was significantly reduced to the range of 7.34:1 to 51.70:1. The reduction in this ratio indicates the significant increase in female flower number in comparison to the number of male flower during the third year. The CV% found here was 40.14.

For physiological data the parameters *viz.* total leaf area; photosynthetic rate; stomatal conductance and transpiration rate were studied. Variation in leaf area among accessions reflects the extent or seasonal integral of light interception, which may be directly correlated with yield. Accession *Jc-14* (Kohima) showed highest measurements for all the parameters in the third year record (Table 4.3). There was a vast difference between the total leaf area size of first year and third year data. It was ranged from 3687.42 (*Jc-18*) to 5683.49 cm^2/plant (*Jc-14*). Thus the average total leaf area size recorded as 4641.25 (±112.97) cm^2/plant for the third year with CV% 14.38. Highest photosynthetic rate recorded in the first year was 11.48 µmol CO_2 $m^{-2}s^{-1}$ for *Jc-10*, which was followed by 11.27 and 11.23 of *Jc-14* and *Jc-04* respectively (Fig. 4.5 A). While in the second year highest rate was recorded in the accession *Jc-08* (13.67 µmol CO_2 $m^{-2}s^{-1}$) and then second highest is of 13.33 for *Jc-04*. But in the third year the highest photosynthetic rate was counted in the accession *Jc-14* (15.50) followed by *Jc-08* & *Jc-09* with the rate of 15.30 & 15.48 µmol CO_2 $m^{-2}s^{-1}$

respectively. Though the highest rate recorded was 15.50 µmol CO_2 $m^{-2}s^{-1}$ for Jc-14, but in the same time there were many accessions (*eg. Jc-02, Jc-16, Jc-21, Jc-22* & *Jc-24*) found with photosynthetic rate with around 1 µmol CO_2 $m^{-2}s^{-1}$ only. The average rate recorded in the third year was of 6.96 µmol CO_2 $m^{-2}s^{-1}$ (±1.09) with CV% 76.58. In case of stomatal conductance there was no significant differences between the measurements of all the three years recorded. From the first year to the third year the average rate increases from 0.37 cm/s to 0.56 cm/s, where the CV% in fact decreases from 56.75 in the first year to 30.36 in the third year (Fig. 3.5 B). In the third year *Jc-14* and *Jc-15* both showed highest stomatal conductance of 0.95 cm/s, which was followed by *Jc-10* (0.93) and *Jc-08* (0.90) whereas lowest recorded in *Jc-21* with 0.11 cm/s. On the other hand the maximum transpiration rate in the first year was recorded in the accessions *Jc-09* and *Jc-14* which was of 1.65 mmol/m^2/s with average of 1.33±0.03 and CV% of 11.28 (Fig. 3.5 C). In the second year this measurement was significantly increases to 2.73 mmol/m^2/s in the accession *Jc-14*, where the average was recorded as 1.80 mmol/m^2/s with CV% 23.33. But in the third year this rate was slightly increased upto 2.95 as the highest measurement in the same accession (*Jc-14*). The average transpiration rate measured in the third year was 2.12±0.06 mmol/m^2/s with CV% of 15.09.

5.3 Study of Seed and oil yield data

The seed is the only important product that is required from the *J. curcas* plant, therefore the seed yield measurement for each and every accessions in all the three years were undertaken. Significant increase in seed yield from first year to the third year had been found (Table 4.4). The range of seed yield in the first year had been measured from 20 gm to 40 gm of seeds per accession in almost all the accessions, except accession *Jc-19* gave 19.06 gm and *Jc-21* gave 18.72 gm. In 2nd year, with the

increase in inflorescence and female flower number, the seed yield also increased more significantly. Besides the six accessions (*viz. Jc- 04, Jc-08, Jc-18, Jc-20, Jc-23* and *Jc-24*), remaining all gave seed yield above 100 gms each. In this year *Jc-05, Jc-14* and *Jc-21* gave highest seed yield of about 180 gm each and *Jc-23* gave the lowest seed yield of only 55.58 gms only. The average seed yield for second year had recorded at 127.00 gm with CV% 27.38. In the last year of this experiment *i.e.* in the third year the seed yield per accession was much speculated. Only four accessions *viz. Jc-02, Jc-04, Jc-18* and *Jc-23* gave less than 250 gm of seeds per accession, where *Jc-04* and *Jc-23* gave only 160.78 gm and 113.19 gm of seeds each respectively. *Jc-23* was again found lowest seed yielded accession as in the second year too. Besides this, *Jc-10* and *Jc-14* showed highest seed yield in the third year with about 389 gm each. These two accessions were followed by accession nos. *Jc-17, Jc-15* and *Jc-11* which gave yield of 387.28 gm, 383.83 gm and 381.83 gm of seeds respectively each. The average seed yield for the third year had been recorded as 301.72 gm (±14.93) with CV% 24.24. Moreover, we also did an approximate calculation from our experiment for seed yield/ha, where we found that there will be an approximate yield of 0.402 t/ha if we took the third year's average yield as the basis and on the other hand if we took 389 gm/accession, then per ha. yield will be 0.518 t.

5.4 Estimation of Oil % and Biochemical analysis of oil constituents

(Quantification of FFA, Phorbolesters & Tocopherols)

The overall quantification for all the biochemical parameters *viz.* 100 seed weight, oil%, oil density, FFA%, tocopherol and phorbolester were done in the third year only and we got the following result for the parameters. The weight of 100 dry and healthyseeds was highest in *Jc-05* (88.86 gm), which was at par with *Jc-09, Jc-10, Jc-13* and *Jc-14* (Table 4.4). The consideration of seed weight in selection and

understanding the geographical variation has been advocated because of the least plasticity in this character (Harper *et al.,* 1970). Growth traits, *viz.* height, stem girth, 100 seed weight and field survival have significant inter-correlation with each other. It was found that heavier seeds have better seedling growth in the field (Aslan, 1975). The correlation suggests that following the completion of germination, seedlings allocate much of their energy for root and shoot development. Such relationship can be explored for early screening of genotypes for oil yield and growth performance. The inter-correlation found among seed weight and seedling characters in *J. curcas* is consistent with that of earlier studies (Palmberg, 1975; Iktueren, 1977; Isik, 1986 and Ginwal *et al.,* 2004). The patterns of variation exhibited for various characters were substantially different and varied with age. The presence of such difference among populations is probably due to different intensities of natural selection acting upon these traits in their natural habitat. Some of the variation found may be associated with the discrete populations from which accession was collected. Variation in accessions of *J. curcas* with respect to morpho-physiological characters and growth performance could be mainly due to the fact that this species grows over a wide range of rainfall, temperature and soil types. Populations might have also experienced marked differences in selective pressure. Crown exposure and genotype of mother tree, and soil and climate of the place of origin are important factors affecting the morpho-physiological characters and growth performance. The weight of 100 seeds varied from 71.15 gm (*Jc-15*) to 88.86 gm and hence the mean 100 seed weight was recorded as 78.46 gm (±0.89) with CV% 5.58 (Table 4.4).

Seeds were removed from ripe fruits, cleaned, and dried in open air. Plate 4.1 showing the detail process of seed harvesting and different parts of the seeds as well. Seed, kernel and shell were ground and immediately subjected to oil extraction in

Soxhlet apparatus using hexane as solvent (5 g seeds/ 50 mL hexane) with at least 5 refluxing of solvent. Three replicates were extracted for each seed lot. Density and percentage of seed oil was estimated on weight basis after total evaporation of hexane under nitrogen stream at room temperature (Table 4.4) and used for subsequent biochemical analysis. Generally said that the accession with highest seed weight have the highest oil content, but the correlation between seed weight and oil content is however not quite straight forward considering the wide variation within several accessions and the limited number of accessions tested. In this study though we had highest seed weight in *Jc-05*, but the highest oil content of 35.17% found in *Jc-14* and while that of *Jc-05* was only 25.64% (Fig. 4.6). Following the accession *Jc-14*, the second highest oil % was found in *Jc-04* and *Jc-10* which was of about 33%. The lowest oil% recorded among the studied accessions is 22.15 in *Jc-24*. All the accessions had oil density ranging from 0.91-0.93, but only *Jc-23* showed oil density of only 0.89.

Colour is also an indication of the difference in composition of oil in different accessions (Plate 4.3). It is quite evident that samples with dark-orange, brownish colour (*Jc-05* and *Jc-10*) can reflect a different degree of oxidative process, or the presence of different quality or quantity of pigments. Three oil samples from each accession have been used for biochemical composition analyses. The analyses carried out included: tocopherols, phorbol esters, free fatty acids and composition of triglygerides. The tocopherols or Vitamin E are lipo-soluble and are totally hexane extracted together with oil. Tocopherols are 4 isomers (α, β, γ and δ) (Fig. 4.7), the presence of one or more isomers can be a characteristic of the biological source (Kornsteiner *et al.,* 2006). Due to their molecular structure tocopherols are strong antioxidant and reactive oxygen scavengers (Arranz *et al*., 2008). Tocopherols are analyzed in HPLC by direct injection of oil (Malvolti *et al.*, 2010) (Fig. 4.8).

The tocopherol isomers were separated using HPLC JascoTritotar III pump and Jasco MD910, photodiodearray detector. 5-10 μL of pure oil were loaded into a Merck Chromolith RP-18e column (100 × 4.6 mm), and eluted with 1.5 mL/min MeOH 95% with 20 min run between each sample analysis. The data at 280 nm were acquired and elaborated by the Borwin software system and determined by comparison with tocopherol standards. It is reported that tocopherol is absent or available in low concentration in Jatropha oil (Abigor and Uadia, 2001). In technical industrial process to transform oil in biofuel frequently α-tocopherol isomer is added (Diwanin *et al*., 2009). From jatropha oil analysis reported in Table 8 the low level of tocopherol is confirmed also for our samples. The α-tocopherol and γ-tocopherol isomers are present in jatropha oil analysed though in some oil samples only traces of one or other isomers are detected. The range of concentration seems wide, ranging from 74 to 908 ng/g of oil in accession *Jc-06* and *Jc-24* respectively (Table 4.4). However, if it is compared to other oil sources tocopherols remain at negligible level. Indeed, for example~~s~~ in walnut oil the range of tocopherol is between 500×10^3 to 1000×10^3 ng/g of oil (Malvolti *et al*., 2010 and Amaral *et al*., 2003) and in *Camelina sativa*, a herbacious species under exploitation for biofuel production, the range is between 700×10^3 to 2000×10^3 ng/g of oil (Abramovic *et al*., 2007 and Pecchia *et al*., 2014). Analyses of oil from jatropha seed~~s~~ collected in experimental field at CSIR-NEIST confirmed that this species has negligible level of tocopherol content. Also the isomer~~s~~ composition is different, *Juglans regia* L. (walnut) and camelina have γ-tocopherol as the main component and α-tocopherol is normally absent in those species.

Jatropha oil is characterized by the presence of toxic substances *i.e.* phorbol esters, which make the oil non edible. Few jatropha accessions, mainly from Central America, have been characterized by absence or low level of the phorbols (Makkar *et al*., 1998 and Martìnez *et al*., 2006). The term 'phorbol esters' is used to describe a naturally occurring family of compounds widely distributed in plants of the families

Euphorbiaceae and Thymelaeceae. Haas *et al.* (2002) identified six phorbol esters from *J. curcas* seed oil, where all compounds possess the same diterpene moiety, 12-deoxy-16-hydroxyphorbol (Fig. 4.9A), the dicarboxylic acid moieties of 2-5 contain a bicyclohexane unit, and those of 6 and 7 a cyclobutane unit, which is described for the first time within this compound class (Fig. 4.9B). Fig.4.10. showing the comparison of HPLC phorbol esters separation obtained by Makkar *et al*., 2009 (left panel) and with Kinetex column used in this work (right panel). In all jatropha accession oil analyzed, the phorbols have been detected (Table 4.5) which was in the range between 1.09 (*Jc-21*) to 3.25 (*Jc-16*) mg/g of oil. The values measured in the 24 accessions investigated fit in the reported mean value of phorbols in jatropha oils which is 2.9 mg/g. Also the composition of phorbol esters has been evaluated (Table 4.5). The Peak I is always the minor component (form 0 to 8% of total phorbols). But in two accessions *Jc-08* and *Jc-13* we found exceptionally phorbol amount of 10.59% and 11.61% in Peak I. The Peak II is the main constituent of phorbols (40-50%) with two exceptions *Jc-05* and *Jc-10*, where Peak II is about 25%. The remaining part of phorbols is equally distributed between Peak III and Peak IV. In *Jc-05* and *Jc-10*, Peak III and Peak IV are the predominant phorbols detected. According to HPLC separation (Fig. 3.10) and UV spectra (Fig 4.9) the phorbols in oils, extracted from Jorhat samples, can be tentatively identified using Makkar (2009) and Haas (2002) data. Peak I as DHPB (12-deoxy-16-hydroxyphorbol) (Fig. 4.9) or jatropha factor C_1, Peak II as jatropha factor C_2, Peak III as jatropha factor C_6 and Peak IV as jatropha factor $C_4 – C_5$.

Wide variability is reported in free fatty acid content in jatropha oil from different accessions. Kumar *et al.* (2007) reported a possible range of 1 to 14% free fatty acids in extracted oil. However, it is not clear if the free fatty acid originates from within the growing seed or due to processing after harvest. In Table 4.6 the percentage of free fatty acids content in hexane extracted oil and fatty acid composition are

presented. The 24 accessions growing in Jorhat show in large part the free fatty acids in the range reported for jatropha. The free fatty acid in oil pose a technical problem in trans-esterification process to produce biodiesel. Free fatty acids consume the base catalyst (sodium or potassium hydroxide) and block or at least, slow down the reaction rate with consequent increase of time necessary to obtain the fatty acid esters (Waled and Jummat, 2009 Kumar *et al.,* 2007). Among the accessions, *Jc-14* which has the highest oil content possess low free fatty acid content (1.43%), slightly higher than the lowest reported value for Jatropha. The accession *Jc-10* showed maximum FFA of 38.73%, which was significantly higher of reported value for Jatropha. On the other hand the accessions *Jc-01, Jc-02, Jc-05, Jc-06* and *Jc-12* had FFA amount of more than 20% (Table 4.6). The *Jc-05* and *Jc-10* were already evidenced as the accessions with dark-orange, brownish colour and with higher Peak III and Peak IV in phorbol esters composition too.

Analysing the fatty acid composition of triglyceride oil fraction revealed the presence of four main components – linoleic, oleic, stearic and palmitic acids (Table 4.6). Fig. 4.11., showing the detail HPLC separation of different FFA in jatropha oil (bottom) along with the standard chromatogram for FFA analysis (Top panel). The oleic acid (50-60%) is the main component followed by linoleic acid (24-32%), palmitic acid (8-16%) and stearic acid (2-5%). But in this study exceptions occurred in all the cases. Besides the range of 50-60% in case of oleic acid most of the accessions showed its presence in the range of around 25-50% also. The highest oleic acid % found in the accession *Jc-24* (63.66%) which is followed by *Jc-06* with slight difference (66.61%). Jc-20 recorded the lowest amount of oleic acid % with 25.13. On the other hand *Jc-06* showed highest linoleic acid % of 27.61, whereas *Jc-20* possesses only 11.73% which is the lowest % of linoleic acid in this study. The range of

presence of stearic acid is 1.40% (Jc-06) to 20.54% (*Jc-19*) and thus the mean value for this acid is 10.92% (±0.93) with CV% of 41.67. Similarly the presence of palmitic acid among the 24 accessions studied has been recorded in the range of 4.19% (*Jc-17*) to 45.40% (*Jc-08*). The average value recorded for palmitic acid in this study is 21.44% (±2.74) with CV% of 62.55.

5.5 Genetic variability analysis of the accessions

J. curcas has been found highly promising species which can yield oilseed as a source of energy in the form of bio-diesel owing to its short gestation period, hardy nature, high quality oil content etc. Considering vast semi-wild distribution of *J. curcas* in different parts of India, it would be expected to have considerable genetic variation. The first possibility was mainly investigated through the use of the TBP molecular marker while the second through studies performed on DNA methylation. Different versions of the TBP method (Bardini *et al*., 2004; Breviario *et al*., 2007 and Galasso *et al*., 2011) were performed on the genomic DNA extracted from all the accessions (Plate 4.3). Plate 4.4 reports the results of TBP amplification experiments where it can be easily appreciated that no DNA polymorphism can be detected in any of the 24 accessions. The same result, that is absence of any relevant DNA polymorphism, was obtained when the hTBP method was applied (Plate 4.5). Since the hTBP method amplifies both introns present in the coding sequence of the beta-tubulin genes, thus it also confirmed the absence of any polymorphism in length in any of the 24 analyzed accessions of this study. This is consistent with the lack of polymorphism in the second intron found when applying the c-TBP version. Overall, these data confirm the low level of DNA polymorphism that is present in different accessions of *J. curcas*. These data are consistent with the finding of previously published works performed with the use of different molecular markers such as AFLP and SSRs (Sudheer *et al*., 2010; Shen *et al*., 2010 and Vischi *et al*., 2013).

Nevertheless the 24 accessions were characterized by an astonishing variability in several morphological, physiological and biochemical parameters. Therefore, phenotypic diversity doesn't seem to depend upon polymorphic genomic DNA traits as far as it can be inferred by the use of molecular markers. Such a strict conservation in beta-tubulin intron length is a rare feature when analyzing wild accessions of a plant species. In fact, under similar circumstances we have always found Intron Length Polymorphism (ILP) (Braglia *et al*., 2010) that may be present even in highly selected and cultivated species such as wheat (*T. aestivum*) (Casazza *et al*., 2011).

The evidence of a large phenotypic variation in *J. curcas* not sustained by a similar wide level of genetic diversity, 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 some reports are published on this matter and yet they do not convey a clearly straight forward message. This is the reason why the status of DNA methylation was investigated in some of our *J. curcas* accessions to verify the presence of polymorphism. DNA methylation was studied at two levels: at the level of beta-tubulin genomic loci and with a more randomized system that makes use of Random Amplified Polymorphic DNA (RAPD) markers.

The activity of restriction enzymes such as *Hha*I, *Aat*II, *Stu*I and *Eco*RI are known to be influenced by DNA methylation method as their recognition site contains a CpG dinucleotide which is a target for DNA methylation, present either within the target sequence (*Hha*I and *Aat*II) or slightly adjoined to it (*Stu*I and *Eco*RI). Among these enzymes, the *Aat*II enzymes was used to perform experiments on the genomic

DNA extracted from all the 24 *J. curcas* accessions. Once digested, the beta-tubulin loci were amplified with TBP and the restriction pattern was compared with that of a straight forward TBP experiment (control) and the pattern obtained by cutting the TBP fragments with *Aat*II after their amplification that is in the absence of methylation. As shown in Plate: 4.6, no evidences for polymorphism were clearly detected in any of the 24 *J. curcas* accessions.

Then in another approach, based on the use of the more versatile, wide spread, targeted to anonymous sequences, RAPD markers was enforced. RAPD primer LBJ6 has been chosen to assess the presence of a differential status of DNA methylation across the 24 *J. curcas* accessions. RAPD amplification was performed after restricting the genomic DNA with either the *Hpa*II or the *Msp*I enzyme. As shown in the Plate: 4.7 the pattern of amplification with *Hpa*II was not different from that obtained with *Msp*I. No polymorphic fragment was visualized suggesting that epigenetic variation is not involved in species differentiation in this study.

5.6 Study of correlation coefficient between the studied parameters

It has been observed from the calculation of coefficient of correlation (by Karl Pearlson's method) between the studied parameters in this study that there are very little correlations between the characteristics of the parameters (Table 4.7). The coefficient of correlation between the parameters was calculated by Pearson's method. Though this statistical analysis showed positive correlations among almost all of the parameters, however, the noticeable correlation has been found between the leaf area and the photosynthetic rate of the plants, which is of $r = 0.66$. Oil% showed positive correlation at around $r = 0.50$ with plant height and leaf area. Coefficient of correlation

for female flower no. with plant height and photosynthetic rate though found positive, but were very negligible with *r*=0.08 and 0.03 respectively. Number of female flower also gave one negative correlation with primary branch no., where *r*=-0.15. Correlation coefficient for all the remaining parameters has been recorded at the range of *r* = 0.1 to 0.44. On the other hand, the seed yield data gave significantly interesting result of coefficient of correlation with all the other parameters. It showed highest positive correlation with number of female flower, where *r*=12.59. This followed by the correlation with inflorescence no. (*r*=3.09) and then that of with leaf area (*r*=1.92). Correlation coefficient of seed yield data with oil% is 0.83 and with plant height is 0.12. Lastly the seed yield data gave negative correlation with primary branch no. (*r*= -0.13) and with photosynthetic rate (*r*= -0.02).