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Original Research Article

A Study on Effect of Genetic Variations of *Jatropha Curcas* on the Quality of Extracted Oil

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Abstract

Jatropha oil is considered one of the most important vegetable oil can use as biodiesel. This study was studied the genetic variations of Egyptian *Jatropha curcas*. Analysis of the five SSR loci with the ten of genotypes detected a total of 10 bands or alleles with 60 % polymorphism. The results showed that genotypes having the allele with the JCDS10 SSR locus (116bp) were 42 times more likely to have a high (above 10%) oleic acid content compared with these without this allele. The same results were obtained with the SSR loci JMDB57 and JCPS20 (alleles 82bp and 108bp) and increased odds to 3.8 and 3.6, respectively). While, allele at the JEM100 (140bp) SSR locus was showed negatively correlated with oleic acid content and exhibits a decreased odds (0.035) of having high (above 10%) oleic acid content. On other hand, Oleic acid content might be independent from the alleles at JCKASII SSR locus (OR=1.0). Moreover, the G9 oil specification was matched with European's standard. The Values of Oil density, Viscosity and Iodine Value were 0.85 gm/cc, 34 cp and 96.5 mg. I_2. g^{-1}, respectively. Altogether, we can conculcated that the 5 SSR loci can use for study the genetic variations of Egyptian *Jatropha curcas* on a molecular basis. In addition, genotype 9 could be useful parental line for developing mapping population to map EST-SSRs (transcribed regions).

Key words: *Jatropha curcas*, SSRs markers, specific alleles, genetic diversity, biochemical properties.

INTRODUCTION

Over than 170 species are classified under *Jatropha* genus which is a widespread in the world. The species can be monoecious or dioecious, trees, shrubs rhizomatous subshrubs, or geophytes, and herbs, including some annual taxa [1,2,3] and can be used for basket making, as living hedgerow, in folk medicine, as ornamental and the oil of some species is used for candle and soap making as well as a biofuel [2]. Moreover, some species may be toxic to humans and animals and can't use in nutrition. *Jatropha curcas* L. is considered one of the most famous and economically important species in the Jatropha genus. Mexico and Central America are the original home of *J. curcas*. Then. It was distributed in Latin America, Africa, India and South East Asia and was later on introduced in many parts of tropics and subtropics and grows without any human care [4,5,6]. *J. curcas* is generally known to be a poisonous plant. So, it can't use for feeding the human or animals. It is a semi-evergreen shrub or small tree reaching a height of 6 mt (20 ft). *J. curcas* is strong tree and can grow well under any unsuitable agro-climatic circumstances for its low moisture demand, low fertility requirement and tolerance to high temperature So, it can cultivated in arid lands.

Almost every part in Jatropha are used in traditional folk medicine, Its seed are used in soap oil and biodiesel production. *J. curcas* is a diploid plant with 2n= 2x= 22 [7] and a genome size is approximately 370 Mb [8]. Jatropha genome sequences have been previously obtained by whole-genome sequencing [9] and have been updated with newly assembled nonredundant sequences of approximately 298 Mbp from 39,277 contigs including 25,433 predicted genes [10].

Before, the scientists were used the morphological study and chemical parameters for identified the differences in the same species in breeding experiments. But, traditionally used

morphological and chemical parameters have not been found to be discriminative enough, warranting more precise techniques. Recently, Molecular markers successfully developed during the last three decades have largely overcome the problems that are associated with phenotype-based classification. DNA markers have proved worthy in crop breeding, especially in studies on genetic diversity and gene mapping. The commonly used polymerase chain reaction (PCR)-based DNA marker systems are random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and more recently simple sequence repeats (SSRs) or microsatellites [11,12]. The major limitations of these methods are low reproducibility of RAPD, high cost of AFLP and the need to know the flanking sequences to develop species specific primers for SSR polymorphism.

SSR markers are efficient and cost-effective and detect a significantly higher degree of polymorphism [13,14]. An alternative method to SSR, called inter-SSR (ISSR)–PCR which is a technique that overcomes most of these limitations [15-18]. Now, It is rapidly being used by the research community in diverse fields of plant amelioration [19]. Moreover, the technique is useful in areas of genetic diversity, phylogenetic studies, gene tagging, genome mapping and evolutionary biology in a wide range of crop species.

In this study, we investigated the effect of genetic variations among some Egyptian Jatropha genotypes on the quality of extracted oil using SSR markers, through association of the amplified alleles from SSR loci with unsaturated fatty acids genes. In addition, measuring the concentration of unsaturated fatty acids in Jatropha oil samples as biofuel and evaluation of the efficiency of Egyptian Jatropha oil as an ideal biofuel by estimating physicochemical properties.

MATERIAL AND METHODS

Plant Materials

Ten genotypes were collected from individual plant selection program based on the vigorous growth parameters with some morphological and yield differences, seeds were collected from each tree (which represents a single genotype) in the parental population were sown in rows (3*3m) in the orchard of Agricultural Research Station in Ismailia Governorate, Egypt.

DNA extraction

The 10 genotypes of the Egyptian Jatropha under study are located under Ismailia Governorate conditions, which not selected as protected area. The young leaf samples of them referred to in this study by G1 to G10 were collected for purposes of DNA analyzes. It is noteworthy that these genotypes originated from seed propagation, characterized by good productive properties. Thus, the vegetative samples for DNA tasks were stored at –20°C until use. By using liquid nitrogen for helping to break the cells, the samples were physically ground to powder using a mortar and pestle. The total DNA was extracted from frozen young leaves of a single plant of each genotype, according to the CTAB method [20,21]. The quantity of genomic DNA (gDNA) for each sample was determined by using the Nanodrop (NanoVue Plus, GE Healthcare Life Sciences).

Selection of SSRs Primers and preparing microsatellite primers for PCR-reactions

The primers for microsatellite loci were prepared for PCR reaction including design, dilution and storage. The sequence of the forward and reverse primer and the selected reference., in addition to Ta for each primer were given in Table 1.

Table-1: A list of 5 microsatellite (SSRs) markers included forward and reverse sequence, selected references and range of annealing temperature

Loci code	SSR type	Sequence (5→3)	Reference	Ta
JEM_100T	EST	F: CGGCAGATGGAGGATGTAAC R: AATGTAACGGCATCGGTAT	[23]	52
JMD_B57	EST	F: TGGCAGAGCAACTGCAAATA R: TCTCACACACCCCAAATTCA	[23]	56
Jcds_10	Genomic	F: CATCAAATGCTAATGAAAGTACA R: CACACCTAGCAAACTACTTGCA	[24]	46
Jcps_20	Genomic	F: ACAGCAAGTGCACAACAATCTCA R: TACTGCAGATGGATGGCATGA	[24]	55
JCKAS_II	EST	F: ACAGTCACCTTGTTTTTGTTCC R: ATGCTAATTTACCCTGATAAGG	[25]	52

Ta refers to temperature of annealing

PCR amplification and Detection of SSR products

PCR amplification was carried out in 25 µl reaction mixture composed of 2µl DNA (30 ng/µl), 5 µl of 5x PCR Buffer, 2.5µl dNTPs (2 mM), 2µl of each primer (10 pmol/µl), and 0.2µl Taq DNA polymerase and then the final volume was adjusted using ddH_2O. PCR amplification was performed in a thermos cycler (Eppendorf Master Cycler Gradient Eppendorf,

Hamburg, Germany) programmed to fulfill 35 cycles after an initial denaturation cycle for 5 min at 94 °C. Each cycle consisted of a denaturation step at 94 °C for 45s, an annealing step at 46 °C- 56 °C for 50s, and an elongation step at 72 °C for 60s. The primer extension segment was extended to 7 min at 72 °C in the final cycle. Subsequently followed by 10 cycles of denaturation for 30 s at 94 °C, annealing for 45 s at 53 °C, extension for 45s at 72 °C followed by final extension for 12 min at 72 °C. Every reaction was repeated two times to guarantee the reproducibility of the results.

PCR mixer and cycling PCR products were separated on agarose gel (2%) and ethidium bromide was used for staining to ensure the PCR amplification and determine approximately the size of the amplified fragments. Then, products were separated on Polyacrylamide gels (7%) to confirm allele sizing of the SSR loci, and then stained with ethidium bromide solution and visualized using gel documentation model (Gel-Doc 2000 with Diversity Database software Ver. 2.1, Bio-Rad Laboratories, Hercules, California, USA) for gel analysis. It was not possible to differentiate between simplex, diallelic duplex and among different types of triallelic grouping of SSR loci. So, the fragment frequencies were analyzed as multiloci and each allele was scored as present or absent (1/0).

Molecular data analysis

The computations were performed with the programs, GENEPOP version 1.31 [25], SPSS version 16, Irfan view and Microsoft Excel. In addition, Odds ratio data were generated by using MEDCALC™ statistical software.

Extraction of crude oil from the Jatropha Seeds

To ensure a higher oil quality, mechanical pressing is preferred taking into account, the low temperature of the process and non-use of solvents. In the present study we used cold pressing method to extract the oil from the ten Jatropha genotypes under study. To achieve this, a simple mechanical cracking machine and screw-press was used for the oil extraction process. The harvested Jatropha fruit was dried for three to four days before cracking according to [26]. About 600 grams from seeds of each sample was obtained after cracking. Then, each sample was separately squeezed using high pressure machine.

Estimation of the unsaturated fatty acids concentration in Jatropha seeds oil.

The concentrations were estimated by Gas Liquid Chromatography Trace GC Ultra Thermo Scientific according to [27]. Modification of the oil to its ethyl esters was made using 2% H_2SO_4 as catalyst in the presence of dry ethyl alcohol in excess. The chromatographic analysis was made using Hewlett Packard Model 6890 Chromatograph.

Conversion of crude oil into biofuel

200 ml of Jatropha oil has been used to convert it into biofuel. Then, 200 ml methanol was added to Jatropha oil (200 ml). Under temperature above 60 degrees for 1 to 2 min, all contains were mixed well until the glycerol was deposited in the bottom of the tester. 3 g of sodium hydroxide and 10 ml of phosphoric acid were added to mixture and stirred for 10 min. Then, the mixture was left for 2-3 hours. Two layers were observed. The top layer is the biofuels, while the bottom layer is glycerol.

RESULTS AND DISCUSSION

Analysis of the five SSR loci with the ten of genotypes detected a total of 10 bands or alleles only across the ten of genotypes. Out of 10 bands, 6 bands were (scorable polymorphic markers) polymorphic with a rather moderate level of polymorphism (60%) among the genotypes.

The profiles of SSR loci were combined and compared to elucidate whether any genotypes were genetically identical or not. About this, six of the genotypes (60%) 1, 2, 3, 4, 6 and number 7 had 2 identical loci. While, genotypes numbered 8, 9, and 10 identical in 3 loci. On the other hand, none of the genotypes were different or similar at all loci with any of the rest genotypes. The genetic analysis of the 10 Jatropha genotypes based on 5 SSR markers detected 3 distinct specific alleles, two genotypes (G5 and G9) had alleles were unique. So these two genotypes did not give identical DNA-fingerprints and largest number recorded of the specific/unique alleles were in the genotype number 9. It is clear that these specific alleles are closely related to the fact that genotype 9 in the present study was the best from where seeds oil content of unsaturated fatty acids. Especially, monounsaturated fatty acids like palmitic, stearic and oleic acids. While, the perfect biodiesel composition should have more monounsaturated fatty acids and less polyunsaturated acids [28]. The developed Expressed Sequence Tag-SSR (EST-SSR) markers by cDNA libraries have less polymorphism .Whereas, the transcriptome are more conserved and less variable, in addition more linked to genes of agronomic importance [29].

Table-2: Frequencies of alleles with Oleic acid

	Genotypes		**Alleles**	
JEM 100	140/140	140/370	140	370
Low Oleic	6	0	1	0
High Oleic	3	1	0.875	0.125
Allelic odds ratio 140 OR = 0.035				
95% CI = 0.002 – 0.603 at P = 0.02				
	Genotypes		**Alleles**	
JMDB57	82/196	196/196	82	196
Low Oleic	1	5	0.08	0.92
High Oleic	2	2	0.25	0.75
Allelic odds ratio 82 OR = 3.8				
95% CI = 1.63 – 8.99 at P = 0.002				
	Genotypes		**Alleles**	
JCDS10	116/116	158/158	116	158
Low Oleic	5	1	0.83	0.17
High Oleic	4	0	1	0
Allelic odds ratio 116 OR= 42.1				
95% CI = 2.49 – 711.07 at P = 0.0095				
	Genotypes		**Alleles**	
JCPS20	108/108	108/270	108	270
Low Oleic	2	4	0.66	0.33
High Oleic	3	1	0.875	0.125
Allelic odds ratio108 OR = 3.6				
95% CI = 1.73 – 7.51 at P = 0.0006				
	Genotypes		**Alleles**	
JCKAS II	192/192	192/444	192	444
Low Oleic	3	3	0.75	0.25
High Oleic	2	2	0.75	0.25
Allelic odds ratio192 OR = 1.0				
95% CI = 0.52 – 1.89 at P = 1.00				

The amplified alleles from SSR loci and association with genes responsible for oleic acid content

According to Table 2, genotypes having the allele 116bp at the JCDS10 SSR locus were 42 times more likely to have higher (above 10%) oleic acid content, as compared to those without this allele. It is worth mentioning that this allele (Jcds10_116bp) was recorded as a specific allele with genotype 9. Likewise, alleles 82bp and 108bp at the SSR loci JMDB57 and JCPS20, respectively Fig-1, confer increased odds (3.8 and 3.6, respectively) of having high (above 10%) oleic acid content. On the other hand, allele 140 at the JEM100 SSR locus seems to be negatively correlated with oleic acid content and exhibits a decreased odds (0.035) of having high (above 10%) oleic acid content. Finally, oleic acid content might be independent of the alleles at JCKASII SSR locus (OR=1.0).

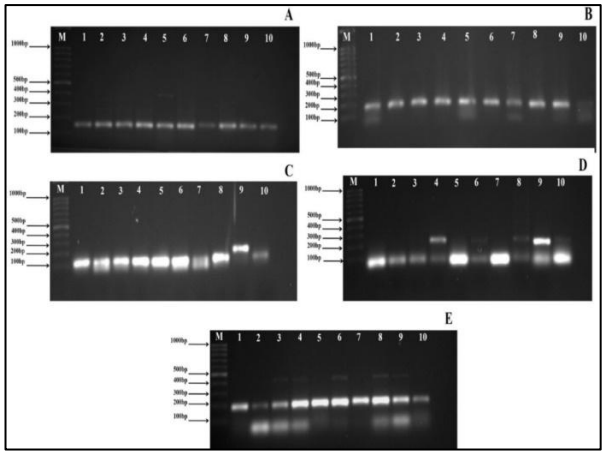

Fig-1: SSR profiles as detected with loci JEM_100T (A), JMDB_57 (B), Jcds_10 (C), Jcps_20 (D) and JCKAS_II (E). Whereas, M. refers to DNA ladder, lane 1 to 10 refers to Egyptian Jatropha genotypes in the present study

Characterization of crude *Jatropha curcas* oil.

In the present study, Jatropha seeds were pressed by screw-press resulting in a yield of 168 gm Jatropha oil and 432gm press cake. This means that Jatropha oil represented about 28% of crude oil by weight per kg of the dry Jatropha seeds. fatty acids compositions were determined in the ten Egyptian Jatropha genotypes for their importance Table 3. The properties of the triglyceride and the biodiesel fuel are determined by the amounts of each fatty acid that are present in the molecules. Chain length and number of double bonds determine. The physical characteristics of both fatty acids and triglycerides [30]. Transesterification does not alter the fatty acid composition of the feed stocks and this composition play an important role in some critical parameters of the biodiesel, as cetane number (cn) and cold flow properties [31]. Eight of fatty acids were detected (Capric acid, Undecylic acid, Lauric acid, Tridecylic acid, Mirisitic acid, Palmitic acid, Stearic acid and Oleic acid) in the seeds of ten Jatropha genotypes. The results showed that the genotypes were different in concentrations of fatty acids. For example, genotype No. 9 (G. 9) showed a highest concentrations of stearic acid and palmitic acid (46.97% and 8.04%), respectively. While, the same genotype showed low concentrations of Capric acid, Undecylic acid, and oleic acid (1.02%, 2.09% and 2.66%, respectively). Also, genotype No. 4 was showed high values with (Undecylic acid, Lauric acid and Tridecylic acid 31.87%, 12.26% and 40.84%), respectively. The same results with genotype 4, No Palmitic acid (16:0) was detected. While the comparison between the ten genotypes in the concentrations of different fatty acids showed that the highest concentration of Tridecylic (23:0) was 50.66% in genotype No. 7. On the other hand, the lowest concentration was 22.63% with genotype 4. At the same time, the lowest value of Lauric (12:0) was 8.63% in G9 but genotype number 8 showed the highest value (20.14%). Gubitz reported existence of variation in the concentration of fatty acids

(Miristic acid 0.0–0.1, Palmitic acid 0.0%– 1.3%, Stearic acid, 3.7%– 9.8%, and Oleic acid 34.3%–45.8%) [32]**.** from above it can be conclude that the concentrations of fatty acids in Jatropha seeds changed depending on different factors, but, here in this study, We can return the difference in the concentrations of fatty acids to the genetic diversity between the genotypes. In this context, Condro reported the major role of *FAD2* gene for the synthesis of unsaturated fatty acids, where the *FAD2* gene has a role in the formation of Alolinic, Linoleic and Palmitic acids in Jatropha plant [33].

Table-3: Main ± standard deviation (SD) of the estimated unsaturated fatty acids in seeds of ten Egyptian Jatropha genotypes

Genotypes / Fatty acids*	G1***	G2	G3	G4	G5	G6	G7	G8	G9	G10
Capric 6:0**	2.91%±0.12	2.79% ± 0.24	2.47% ±0.22	3.77% ±0.32	3.25% ±0.23	1.78% ±0.11	2.57% ±0.25	2.97% 0.±33	1.02% ±0.12	1.88% ±0.24
Undecylic 11:0	27.07% ±0.43	27.21% ±0.33	18.51% ±0.34	31.87% ±0.62	15.63% ±0.54	3.83% ±0.23	5.89% ±0.43	6.15% ±33	2.09% ±0.21	3.95% ±0.21
Lauric 12:0	9.77% ±0.23	9.28% ±0.44	8.22% ±0.23	12.26% ±0.12	17.37% ±0.33	17.73% ±0.32	19.07% ±0.43	20.14% ±0.33	8.63% ±11	12.99% ±0.21
Tridecylic 23:0	30.24% ±45	39.48% ±0.54	24.93% ±34	40.84% ±0.56	22.63% ±0.34	27.67% ±0.44	50.66% ±0.67	35.13% ±0.53	25.71% ±0.32	34.80% ±0.54
Myristic 14:0	7.05% ±0.22	8.33% ±12	2.19% ±0.11	3.13% ±0.21	2.35% ±0.13	4.15% ±0.32	8.35% ±0.14	6.61% ±0.23	4.88% ±0.33	4.50% ±0.32
Palmitic 16:0	1.30% ±0.10	3.04% ±0.23	4.37% ±0.23	0.0% ±0.0	3.34% ±0.11	4.62% ±0.22	0.70% ±0.04	2.35% ±0.15	8.04% ±0.22	5.19% ±0.22
Stearic 18:0	5.37% ±0.37	3.89% ±0.21	9.25% ±0.23	3.59% ±0.22	15.55% ±0.44	33.14% ±0.66	7.39% ±0.22	4.61% ±0.33	46.97% ±0.67	32.06% ±0.56
Oleic 18:1	16.26% ±0.43	5.944% ±0.43	11.48% ±0.33	4.52% ±0.12	18.37% ±0.43	7.05% ±0.33	5.33% ±0.22	16.19% ±0.34	2.66% ±0.21	4.59% ±0.22

*=Detection method According to: Fatty acids Gas Liquid Chromatography Trace GC Ultra Thermo Scientific.
**=Lipid Numbers.
***=Number of the genotype.

Conversion of crude oil into biofuel

In experiment to conversed the Jatropha crude oil into Biofuel. Crude *J. curcas* oil was used without having undergone any further refining for use as a biodiesel feedstock. Its properties were established to ascertain suitability for biodiesel production and to determine a suitable production process for the feedstock. Table 4 shows some physicochemical properties of Jatropha oil which can be biodiesel, the results showed that oil density, viscosity, and Iodine value were 0.85 gm/cc, 34cp and 96.5 mg. I2. g^{-1}, respectively.

Table-4: Physicochemical properties of Jatropha oil

Measurements	Value
Oil density (gm/cc), 30 C	0.85
Viscosity (cp)	34
Iodine Value (mg. I_2. g^{-1})	96.5
Physical state at room temperature	Liquid

While, Physical state at room temperature was liquid. Our results are matching with previous work cover the same points [36]**.** The iodine value is a measured of the unsaturation of fats and oils. Higher iodine value indicated that higher unsaturation of fats and oils [35,36]. The iodine value of Jatropha oil was determined at 96.5 mg. I2. g^{-1} Table 4, and the standard iodine value for biodiesel was 120 mg. I2. g^{-1} according to Europe's EN 14214 specification. The limitation of unsaturated fatty acids is necessary due to the fact that heating higher unsaturated fatty acids results in polymerization of glycerides. This can lead to the formation of deposits or to deterioration of the lubricating [37]. Fuels with this characteristic (e.g Sunflower oil, soybean oil and safflower oil) also likely to produce thick sludges in the sump of the engine, when fuel seeps down the sides of the cylinder into crankcase [38]. The iodine values of *J. curcas* place them in the semi-drying oil group. High iodine value of Jatropha are caused by high content of unsaturation fatty acid such as oleic acid and linoleic acid. The iodine values of Jatropha oil seed of suggest their use in production of alkyd resin, shoe polish, varnishes etc [39].

Viscosity defined as resistance liquid to flow. Viscosity increased with molecular weight but decreased with increasing unsaturated level and temperature [40]. At room temperature kinematic viscosity of the sample were detected at 34 cp Table 4. The viscosity of Jatropha oil seed must be reduced for biodiesel application since the kinematic viscosity of biodiesel were very low compared to vegetable oils. High viscosity of the Jatropha oil seed are not suitable if its use directly as engine fuel, often results in operational problems such as carbon deposits, oil ring sticking, and thickening and gelling of lubricating oil as a result of contamination by the vegetable oils.

Different methods such as preheating, blending, ultrasonically assisted methanol transesterification and supercritical methanol transesterification are being used to reduce the viscosity and make them suitable for engine applications [41, 42]. Also, the density of Jatropha oil was 0.85 which was matched with the other previous reference. In 2003, Pramanik was reported that the density in his study was 0.93, the study was compared the density of the Diesel which was 0.836 Table 4.

So, our results are close to density of the diesel. Ogunleye and Eletta used two solvents to extract the Oil (n-hexane and Isopropanol) at a powder weight to solvent volume of 1:5 and particle size of between 0.5mm and 0.75 mm [43]. A randomized 31 set of central composite design comprising three factors (solvent composition [0 - 100% n-haxane], time of extraction [1-5 hours] and extraction temperatures (40–60℃) at five levels were experimented, The characteristics of Biofuels were as follows: viscosity 39.7710 cp, FFA of 2.1185 % and Iodine value of 101.51 g/g.

Fig-2: Biofuel oil is at the up layer of the flask in translucent yellow color while the bottom layer is the glycerol layer (The experience was done at the Faculty of Engineering at Port Said University)

In conclusion, genotype 9 showed a moderate to slightly high degree of variation with most of the rest of the genotypes. This was evidenced by the recording of specific alleles in this genotype, most of which are linked to functional genes. Which in turn may be associated with inheriting or participating in the inheritance of the active substances found in the extract of this genotype as compared to others. Where, the high content of seed oil of acids helped to transform it into a perfect biofuel. Therefore, this genotype could be useful parental line for developing mapping population to map EST-SSRs (transcribed regions). In addition, the efficient use of available germplasm of Egyptian Jatropha *(J. curcas*) for breeding and the genetic improvement purposes.

CONCLUSION

The molecular characterization and the amount of information available from the amplification of 5 SSR loci among 10 genotypes of the Egyptian Jatropha were extremely useful for distinguishing between genotypes on a molecular basis and accurately. The results showed a slightly moderate degree of the genetic diversity between genotypes. In addition, the results showed that Genetic differences have a high effect on the concentrations of fatty acids in Egyptian Jatropha genotypes. Moreover, the extracted oil from Egyptian Jatropha is appropriate for producing biofuel.

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