Comparative studies for selection of *Jatropha curcas* L. capable of high yield and oil quality in Assam environment

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***Jatropha curcas* L., a multipurpose shrub, originated in Central America, is present worldwide throughout tropical and subtropical regions. In India, *J. curcas* had recently been promoted as a potential source to reduce dependence on crude oil. However, our knowledge concerning genotype, phenotype and environmental interaction is limited. In the present study the magnitude of phenotypic growth, oil yield and quality of promising jatropha sources from India have been evaluated at Jorhat in Assam. The molecular basis of the phenotypic diversity present in different accessions predominantly recovered from different locations in India was also verified. After 36 months of field planting, significant differences were noticed among all accessions tested for agronomical and physiological parameters. Free fatty acids, triglyceride acid composition and the presence of phorbol esters and tocopherols have been studied as they influence oil quality. Integration of biochemical parameters with physiological and agronomical data shows that, under the Jorhat environment, accessions expressing the best performance in the field are also the best in oil yield and quality. Genetic diversity among all 31 jatropha accessions has also been studied using different molecular markers. Our results suggest that phenotypic diversity does not seem to rely on polymorphic genomic DNA traits as inferred by the use of the Tubulin Based Polymorphism and the Random Amplified Polymorphic DNA molecular markers.**

Keywords: Genetic improvement, *Jatropha curcas*, morphological traits, oil, phorbol esters, seed source.

FINDING an alternative fuel source for energy production is a major challenge of the 21st century. Indiscriminate land use over several years has resulted in extensive degradation of agricultural land in the country. Of the estimated 130 million hectares of wasteland in India, about 33 million hectares are available for reclamation through tree plantation according to the Planning Commission of India. *Jatropha curcas* L. plant is an appropriate alternative source, capable of growing on marginal soils, and plays a vital role in helping the rural economy and the demands of renewable energy in many developing countries. The Government of India launched a 'National Mission on Biodiesel' with a view to finding cheap and renewable liquid fuel based on vegetable oil[1]. India has a dearth of edible oil (6.31 million tonnes) and cannot afford to use edible oils for production of biodiesel. India is home to a billion people, which is about a sixth of the world human population. One factor that has decelerated India's rate of economic development is the need to import about 70% of its petroleum demand. The annual consumption of diesel oil in India is approximately 40 million tonnes forming about 40% of the total petroleum products consumption. The ongoing economic development (GDP growth) would further increase the demand for transportation fuel in short and medium term at a higher rate. India's developmental objectives are based on economic models that require a per capita consumption of fuel oil several folds higher than the current Indian consumption levels. Environmental problems that might crop up from such increased fuel consumption also need to be taken into account. In this backdrop, *J. curcas* has been identified as the potential biodiesel crop without compromising on food and fodder security and to improve livelihoods in the arid regions of the country[2]. The present study was undertaken to identify the best seed source based on morphological, physiological, biochemical and genetic variation in growth and adaptability of *J. curcas* in North East India.

Material and methods

Morphological and physiological evolution

The experiment was conducted at the CSIR-North East Institute of Science and Technology (CSIR-NEIST), Jorhat which lies between 27.35′N and 26.30′N and 93.45′E and 94.30′E. The area enjoys a moderate climate; with mean annual rainfall of 2244 mm. Seeds were collected from

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Table 1. Seed sources of *Jatropha curcas* and their geographical locations

Accession code	State	Locality	Latitude (°N)	Longitude (°E)	Altitude (m)	Av. R/F (mm)	Temp. (°C)	
							Minimum	Maximum
Jc-1	Mizoram	Kolasib	24.13	92.40	660.54	2860	7	32
Jc-2	Mizoram	Aizawl	23.36	93.00	1132	3000	11	30
Jc-3	Assam	Dibrugarh	27.28	94.55	108	2758	10	31
Jc-4	Assam	Dhubri	26.02	89.58	34	1600	8	30
Jc-5	Assam	Lakhimpur	27.65	96.25	87	2635	8	31.5
Jc-6	Assam	Sivsagar	27.00	94.36	97	2504	7	29
Jc-7	Assam	Tezpur	26.37	92.47	79	1600	7	36
Jc-8	Assam	Hajo	25.31	23.11	55	1800	10	38
Jc-9	Assam	Jorhat	26.30	94.30	116	2244	9	39
Jc-10	Manipur	Imphal	24.44	93.65	790	990	5	35
Jc-11	Nagaland	Kohima	25.40	94.08	1433	2300	4	31
Jc-12	Gujarat	Bhavnagar	21.45	72.10	24	454	21.4	33.6
Jc-13	Arunachal Pradesh	Naharlagun	27.00	93.42	200	2688	8	32
Jc-14	Arunachal Pradesh	Roing	28.05	95.89	300	2800	5	29
Jc-15	Assam	Sonitpur	26.60	92.78	86	1563	11	31
Jc-16	Arunachal Pradesh	Itanagar	27.06	93.41	146	3000	8	32
Jc-17	Tripura	Agartala	23.50	91.25	12.80	2240	10	35
Jc-18	Tripura	Udaipur	23.31	91.31	24.68	2100	12	35
Jc-19	Manipur	Loktak	24.30	93.55	768	1183	6	32
Jc-20	Assam	Nagaon	26.45	92.41	69	1745	10	35
Jc-21	Meghalaya	Garo hills	25.30	90.13	870	2600	7	30
Jc-22	Orissa	Banki	20.21	85.33	48	1400	8	33
Jc-23	Gujarat	Jafarabad	20.52	71.25	30	550	8	45
Jc-24	Nagaland	Mokokchang	26.44	94.65	1325	2330	9	25
Jc-25	Orissa	Bhubaneswar	20.15	85.52	35	1500	7	45
Jc-26	Orissa	Baleshwar	21.30	86.54	25	1568	10.8	42.8
Jc-27	Gujarat	Baroda	25.25	76.70	38	129	11	42
Jc-28	Uttarakhand	Ranchi	23.28	85.32	625.00	1530	10.3	37.2
Jc-29	Tamil Nadu	Coimbatore	11.00	76.97	426.00	557	20.7	39.4
Jc-30	West Bengal	Midnapur	22.15	87.39	159.00	1450	24.5	40.1
Jc-31	Assam	Bongaigaon	26.28	90.34	53	3500	12.9	31.7

31 sources representing different states of India (Table 1). From each of the sources, seeds were collected from 10 average trees, located about 100 m apart from each other to avoid relatedness or inbreeding[3]. Seeds from each plant were labelled to maintain their identity. Six-month-old seedlings were planted in the field (pit size $50 \times 50 \times 50$ cm) in a randomized complete block design with three replications, and the spacing between plants was 2.5×2.5 m. Observations were recorded 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 24 months after field planting and subsequently after 36 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. Leaf area was multiplied by the number of leaves occurring in the plant and expressed as total leaf area per plant. The jatropha trees began to produce seeds in the second growing season, but only a part of accessions bore seeds in spring-summer period. However, all accessions yielded seeds in autumn. So the seeds collected in this season from all the jatropha accessions were utilized for biochemical analyses. Considering that jatropha oil is toxic due to the presence of phorbol esters[3], the presence of phorbol esters in plant exudates was analysed. Plant exudates were collected, frozen-dried and subjected to phorbol extraction with methanol and HPLC analysis according to the methods decribed earlier[4,5]. Different versions of the Tubulin Based Polymorphism (TBP) method[6–8] were performed on the genomic DNA extracted from all of these accessions. Statistical analysis was done according to the standard procedure[4]. The entire percentage data was suitably transformed and analysed in a completely randomized design (CRD).

J. curcas is being explored for its oil-yield potential throughout the world. Sources used in this study had mean annual rainfall ranging from 129 to 3500 mm. The corresponding mean performance values are presented in Tables 2 and 3. After 24 months of field planting, significant differences ($P < 0.05$) were noticed among accession sources in height, stem girth, number of branches, leaf area, photosynthesis rate, stomatal conductance and

Table 2. Growth performance of various accessions after 2nd and 3rd year

Accession code	Plant height (cm)		Stem girth (cm)		Number of branches/plant		100 seed weight (g)		1000 seed weight (g) 3rd year Nov.
	2nd year	3rd year	2nd year	3rd year	2nd year	3rd year	2nd year June	2nd year Nov.	
Jc-1	80.0	159.81	12.46	20.00	2.52	4.62	79.5	51.83	538.29
Jc-2	37.5	120.5	5.83	12.98	0.00	2.83	0.00	38.90	539.43
Jc-3	37.83	71.17	9.47	17.59	0.83	2.17	0.00	46.65	534.48
Jc-4	31.5	101.72	9.00	16.56	0.31	1.83	68.1	36.5	540.75
Jc-5	124.5	175.5	11.74	19.63	1.7	4.43	76.1	36.45	541.61
Jc-6	31.9	102.5	10.6	18.11	0.00	3.5	0.00	38.15	551.11
Jc-7	26.5	105.17	7.82	15.67	1.00	1.75	71.4	48.75	472.38
Jc-8	96.25	198.0	13.61	21.07	3.51	4.56	137.7	56.50	542.50
Jc-9	23.5	118.31	8.4	16.50	0.52	2.91	85.67	55.95	620.00
Jc-10	25.5	82.17	4.5	12.95	0.00	2.17	0.00	52.26	551.11
Jc-11	94.75	278.5	26.0	33.89	1.75	7.25	140.5	57.55	783.53
Jc-12	51.5	149.5	8.0	15.16	0.00	5.5	50.78	29.15	708.57
Jc-13	42.5	108.0	6.0	13.95	0.00	2.5	49.43	55.28	507.69
Jc-14	45.0	112.83	7.5	24.16	0.00	3.5	84.57	53.15	750.00
Jc-15	59.5	155.83	7.17	14.43	0.00	3.83	64.14	38.86	688.89
Jc-16	40.5	124.5	6.5	13.98	0.00	2	43.04	30.54	414.28
Jc-17	33.5	152.83	11.0	19.10	0.00	3.5	61.28	46.75	533.33
Jc-18	127.5	174.5	13.72	20.96	0.00	9.5	0.00	46.30	540.51
Jc-19	98.5	177.5	16.5	24.31	0.00	3.5	66.43	45.15	538.58
Jc-20	37.0	86.5	6.5	13.78	0.00	0.5	36.5	33.65	539.76
Jc-21	28.5	92.0	4.0	12.64	0.00	1.5	0.00	41.95	540.33
Jc-22	108.5	178.5	24.5	31.99	0.00	3.5	0.00	46.15	538.23
Jc-23	84.5	152.5	4.5	11.75	0.00	6.5	38.5	43.25	548.22
Jc-24	36.5	119.5	5.5	12.84	0.00	1.5	51.83	34.75	540.79
Jc-25	24.5	87.17	4.5	12.03	0.00	2.17	0.00	31.15	537.57
Jc-26	26.5	73.0	6.0	13.86	0.00	3	60.72	42.00	533.86
Jc-27	26.5	73.0	6.0	14.10	0.00	3	60.72	47.85	520.69
Jc-28	56.5	105.0	11.0	18.61	0.00	2	0.00	30.15	539.45
Jc-29	44.8	132.5	9.5	17.18	0.00	1.5	72.68	27.25	522.28
Jc-30	58.04	254.5	12.5	19.86	1	4.5	52.5	46.95	539.26
Jc-31	113.0	178.37	14.61	22.47	5.68	5.94	124.9	52.65	521.05
CD (5%)	7.39	3.35	9.27	2.15	NS	2.05	NS	3.63	3.57

NS, Non-significant; CD, Critical difference; 2nd year = 24 months growth; 3rd year = 36 months growth.

survival percentage. The apparent variability in growth performance indicates that economic benefits may be obtained. The results of the present study will be valuable for conserving genetic variation, prospects of improvement and assessment of the potential of the locally adapted accession source. A clear distinction in the performance of accession sources was observed during two years, in which the Jc-11 source outranked the remaining in height (94.75 cm), stem girth (26.0 cm), 100 seed weight (140.5 g) and field survival (100%). This distinction remained at three years with regard to height (278.50 cm), stem girth (33.89 cm), 1000 seed weight (783.53 g) and field survival (100%). The number of branches was maximum after the 2nd year of growth in Jc-31 followed by Jc-81, Jc-1 and Jc-11, and they were statistically at par with each other. However, the number of branches was maximum after the 3rd year of growth in Jc-11 followed by Jc-31, Jc-1 and Jc-5. At the end of three years 100% survival was recorded in 12 out of 31 sources, the lowest survival was recorded in Jc-26 (Table 3). The average leaf area, photosynthesis rate and stomatal conductance ranged between 90.4 and 126.1 cm^2, 0.07 and 11.48 μmol CO_2 m^{-2} s^{-1}, and 0.01 and 0.91 cm s^{-1} respectively, at the end of 2nd year, whereas it ranged between 96.52 and 132.08 cm^2, 1.01 and 13.48 μmol CO_2 m^{-2} s^{-1}, and 0.06 and 0.94 cm s^{-1} respectively, at the end of 3rd year. The total leaf area per plant was maximum in Jc-11, at the end of the 2nd and 3rd year (Table 4). Variation in leaf area among accessions reflects the extent or seasonal integral of light interception, which may be directly correlated with the yield. Similarly, the photosynthetic rate and stomatal conductance was maximum in Jc-11 whereas Jc-2 showed minimum values at two and three years (Table 5). The 100 seed weight was highest in Jc-11, which was at par with Jc-8 and Jc-31 at the end of 2nd year growth (Table 2). Jc-11 also recorded the highest 1000 seed weight at the end of 3rd year growth (Table 2). Similarly the inflorescence number was found to be higher in Jc-11 at three years (Table 6). The consideration of seed weight in

selection and understanding the geographical variations have been advocated because of the least plasticity in this character[5]. 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[9]. 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[10–13]. 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.

Table 3. Survival rate of various accessions after 2nd and 3rd year

Accessions code	Field survival (%) 2nd year	Field survival (%) 3rd year
Jc-1	100.00	100.00
Jc-2	88.5	88.5
Jc-3	18.5	18.5
Jc-4	44.33	44.33
Jc-5	100.00	100.00
Jc-6	42.5	42.5
Jc-7	79.75	79.75
Jc-8	100.00	100.00
Jc-9	31.83	31.83
Jc-10	54.05	54.05
Jc-11	100.00	100.00
Jc-12	100.00	100.00
Jc-13	100.00	100.00
Jc-14	100.00	100.00
Jc-15	100.00	100.00
Jc-16	100.00	100.00
Jc-17	100.00	100.00
Jc-18	87.38	87.38
Jc-19	42.94	42.94
Jc-20	42.94	42.94
Jc-21	100.00	100.00
Jc-22	54.05	54.05
Jc-23	65.16	65.16
Jc-24	65.16	65.16
Jc-25	20.72	20.72
Jc-26	8.5	8.5
Jc-27	65.16	65.16
Jc-28	65.16	65.16
Jc-29	65.16	65.16
Jc-30	65.16	65.16
Jc-31	100.00	100.00
CD (5%)	12.05	12.05

CD, Critical difference; 2nd year = 24 months growth; 3rd year = 36 months growth.

Biochemical evaluation

Seeds were removed from ripe fruits, cleaned and dried in open air. 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 batch of seeds. Density and percentage of

Table 4. Total leaf area of various accessions after 2nd and 3rd year growth

Accession code	Area of single leaf (cm^2) 2nd year	Area of single leaf (cm^2) 3rd year	Total leaf area (cm^2 $plant^{-1}$) 2nd year	Total leaf area (cm^2 $plant^{-1}$) 3rd year
Jc-1	91.7	97.39	3912.9	4111.1
Jc-2	96.3	102.88	3812.7	4286.0
Jc-3	110	115.67	4347	4832.3
Jc-4	90.4	96.52	3490.7	4033.1
Jc-5	93.3	100.05	3885.3	4129.0
Jc-6	96.7	102.59	4024.7	4335.8
Jc-7	90.6	97.05	3866.7	3941.2
Jc-8	125.3	131.08	5197.3	5605.8
Jc-9	113.3	118.66	4558.5	4896.2
Jc-10	107.2	113.35	4346.5	4750.3
Jc-11	126.1	132.08	5230.1	5682.2
Jc-12	105.4	111.55	4060.7	4560.7
Jc-13	120.1	125.55	4740.9	5244.3
Jc-14	123	128.69	4978.5	5360.1
Jc-15	100.1	106.42	4240.5	4436.3
Jc-16	93.2	99.78	3691.8	4059.2
Jc-17	96.8	102.06	4028.8	4288.0
Jc-18	119.5	125.28	4717.5	5282.5
Jc-19	122.6	128.73	4962.5	5302.2
Jc-20	95.4	101.26	3777.6	4264.1
Jc-21	97.5	102.91	4057.5	4251.4
Jc-22	95.8	101.47	3987.8	4298.3
Jc-23	109.6	115.56	4331.4	4835.0
Jc-24	106.7	113.68	4001.9	4774.2
Jc-25	105.1	111.97	3836.1	4698.2
Jc-26	111.4	117.09	4288.7	4880.6
Jc-27	113.6	119.79	4142.1	4989.7
Jc-28	97.3	103.60	3851.7	4351.1
Jc-29	92.7	99.15	3483.9	4049.4
Jc-30	91.1	97.83	3795.1	4025.1
Jc-31	123.3	129.47	5115.3	5433.2
CD (5%)	5.97	5.73	11.67	13.15

CD, Critical difference; 2nd year = 24 months growth; 3rd year = 36 months growth.

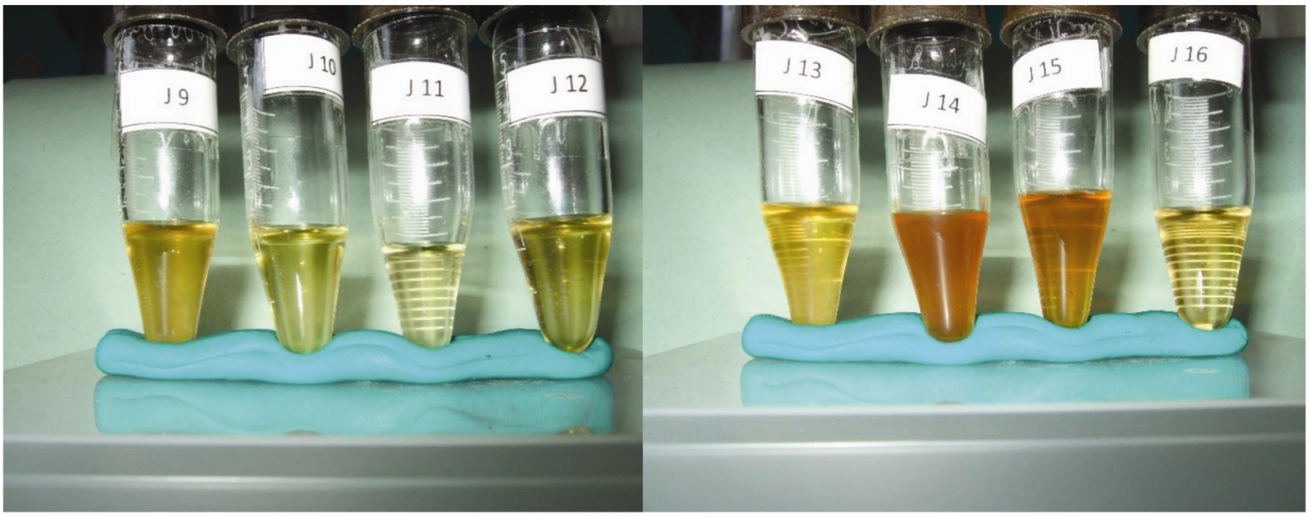

Figure 1. Visible evidence of *Jatropha curcas* oil extracted from the accessions analysed.

Table 5. Photosynthetic rate and stomatal conductance of various accessions after 2nd and 3rd year of growth

Accessions code	Photosynthetic rate (µmol CO_2 m^{-2} s^{-1}) 2nd year	Photosynthetic rate (µmol CO^2 m^{-2} s^{-1}) 3rd year	Stomatal conductance (cm s^{-1}) 2nd year	Stomatal conductance (cm s^{-1}) 3rd year
Jc-1	1.12	1.61	0.05	0.14
Jc-2	1.00	1.01	0.01	0.06
Jc-3	0.82	2.15	0.06	0.17
Jc-4	1.23	1.2	0.06	0.15
Jc-5	1.19	2.46	0.19	0.29
Jc-6	1.33	1.47	0.12	0.29
Jc-7	2.2	3.9	0.02	0.44
Jc-8	10.82	13.39	0.86	0.15
Jc-9	2.96	4.71	0.05	0.82
Jc-10	2.54	4.29	0.04	0.11
Jc-11	11.48	13.48	0.91	0.94
Jc-12	9.25	11.06	0.14	0.29
Jc-13	2.2	9.67	0.18	0.34
Jc-14	10.25	12.48	0.74	0.93
Jc-15	5.4	2.78	0.03	0.35
Jc-16	0.07	1.39	0.04	0.09
Jc-17	1.26	1.37	0.06	0.32
Jc-18	0.85	1.62	0.11	0.43
Jc-19	1.77	3.39	0.24	0.64
Jc-20	2.63	3.26	0.17	0.29
Jc-21	1.27	1.17	0.07	0.20
Jc-22	1.17	1.39	0.12	0.40
Jc-23	0.98	2.42	0.29	0.51
Jc-24	0.07	2.41	0.23	0.62
Jc-25	1.48	1.83	0.09	0.19
Jc-26	0.26	3.48	0.53	0.65
Jc-27	2.47	4.48	0.65	0.85
Jc-28	1.09	3.47	0.52	0.73
Jc-29	0.26	11.48	0.44	0.92
Jc-30	3.9	1.6	0.02	0.91
Jc-31	10.26	12.78	0.88	0.75
CD (5%)	1.29	1.10	0.95	0.63

CD, Critical difference; 2nd year = 24 months growth; 3rd year = 36 months growth.

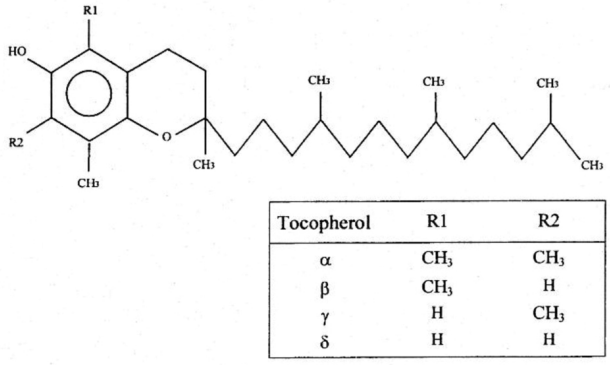

Figure 2. General formula and structure of tocopherols.

seed oil were estimated on weight basis after total evaporation of hexane under nitrogen stream at room temperature (Table 7) and the results were used for subsequent biochemical analysis. The seed weight varied from 27.25 to 57.55 g/100 seeds for Jc-29 and Jc-11 respectively. The local accessions Jc-20, Jc-5, Jc-4, Jc-6 and Jc-15, all originated from Assam had similar seed weight (ranging from 33 to 38 g/100 seeds). Another group (Jc-8, Jc-9, Jc-31, Jc-7 and Jc-11) has the heaviest seed (more than 50 g/100 seed). In general, accessions with heavy seed have higher oil content; Jc-11 has the highest oil content (35%). The correlation between seed weight and oil content is however not straightforward considering the wide variation within several accessions and the limited number of accessions tested. Most of the accessions had oil density ranging from 0.900 to 0.925 and only two accessions Jc-31 (lower) and Jc-26 (higher) did not conform to this range. Colour is also an indication of the difference in composition of oil in different accessions (Figure 1). It is evident that samples with dark-orange, brownish colour (Jc-14 and Jc-15) can reflect a different degree of oxidative

process or the presence of different quality or quantity of pigments.

Three oil samples from each accession were used for biochemical composition analysis. The analysis carried out included: tocopherols, phorbol esters, free fatty acids (FFAs) 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) (Figure 2), the presence of one or more isomers can be a characteristic of the biological source[14]. Due to their molecular structure tocopherols are strong antioxidant and reactive oxygen scavengers[15]. Tocopherols are analysed in HPLC by direct injection of oil[16] (Figure 3). Tocopherol isomers were separated using HPLC Jasco-Tritotar III pump and Jasco MD910, photodiode array detector. Pure oil 5–10 µl was 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[17]. In technical industrial process to transform oil in biofuel, frequently α-tocopherol isomer is added[18]. 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 oils 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. However, if it is compared to other oil sources tocopherols remain at negligible level. Indeed, for example, in walnut oil the range of tocopherol is between 500×10^3 and 1000×10^3 ng/g of oil[16,19] and in *Camelina sativa*, a herbacious species under exploitation for biofuel production, the range is between 700×10^3 and 2000×10^3 ng/g of oil[20,21]. Analysis of oil from jatropha seed collected in experimental field at CSIR-NEIST confirmed that this species has negligible level of tocopherol content. Also, the isomer composition is different,

Table 6. Flowering data of the selected 31 *Jatropha curcas* accessions after 3rd year of growth

Accession code	Inflorescence no.	Male flowers	Female flowers
Jc-1	10	516	32
Jc-2	35	983	53
Jc-3	16	576	38
Jc-4	19	581	23
Jc-5	18	787	48
Jc-6	38	943	91
Jc-7	57	807	110
Jc-8	27	530	41
Jc-9	10	500	35
Jc-10	4	487	18
Jc-11	68	701	113
Jc-12	20	753	37
Jc-13	5	633	27
Jc-14	61	675	93
Jc-15	11	568	33
Jc-16	30	586	43
Jc-17	13	441	38
Jc-18	18	557	13
Jc-19	58	787	48
Jc-20	65	496	113
Jc-21	16	498	35
Jc-22	13	788	43
Jc-23	4	950	24
Jc-24	7	350	53
Jc-25	16	614	23
Jc-26	5	549	25
Jc-27	7	480	27
Jc-28	8	875	13
Jc-29	30	413	91
Jc-30	13	576	131
Jc-31	9	557	73
CD (5%)	2.56	10.73	6.13

CD, Critical difference.

Table 7. Seed weight, percentage of oil content and oil density of the selected 31 *Jatropha curcas* accessions

Accessions code	State of origin	100 Seed weight (g), 2nd year (Nov.)	Oil (% w/w)	Oil density (g/ml)
Jc-01	Mizoram	51.83	27	0.919
Jc-02	Mizoram	38.9	29	0.926
Jc-03	Assam	46.65	24.5	0.921
Jc-04	Assam	36.5	28	0.921
Jc-05	Assam	36.45	28	0.917
Jc-06	Assam	38.15	26	0.922
Jc-07	Assam	48.75	25	0.912
Jc-08	Assam	56.5	32	0.908
Jc-09	Assam	55.95	27	0.913
Jc-10	Manipur	52.26	31	0.912
Jc-11	Nagaland	57.55	35	0.906
Jc-12	Gujarat	29.15	28	0.904
Jc-13	Arunachal Pradesh	55.28	30	0.921
Jc-14	Arunachal Pradesh	53.15	33	0.918
Jc-15	Assam	38.86	25	0.919
Jc-16	Arunachal Pradesh	30.54	29	0.915
Jc-17	Tripura	46.75	29.85	0.917
Jc-18	Tripura	46.3	22	0.916
Jc-19	Manipur	45.15	31	0.903
Jc-20	Assam	33.65	25	0.910
Jc-21	Meghalaya	41.95	25	0.912
Jc-22	Orissa	46.15	26	0.915
Jc-23	Gujarat	43.25	23	0.913
Jc-24	Nagaland	34.75	26	0.921
Jc-25	Orissa	31.15	25	0.913
Jc-26	Orissa	42	22	0.931
Jc-27	Gujarat	47.85	25.5	0.918
Jc-28	Uttarakhand	30.15	27	0.921
Jc-29	Tamil Nadu	27.25	29.96	0.913
Jc-30	West Bengal	46.95	20	0.912
Jc-31	Assam	52.65	33	0.888
	CD (5%)	2.91	1.83	0.58

CD, Critical difference; 2nd year = 24 months growth.

Table 8. Tocopherol (vitamin E) and phorbol esters contents in the oil of 31 selected *Jatropha curcas* accessions from different origins cultivated in the same environment of Jorhat

Accessions code	Tocopherol (ng/mg)	Phorbol (mg/g)	Percentage distribution on total phorbols			
			Peak I	Peak II	Peak III	Peak IV
Jc-01	78.1 ± 5.71	1.94 ± 0.13	7.12 ± 2.75	45.45 ± 1.06	21.83 ± 0.48	25.61 ± 1.27
Jc-02	112.5 ± 11.72	1.21 ± 0.04	8.33 ± 0.31	42.73 ± 0.44	25.72 ± 0.51	23.22 ± 0.16
Jc-03	73.5 ± 9.08	1.85 ± 0.02	8.98 ± 0.37	50.58 ± 0.29	21.38 ± 0.20	19.07 ± 0.05
Jc-04	76.2 ± 2.52	4.30 ± 0.09	8.11 ± 0.22	42.67 ± 0.40	20.43 ± 0.13	28.79 ± 0.35
Jc-05	253.1 ± 50.10	2.74 ± 0.02	10.59 ± 0.21	40.21 ± 0.21	21.61 ± 0.07	27.59 ± 0.06
Jc-06	146.6 ± 13.01	1.80 ± 0.03	5.88 ± 0.41	41.23 ± 0.14	23.84 ± 0.14	29.04 ± 0.14
Jc-07	282.6 ± 15.73	2.35 ± 0.02	8.68 ± 0.30	43.98 ± 0.27	19.99 ± 0.03	27.35 ± 0.10
Jc-08	183.1 ± 20.24	1.61 ± 0.04	2.24 ± 0.10	48.08 ± 0.43	25.44 ± 0.24	24.23 ± 0.18
Jc-09	377.4 ± 14.39	2.89 ± 0.03	7.36 ± 0.27	39.35 ± 0.10	23.15 ± 0.08	30.14 ± 0.26
Jc-10	241.2 ± 4.74	1.09 ± 0.01	6.39 ± 0.08	46.89 ± 0.43	25.06 ± 0.19	21.67 ± 0.33
Jc-11	246.4 ± 12.62	2.30 ± 0.42	7.47 ± 0.25	41.37 ± 0.21	23.11 ± 0.46	28.06 ± 0.01
Jc-12	259.5 ± 2.75	2.43 ± 0.02	6.59 ± 0.08	42.56 ± 0.25	23.14 ± 0.28	27.72 ± 0.03
Jc-13	294.3 ± 1.11	1.59 ± 0.02	5.35 ± 0.47	46.23 ± 0.32	23.67 ± 0.24	24.76 ± 0.31
Jc-14	403.5 ± 15.71	2.61 ± 0.12	1.03 ± 0.03	25.27 ± 0.35	39.16 ± 0.14	35.57 ± 0.23
Jc-15	194.1 ± 1.65	2.49 ± 0.34	3.48 ± 0.19	23.28 ± 0.45	37.01 ± 0.26	36.22 ± 0.49
Jc-16	353.1 ± 36.51	2.52 ± 0.01	6.66 ± 0.11	41.58 ± 0.09	25.14 ± 0.11	26.62 ± 0.06
Jc-17	218.4 ± 5.76	2.82 ± 0.03	6.52 ± 0.32	44.93 ± 0.18	20.71 ± 0.19	27.84 ± 0.23
Jc-18	907.6 ± 39.11	2.72 ± 0.06	5.08 ± 0.04	43.02 ± 0.92	21.68 ± 0.72	30.22 ± 0.18
Jc-19	326.1 ± 78.09	1.27 ± 0.06	5.55 ± 0.10	41.52 ± 0.51	26.37 ± 0.12	26.56 ± 0.48
Jc-20	369.4 ± 69.92	1.86 ± 0.09	4.85 ± 0.35	44.79 ± 0.40	24.44 ± 0.23	25.92 ± 0.42
Jc-21	428.4 ± 11.59	3.25 ± 0.12	4.74 ± 0.20	44.92 ± 0.44	21.66 ± 0.40	28.68 ± 0.24
Jc-22	238.0 ± 9.14	3.30 ± 0.04	7.01 ± 0.03	42.61 ± 0.50	21.64 ± 0.37	28.73 ± 0.18
Jc-23	135.4 ± 0.67	2.73 ± 0.09	4.74 ± 0.41	45.81 ± 0.41	20.62 ± 0.40	28.82 ± 0.15
Jc-24	328.5 ± 11.62	2.12 ± 0.06	6.47 ± 0.15	43.72 ± 0.17	21.73 ± 0.14	28.08 ± 0.09
Jc-25	217.5 ± 5.02	1.73 ± 0.04	11.71 ± 0.19	43.40 ± 0.54	20.24 ± 0.31	24.65 ± 0.27
Jc-26	249.6 ± 0.72	2.19 ± 0.14	4.33 ± 0.18	44.79 ± 0.21	22.27 ± 0.49	28.61 ± 0.39
Jc-27	458.2 ± 83.93	1.91 ± 0.04	4.56 ± 0.13	38.33 ± 0.47	26.93 ± 0.23	30.19 ± 0.15
Jc-28	458.1 ± 83.95	2.50 ± 0.06	7.85 ± 0.23	41.21 ± 0.42	23.65 ± 0.23	27.28 ± 0.13
Jc-29	267.4 ± 35.01	2.06 ± 0.02	7.16 ± 0.25	50.33 ± 0.28	22.48 ± 0.46	20.03 ± 0.40
Jc-30	303.4 ± 12.72	2.10 ± 0.05	7.98 ± 0.27	51.43 ± 0.54	21.79 ± 0.07	18.80 ± 0.46
Jc-31	573.0 ± 18.11	1.64 ± 0.21	5.60 ± 0.84	41.24 ± 3.12	29.28 ± 2.47	23.89 ± 1.43
CD (5%)	10.91	1.37	7.45	12.17	14.51	12.43

CD, Critical difference.

Juglans regia L. (walnut) and camelina have γ-tocopherol as the main component and α-tocopherol is normally absent in those species.

As mentioned earlier jatropha oil is characterized by the presence of phorbol esters, toxic substances that make the oil non-edible. Few jatropha accessions, mainly from Central America, have been characterized by the absence or low level of phorbols[22,23]. 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.*[24] identified six phorbol esters from *J. curcas* seed oil, where all compounds possess the same diterpene moiety, 12-deoxy-16-hydroxyphorbol (Figure 4 *a*), 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 (Figure 4 *b*). Phorbols have been detected by methods described by Makkar *et al.*[25] and Haas and Mittelbach[26]. Essentially 2 ml of oil, extracted by hexane, is further extracted up to four times with an equal volume of methanol. Then methanol evaporation under nitrogen stream the residue was dissolved in acetonitrile. HPLC was analysed using HPLC JascoTritotar VI pump and Jasco MD910, photodiode array detector. An extract of 20 µl was loaded into a Phenomenex Kinetex 2.6 µ C18 column (100 × 4.6 mm), and eluted with 0.8 ml/min MeOH 93% with 20 min run between each sample analysis. This column and eluent system gives chromatograms resolution and phorbol esters separation similar to the Makkar HPLC system[25] (Figure 5), but in short time and with less solvent. The spectra of phorbol esters of UV samples are comparable to those reported[24,25]. Phorbols are also quantified according to Makkar criteria using phorbol myristate acetate as reference[25]. The spectra data and absorbance at 280 nm were acquired and elaborated by the Borwin software system.

In all jatropha accession oils analysed the phorbols detected (Table 8) were in the range between 1.08 (Jc-10) and 4.29 (Jc-4) mg/g of oil. The values measured in the 31 accessions 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 8). Peak I is always the minor component (form 0–8% of total phorbols). Peak II is the main constituent of phorbols (40–50%) with two exceptions, Jc15 and Jc14, where peak II is about 25%. The remaining part of phorbols is equally distributed between peaks III and IV. In Jc15 and Jc14, peaks III and IV are the predominant phorbols detected. According to HPLC separation (Figure 5) and UV spectra (Figure 6), the phorbols in oils, extracted from Jorhat samples, can be tentatively identified using Makkar *et al.*[25] and Haas and Mittelbach[24] data. Peak I as DHPB (12-deoxy-16-hydroxyphorbol) (Figure 6) 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. The different ratios in phorbol esters composition in jatropha oils from different sources of seeds have also been reported by Waled and Jumat[27] comparing seeds from different countries.

Wide variability is reported in FFA content in jatropha oil from different accessions. Kumar Tiwari *et al.*[28] reported a possible range of 1–14% FFAs in extracted oil. However, it is not clear if the FFA originates from within the growing seed or due to processing after harvest[29]. FFA have been determined by direct injection of oil, after suitable dilution, on a HPLC system Jasco PU2089 pump equipped with Alltech 3300 ELSD detector using a Phenomenex Luna 2 µ C8 column (150 × 4.6 mm), in thermostat at 18°C, eluted with acetonitrile : isopropanol : water (50 : 30 : 20 v/v, 1 ml/min) for 20 min. Between each chromatographic run the column was washed from triglycerides with 10 min of elution of acetonitrile : isopropanol: water (50 : 45:5 v/v, 1 ml/min). Data from detector were recorded, integrated and elaborated by the Borwin software program. In Table 9, the percentage of FFAs content in hexane extracted oil and fatty acid composition is presented. The 31 accessions growing in Jorhat show in large part FFAs in the range reported for jatropha. The FFA in oil poses a technical problem in trans-esterification process to produce biodiesel. FFA 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[27,28]. Among the accessions, Jc-11 which has the heaviest seed has the highest oil content and low free fatty acid content (1.43%), slightly higher than the lowest reported value for Jatropha. Jc-15, Jc-6 and Jc-9 showed high FFA content (20%) and Jc-14 (38%) which was significantly higher than the reported value for Jatropha. Jc-14 and Jc-15 were already evidenced as accessions with

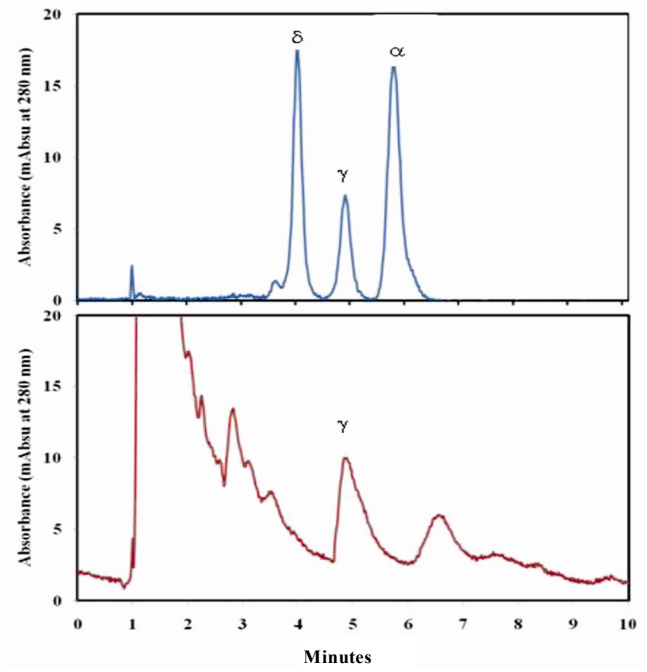

Figure 3. Tocopherol isomers separation analysis on HPLC. Top panel: standard δ, γ and α isomers; Bottom panel: typical tocopherol analysis in jatropha oil. Tocopherol was identified by retention time and online UV spectra.

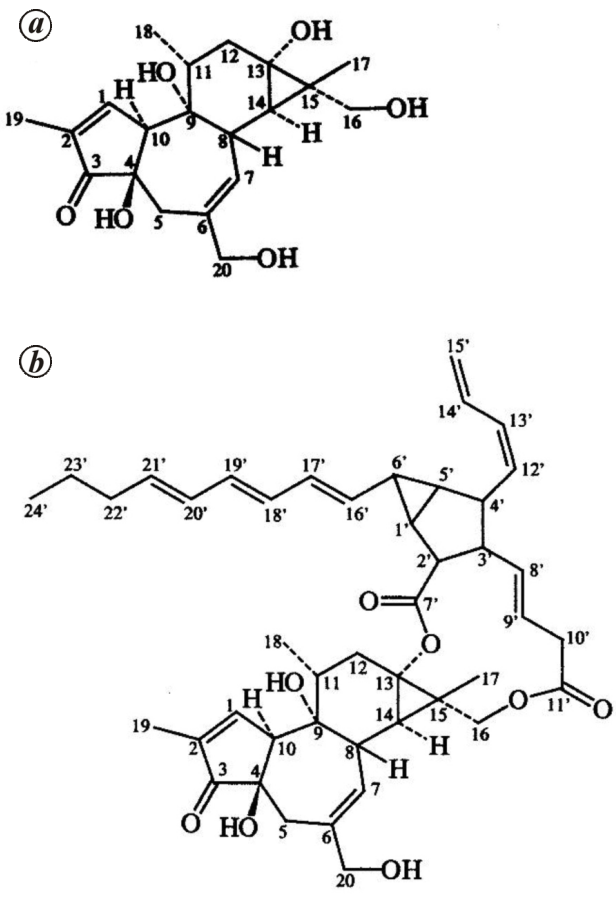

Figure 4. ***a***, 12-deoxy-16-hydroxyphorbol; ***b***, 12-deoxy-16-hydroxyphorbol-4%-[12%,14%-butadienyl]-6%-[16%,18%,20%-nonatrienyl]-bicyclo[3.1.0]hexane-(13-*O*)-2%-[carboxylate]-(16-*O*)-3%-[8%-butenoic-10%]ate (DHPB)[26].

Table 9. Free fatty acids content in the oil of 31 selected *Jatropha curcas* accessions from different origins cultivated in the same environment of Jorhat

Accessions code	FFA/oil (%)	Free fatty acid composition (%)			
		Linoleic acid	Oleic acid	Stearic acid	Palmitic acid
Jc-01	16.44 ± 0.14	16.59 ± 0.21	27.30 ± 0.34	13.68 ± 0.11	42.43 ± 0.02
Jc-02	16.83 ± 0.24	17.10 ± 0.30	34.05 ± 0.37	20.54 ± 0.28	28.31 ± 0.20
Jc-03	4.32 ± 0.02	27.61 ± 0.07	63.61 ± 0.11	1.40 ± 0.15	7.38 ± 0.11
Jc-04	2.37 ± 0.01	31.09 ± 0.13	63.50 ± 0.34	0.73 ± 0.41	4.69 ± 0.06
Jc-05	9.98 ± 0.10	13.96 ± 0.24	30.49 ± 0.53	10.15 ± 0.14	45.40 ± 0.14
Jc-06	21.82 ± 0.27	14.21 ± 0.16	35.03 ± 0.35	9.30 ± 0.01	41.45 ± 0.19
Jc-07	7.48 ± 0.02	28.15 ± 0.01	66.45 ± 0.35	0.70 ± 0.04	4.70 ± 0.04
Jc-08	8.36 ± 0.02	28.80 ± 0.03	58.84 ± 0.69	6.33 ± 0.99	6.03 ± 0.28
Jc-09	22.35 ± 1.07	18.85 ± 0.92	52.52 ± 0.75	10.78 ± 2.73	17.84 ± 1.03
Jc-10	3.92 ± 0.03	19.84 ± 0.22	50.78 ± 0.31	15.52 ± 0.26	13.85 ± 0.17
Jc-11	1.43 ± 0.04	14.16 ± 0.97	32.52 ± 0.74	11.76 ± 0.86	41.56 ± 1.09
Jc-12	7.17 ± 0.64	15.39 ± 6.52	63.44 ± 6.80	8.55 ± 1.86	12.61 ± 1.59
Jc-13	11.14 ± 0.39	22.58 ± 0.88	51.40 ± 1.11	10.15 ± 1.77	15.87 ± 3.77
Jc-14	38.73 ± 0.56	16.19 ± 0.24	48.59 ± 0.34	17.38 ± 0.14	17.84 ± 0.53
Jc-15	20.66 ± 1.35	15.64 ± 1.74	52.39 ± 6.92	10.91 ± 2.06	21.06 ± 3.14
Jc-16	4.27 ± 0.11	23.46 ± 0.27	49.68 ± 1.81	16.57 ± 0.22	10.29 ± 2.31
Jc-17	1.96 ± 0.02	15.43 ± 0.74	36.95 ± 0.79	16.20 ± 0.45	31.42 ± 0.40
Jc-18	14.55 ± 0.01	24.85 ± 0.01	63.66 ± 1.66	4.51 ± 2.39	6.98 ± 0.70
Jc-19	7.60 ± 0.11	22.04 ± 0.36	52.41 ± 0.29	11.94 ± 0.18	13.61 ± 0.25
Jc-20	6.02 ± 0.01	23.85 ± 1.17	53.29 ± 0.36	13.44 ± 0.59	9.42 ± 0.93
Jc-21	9.87 ± 0.01	22.36 ± 0.08	58.37 ± 0.87	10.40 ± 1.21	8.87 ± 0.41
Jc-22	3.40 ± 0.01	20.95 ± 0.31	45.54 ± 0.55	12.02 ± 0.32	21.49 ± 0.08
Jc-23	5.90 ± 0.04	23.31 ± 0.03	55.48 ± 0.98	13.11 ± 0.11	8.10 ± 0.90
Jc-24	6.93 ± 0.05	24.61 ± 0.14	58.06 ± 1.20	8.14 ± 1.89	9.20 ± 0.54
Jc-25	3.16 ± 0.01	18.43 ± 0.38	52.03 ± 0.57	12.29 ± 0.32	17.25 ± 0.13
Jc-26	4.45 ± 0.01	25.65 ± 0.36	53.16 ± 0.43	10.31 ± 0.26	10.89 ± 0.20
Jc-27	13.42 ± 0.16	24.03 ± 0.26	65.16 ± 0.50	4.35 ± 1.31	6.46 ± 0.55
Jc-28	6.24 ± 0.05	22.03 ± 0.28	45.60 ± 0.30	14.46 ± 0.18	17.90 ± 0.16
Jc-29	0.38 ± 0.03	13.74 ± 1.66	18.13 ± 2.23	1.03 ± 0.02	68.14 ± 3.92
Jc-30	0.36 ± 0.06	18.70 ± 2.22	20.87 ± 0.30	3.37 ± 1.63	57.06 ± 1.75
Jc-31	14.93 ± 1.09	19.14 ± 0.45	27.09 ± 2.79	13.59 ± 0.97	40.18 ± 4.22
CD (5%)	12.75	7.55	11.57	17.41	15.83

CD, Critical difference; FFA, Free fatty acids.

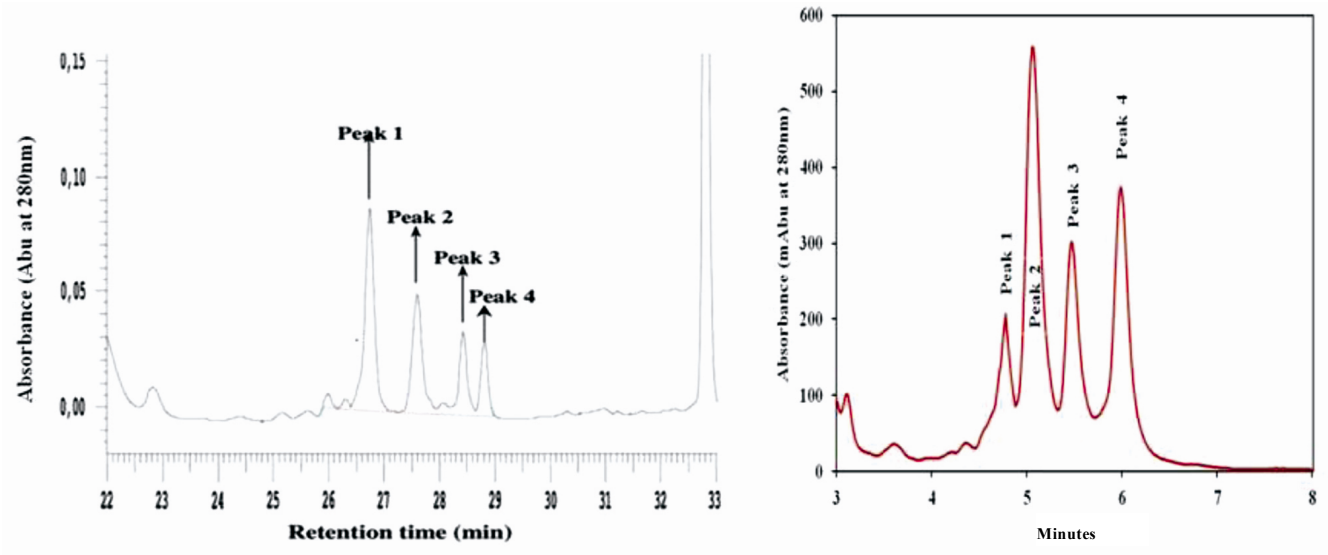

Figure 5. Comparison of HPLC phorbol esters separation obtained by Makkar *et al.*[25] (left panel) and with Kinetex column used in this work (right panel). The reduction of elution time was evident without change in compound resolution.

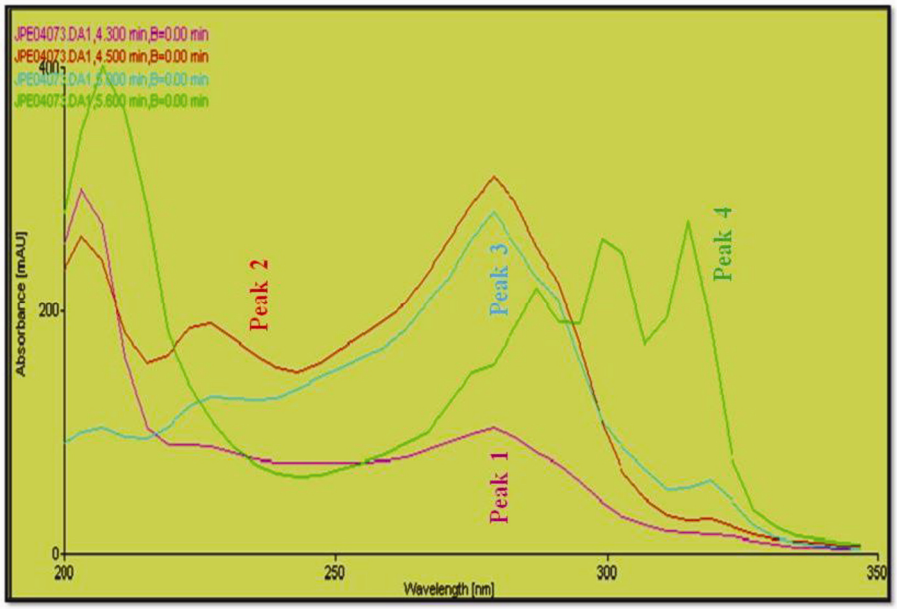

Figure 6. On line UV spectra of phorbol esters from jatropha oils separated by HPLC Kinetex column as shown in Figure 5 right panel.

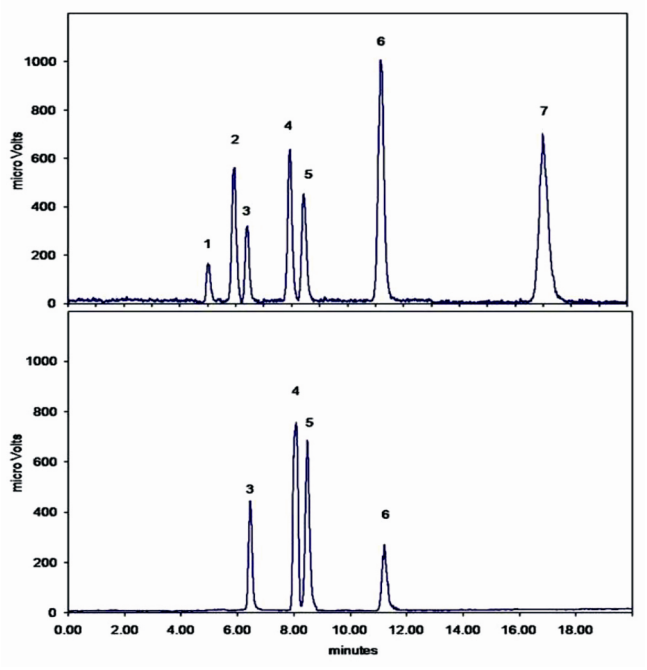

Figure 7. Fatty acid analysis on HPLC ELS detector, standard chromatogram (top), typical fatty acid chromatogram for jatropha oil (bottom). Peak 1, linolenic acid (C18:3); peak 2, palmitoleic acid (C16:1); peak 3, linoleic acid (C18:2); peak 4, eicosatrienooic acid (C20:3); peak 5, palmitic acid (C16:0); peak 6, oleic acid (C18:0); peak 7, stearic acid (C18:0); peak 8, erucic acid (C22:1).

dark-orange, brownish colour and with higher peaks III and IV in phorbol esters composition. Also, Jc-6 and Jc-9 have relative high peak III and peak IV in phorbols.

Analysis of the fatty acid composition of triglyceride oil fraction revealed the presence of four main components – stearic, oleic, linoleic and palmitic acids (Table 9, Figure 7). The seed oils, after removal of phorbols by methanol extraction, were hydrolysed to obtain fatty acids. Oil (20 µl) was hydrolysed in 1 ml of 1% NaOH in MeOH, at 80°C for 60 min. The solution was then dried under vacuum, the residue dissolved in 2 ml of H_2O plus 0.3 ml of 1 N H_2SO_4, and then vigorously shaken. Qualitative and quantitative analysis of fatty acids was done by HPLC system. Oleic acid (50–60%) is the main component, followed by linoleic acid (24–32%), palmitic acid (8–16%) and stearic acid (2–5%). From Table 9, it is evident that Jc-29 has mainly palmitic acid and a saturated fatty acid, as FFAs in oil. Similarly, in Jc-30 and Jc-11, palmitic acid is the main component of FFAs. All these three accessions (Jc-29, Jc-30 and Jc-11) have in common the lower quantity of total FFAs. Furthermore, when accessions with higher FFAs content (Jc-14, Jc-9 and Jc-15) are observed, they are seen to be the ones with

Table 10. Sequence information of RAPD oligonucleotide primers used for amplification

LB Jatropha RAPDs	Operon	Sequence	Primer test
LBJ1	OPD-14	CTTCCCCAAG	Multi locus
LBJ2	OPQ-20	TCGCCCAGTC	Multi locus
LBJ3	OPS-08	TTCAGGGTGG	Multi locus
LBJ4	OPW-18	TTCAGGGCAC	Multi locus
LBJ5	OPB-04	GGACTGGAGT	Multi locus
LBJ6	OPX-06	ACGCCAGAGG	Multi locus
LBJ7	OPF-16	GGAGTACTGG	Single locus
LBJ8	OPI-19	AATGCGGGAG	Multi locus
LBJ9	OPR-03	ACACAGAGGG	Multi locus
LBJ10	OPU-13	GGCTGGTTCC	Multi locus

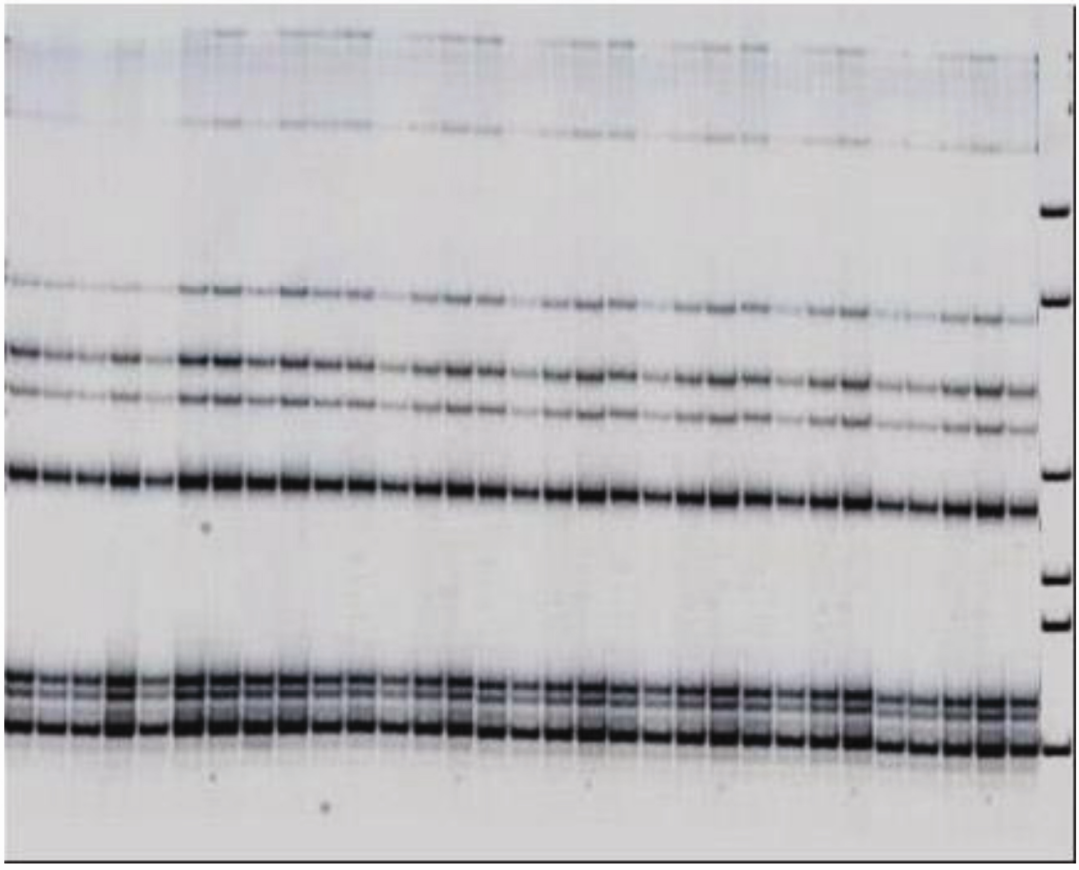

Figure 8. Polyacrylamide gel electrophoresis showing the TBP amplification profile. Samples are loaded in the same order as reported in Table 1. Molecular markers are on the extreme right.

the highest content of unsaturated fatty acids: linoleic and oleic.

Genetic evaluation

Phenotic diversity may result from differences occurring at the genetic or epigenetic level. The first possibility was mainly studied through the use of tubulin-based polymorphism (TBP) molecular marker and the second through studies performed on DNA methylation. Different versions of the TBP method[6–8] were performed on genomic DNA extracted from all the accessions. Figure 8 reports results of one of these experiments where it can be easily appreciated that no DNA polymorphism can be detected in any of the 31 accessions. The same result, i.e. absence of any relevant DNA polymorphism, was obtained when the hTBP method was applied (Figure 9). Since the hTBP method amplifies both introns present in the coding sequence of the beta-tubulin genes, this indicates polymorphic in length in any of the 31 analysed accessions listed in Table 1. This is consistent with the lack of polymorphism in the second intron found when applying the cTBP version (data not shown). Overall, these data confirm the low level of DNA polymorphism that is present in different accessions of *J. curcas*. These data agree with the findings of previously published study performed with the use of different molecular markers such as AFLP and SSRs[30–32]. Nevertheless the 31 accessions were characterized by an astonishing variability in several morphological, physiological and biochemical parameters. Therefore, phenotypic diversity does not seem to depend on polymorphic genomic DNA traits as can be inferred by the use of molecular markers. Such a strict conservation in beta-tubulin intron length is a rare feature when analysing wild accessions of a plant species. In fact, under similar circumstances we have always found Intron length polymorphism (ILP)[33] that may be present even in highly selected and cultivated species such as wheat (*T. aestivum*)[34].

The evidence of large phenotypic variation in *J. curcas* not sustained by a similar wide level of genetic diversity, suggests that epigenetic modifications may play an important role in determining changes at morphological, physiological and developmental level. Up to now, this merely remains a suggestion than a real demonstration. In fact some published reports do not convey a clearly straightforward message. This is the reason why the

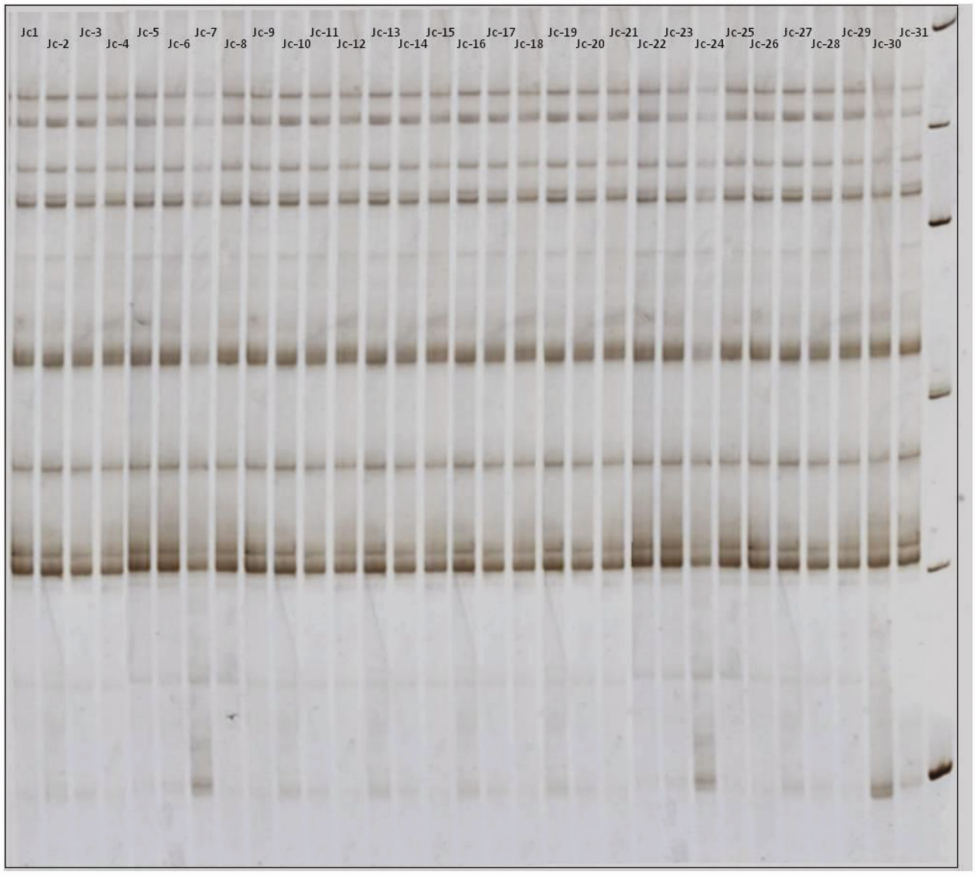

Figure 9. hTBP banding pattern for the 31 *J. curcas* accessions. Introns I and II were amplified together with the second exon of the coding region thus yielding amplicons ranging from 700 to 3000 base pairs in size. No polymorphism in length was detected. Molecular markers are on the right.

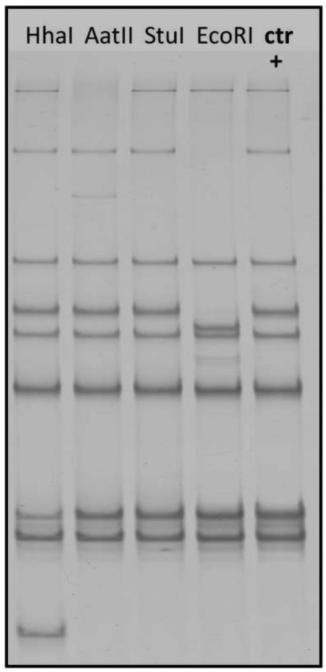

Figure 10. TBP amplification products digested with different restriction enzymes. The undigested products are on the right (TBP control). The *Stu*I cutter did not find any recognition site.

status of DNA methylation was studied 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 presence of 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 was ascertained in the TBP amplified products. Their recognition site contains a CpG dinucleotide that 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). The occurrence of methylation impairs *Eco*RI and *Stu*I cutting while suppressing DNA restriction from the other two selected enzymes. Figure 10 shows that TBP fragments amplified from Jc-11 contain the recognition site for all but one (*Stu*I) of these methylation sensitive cutters and so they are good candidates for epigenetic studies.

*Aat*II enzymes were used to perform experiments on genomic DNA extracted from all the 31 *J. curcas* accessions. Once digested, the beta-tubulin loci were amplified with TBP, the restriction pattern compared with that of a

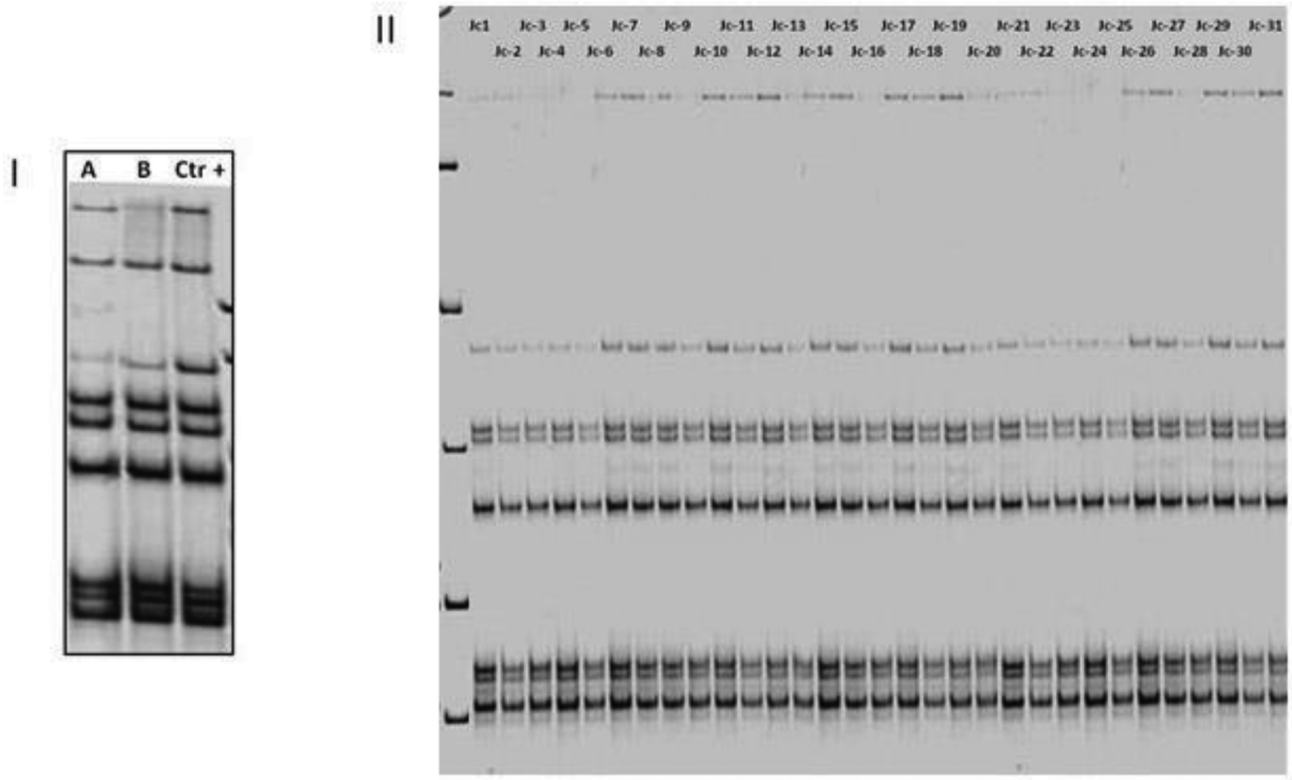

Figure 11. **I**, *Aat*II digestion after (A) and before (B) TBP amplification; Ctr+, TBP amplification product (control). **II**, TBP amplification after genomic DNA digestion with *Aat*II. No polymorphism was observed among the 31 *J. curcas* accessions. Molecular markers are on the extreme left.

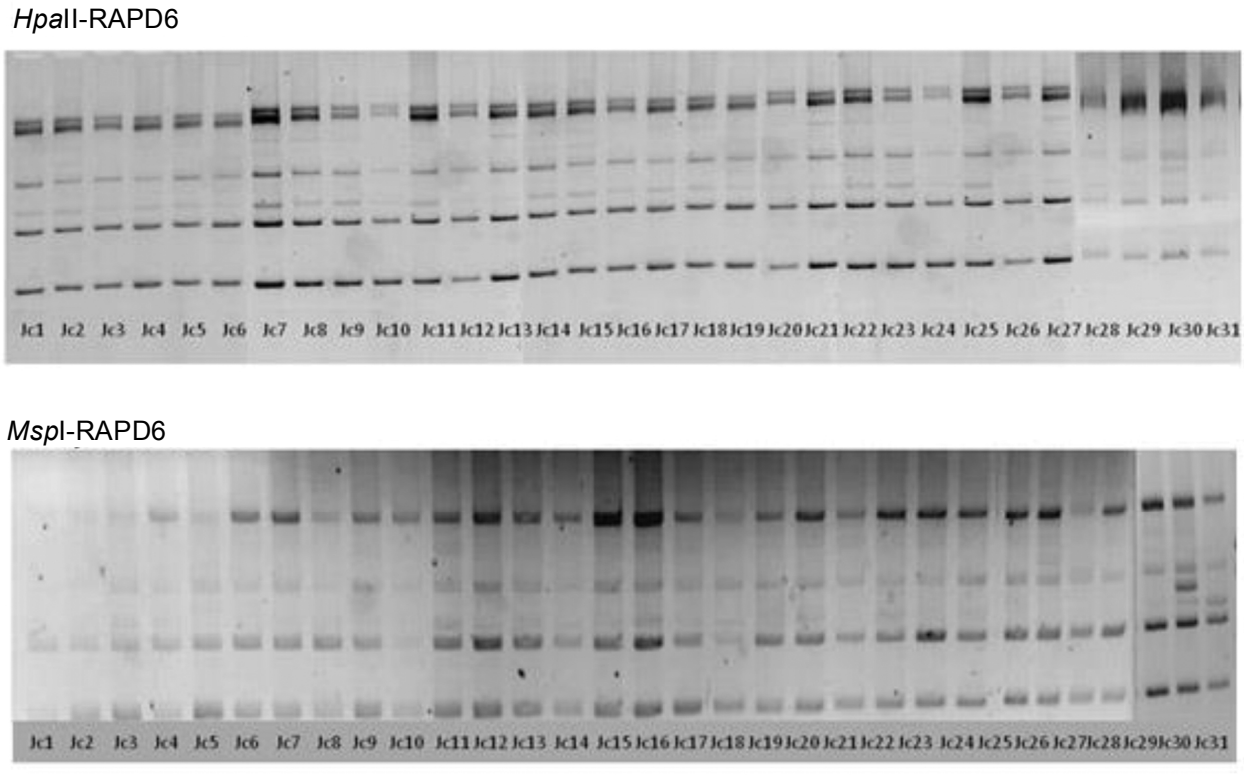

Figure 12. Pattern of RAPD amplification with *Hpa*ll (upper) and *Msp*l (lower).

straightforward 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 Figure 11, no clear evidence for polymorphism was detected in any of the 31 *J. curcas* species. The same data were obtained applying the same protocol to another methylation-sensitive cutter that was *Hpa*I (data not shown).

A different approach based on the use of two isoschizomers (*Hpa*II and *Msp*I), differentially sensitive to methylation in their recognition sequence (CCGG), was then tried. While in the absence of cytosine methylation, both enzymes can cut the CCGG target sequence, *Hpa*II does not cut if the internal cytosine is methylated and *Msp*I digestion is inhibited if the external cytosine residue carries a methyl group. Again, genomic DNA extracted from all the 31 accessions was restricted with both enzymes and the resulting fragments amplified with TBP searching for possible polymorphisms that should arise by a differential methylation status. No polymorphic fragment was visualized suggesting that epigenetic variation is not involved in species differentiation (data not shown).

Because of the monomorphic data obtained while studying DNA methylation at beta-tubulin loci, an approach based on the use of the more versatile, widespread, targeted to anonymous sequences, RAPD markers was employed. Ten different decameric primers were used (Table 10) to first assess their capacity to detect polymorphism within the 31 *J. curcas* accessions. RAPD primer LBJ6 was chosen to preliminary assess the presence of a differential status of DNA methylation across the 31 accessions. RAPD amplification was performed after restricting genomic DNA with either the *Hpa*II or the *Msp*I enzyme. As shown in Figure 12, the pattern of amplification with *Hpa*II was not different from that obtained with *Msp*I, with the exception of accession Jc-30, substantially indicating the absence of DNA methylation.

Conclusion

The agronomical and physiological study shows that considerable genetic variability exists in this species with respect to growth performance, which offers scope for selection and breeding. It is clear that the Kohima (Nagaland) source (Jc-11) is good in growth, particularly in the prevailing conditions at Jorhat. High seed weight observed in this source has been attributed to higher photosynthetic rate, stomatal conductance and leaf area. Further, clay and sandy texture of the soil having level topography might have provided better aeration, facilitating good exchange of gases aiding in increased photosynthetic activity. This source (Jc-11) can be safely used for large-scale reforestation programme in the region for high seed yield and vegetative growth. Germplasm used in afforestation programmes in India and other countries generally utilizes only locally available material. Thus, opportunities for using materials with higher yield potential or with more desirable characteristics might have been missed. This work will facilitate selection of promising accessions for multi-location evaluation and will also hasten the process of utilization of germplasm. It further gives a direction for genetic improvement of this species. The composition of triglyceride oils does not seem to be the main factor that can influence the oil value of the analysed accessions, whereas FFAs or phorbols seem more important factors for selection of best jatropha accessions. Furthermore, integration of biochemical data with physiological and agronomical data shows that the best accessions obtained by one criteria are the same also with the others. Accession Jc-11 seems best suited for further studies, evaluation and propagation in Jorhat habitat. However, it should be inferred that DNA methylation is not the likely mechanism responsible for large phenotypic variability observed in *J. curcas* accessions. Whatever the answer, molecular basis for such a diversity should also be identified at the level of differential expression of those genes that are involved in growth and development. Identifying such genes may represent the key to finally unlock the mystery of *J. curcas* phenotypic variation with respect to a very conserved genetic and epigenetic background status.

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