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Morphological, Biochemical and Molecular Studies on *Jatropha curcas* Seedlings

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Abstract: Pot experiments were carried out in the two seasons of 2014 and 2015 at the greenhouse of National Research Centre, in order to evaluate the morphological, biochemical and molecular studies on *Jatropha curcas* L. seedlings. The results indicate that all morphological plant growth traits as well as stem and root length, stem diameter, number of shoot, leaves and root/plant, leaf area and fresh and dry weight of different plant organs were determined at the end of experiment. Moreover Biochemical study such as Dry matter (DM), Organic matter (OM), Ash and Neutral detergent fiber (NDF) were examined. Organic matter for different parts of *Jatropha* plant; crude protein, crude fiber, ether extracts, nitrogen extract and neutral detergent fiber (CP, CF, EE and NFE) were estimated and varied according to different plant parts. So, the biochemical study provides a reliable account of the endogenic concentrations of such chemicals present in different plant organs. In addition, molecular studies were carried out to compare between Inter-simple sequence repeat (ISSR) and direct amplification of minisatellite-region DNA (DAMD) markers to identify on *J. curcas* L. Ten ISSR and four DAMD markers were applied to identify *J. curcas* L. The ISSR analysis gave a total number of 124 reproducible bands ranging from 100 to 2000 bp scored using ten primers. On the other hand, a total of 33 bands were obtained using four primers DAMD. The number of amplified product varied from 185-1300 bp depending on the minisatellite primers used. This study shows efficiency both ISSR and DAMD markers in characterization of *J. curcas* plant.

Keywords: Morphological, Biochemical, Molecular marker, Microsatellite, Minisatellite.

Introduction:

Jatropha curcas L. (2n = 22) is a drought-resistance perennial a shrubby tree plant that belongs to *Euphorbiaceae* family [1, 2, 3]. Plants sometimes reach height 3 to 5 m, but according to (**4**), under favorite conditions it may grow as tall as 8 to 10 m. It uses for bio-diesel production to provide the energy demands of the industrial section without compromising the agricultural field product. The oil extracted from the seeds generates superior quality biodiesel over fossil diesel and biodiesel from other agricultural yields [5, 6]. Moreover, the high quality its oil has been successfully examined for applying as bio jet fuel, meeting

European and American quality standards [7,1]. However, *jatropha* is still undomesticated and not considered a commercial harvest ascribable the decrease of breeding and genetic improvement. Also, the deficiency of specific agriculture practices [8, 9]. Almost existing farms were originated from seeds produced from wild plants and thus crops are variable [10, 6, 11] and productivity non-uniform [12, 13]. It is better adapted to arid and semi-arid areas. In recent years, there has been increasing benefit in the apply of molecular markers to detect of genetic diversity in *Jatropha* without any effect of environmental variability [14, 15, 16]. Both ISSRs (Inter-simple sequence repeats) and DAMD (Direct amplification of minisatellite-region DNA) markers show much hope in this respect. ISSR analysis includes amplification of DNA fragment existing between two identical microsatellite repeat units oriented in inverse direction [17]. However, DAMD technique was first reported by [18]. It is a dominant marker; the presence and absence of a fragment are known as representing two alleles at a locus. Minisatellite DNAs are tandemly repeated units of genomes, several of which exhibit high levels of length differences referable diversity in the number of repeat regions. It is hypothesized that minisatellites and the DNA sequences flanking them are included in inversions, which produce in their distribution on both strands in inverse orientations [19]. So, the main objective of this study was to evaluate the morphological, biochemical and molecular studies on *J. curcas* L. seedlings. In addition to, compare between DAMD and ISSR markers to identify on *J. curcas* L. plant.

Materials and Methods:

This study was carried out at the one year old seedlings grown in the greenhouse of National Research Centre, Giza Governorate, Dokki, Egypt, during two successive seasons of 2014 and 2015. Seedlings were obtained from nursery of Forestry Department, Horticulture Research Institute, Agriculture Research Centre, and Giza, Egypt. The seedlings were planted on the 15 of March in plastic pots 30 cm diameter, one plants/pot, the average height of seedlings were 30-40 cm, each pot filled with 10 kg from sandy soil. Complete randomized design with six replicates was laid out in the two successive seasons. The following data were recorded at the end of study at the month of October for both seasons as follow:

A- Morphological parameters for different parts of *Jatropha* plant

Stem length (cm), stem diameter (cm), number of shoot, leaves and root/plant, leaf area (cm^2) and fresh and dry weight (g) of different plant organs and root length (cm). Chlorophyll % was determined too.

B- Chemical composition for different parts of *Jatropha* plant

All analyses were done in triplicate. Dry matter was determined by drying at constant weight at 105^{o}C for 3 h in a forced-air oven. Ash was determined by combusting the samples at 550^{o}C for 5 h according to method 942.05 of [20]. Total nitrogen content of the samples was determined using micro-Kjeldahl method (procedure 14.068 in [21], the crude protein (CP) was calculated as nitrogen (N) x 6.25. Ether extract (EE) was determined according to method 920.39 of [21]. Carbohydrates (nitrogen free extract) were calculated by difference (100 - $\sum$ (protein + EE + ash + fiber)). Neutral detergent fiber (NDF) was calculated according to [22] using the following equation: NDF= 28.924 + 0.657 (CF %).

C- Extraction of DNA:

0.5 g of young leaves of *J. curcas* L. plant were collected and soaked in liquid nitrogen for DNA extraction using the 2% Cetyl trimethylammonium bromide (CTAB) procedure as described by[23].

D- ISSR and DAMD assays:

A total of ten ISSR and four DAMD primers (Table 1) were used to amplify DNA (Life Technologies, Gaithersburg, Md.). 25 µl amplification reaction consisted of 2.5 µl 10X PCR buffer, 2.5 µl 25 mM $MgCl_2$; 0.5 µl 40 mM dNTPs; 1 µl *Taq* DNA polymerase (1 unit/µl); 2 µl 0.4 µM primer. Amplification was carried out in DNA thermocycler (Biometra, Germany) under the following conditions: one cycle of 94°C/5 min, followed by 30 cycles for 94°C/45 sec, 52°C/30 sec and 72°C/1 min; a final extension for 72°C/10 min. For DAMD: 1 initial denaturation step at 94^{o}C for 5 min, followed by 35 cycles at 94^{o}C for 30 sec, 36^{o}C for 1 min and 72^{o}C for 1 min and a final extension cycle at 72^{o}C for 10 min.

E - Amplification product analysis:

The amplified DNA (15 µl) for all samples was electrophoresed on 1% agarose containing Ethidium Bromide (0.5 µg/ml) in 1X TBE buffer (89 mM Tris-HCl, 89 mM Boric acid, 2.5 mM EDTA, pH 8.3) at 75 constant volt, and determine with UV transilluminator. The size of each fragment was estimated with reference to a size marker of 10 Kb DNA ladder (BioRoN, Germany). The gels were analyzed by UVI Geltec version 12.4, 1999-2005 (USA).

F- Statistical Analysis:

The design of the experiment was randomized complete blocks with four replicates. All collected data were subjected to the proper statistical analysis as described by [24].

Results and Discussion

A- Morphological parameters for different parts of *Jatropha* plant

Data presented in Figs. (1, 2, 3 and 4) cleared that the morphological parameters for different parts of *Jatropha* plant as well as (shoots, leaves, steam and roots) as means of the two seasons of study. As shown in (Fig. 1), fresh and dry weight/ (gm.) for different plant organs, appeared that fresh weight was (247.67, 98.73 and 58.33 gm.), while dry weight recorded (9.56, 8.51 and 6.58 for *Jatropha* shoots, leaves and roots, respectively. Results concerning the moisture content % for different *Jatropha* plant parts were (96.14, 91.53 and 88.44) for shoots, leaves, and roots. The results illustrated in (Fig. 2) revealed that the numbers of shoots, leaves and roots per *Jatropha* plant ranged 3.33, 15.33 and 11.33, respectively. The Dimensions as length and width of *Jatropha* plant organs presented in (Fig. 3) appeared that plant length, stem diameter and root length were (108.33, 1.83 and 15.67 cm), respectively. Moreover, the green leave properties of *Jatropha* seedling plant (leaf area/cm^2, leaves number and chlorophyll %) were showed in (Fig. 4). These results are in agreement with those obtained by [25, 26, and 27].

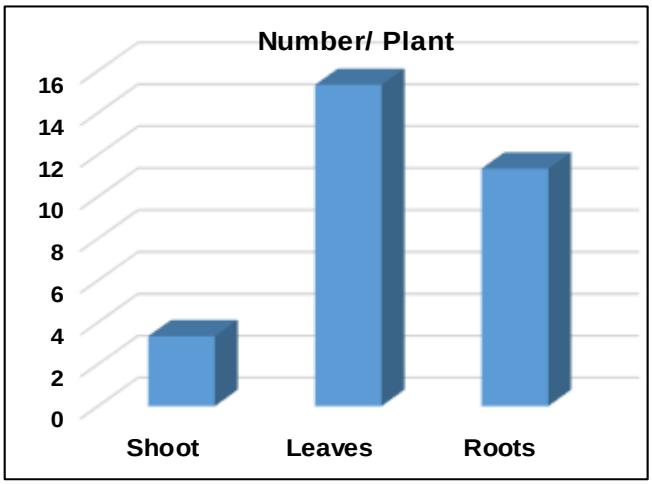

Fig. 1: Morphological analysis of fresh weight, dry weight and moisture content per *Jatropha* seedlings plant. Values are the means of the two seasons.

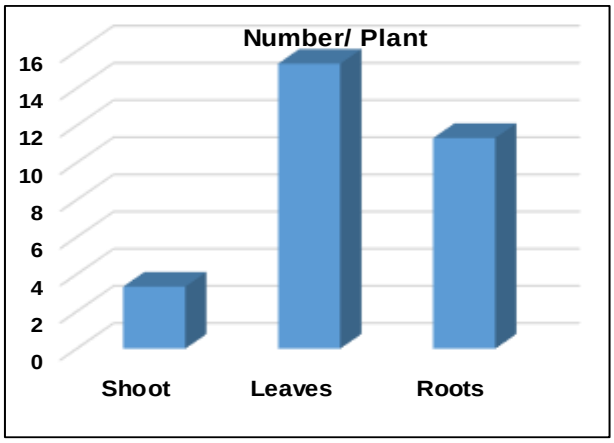

Fig. 2: Morphological analysis of the number of shoots, leaves and roots per *Jatropha* seedlings plant. Values are the means of the two seasons

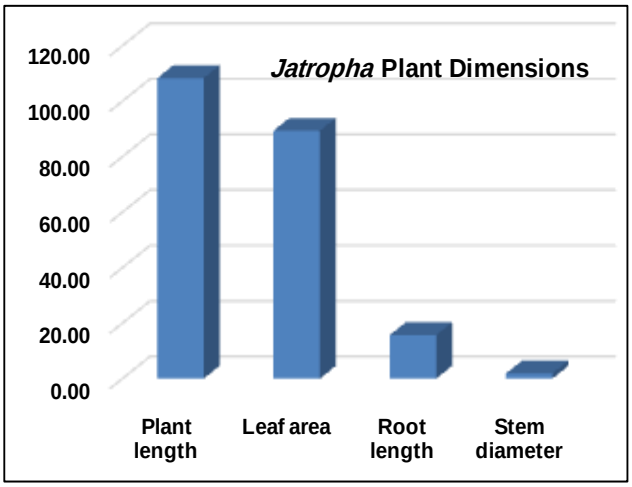

Fig. 3: Morphological analysis of the dimensions of Jatropha seedlings plant parts (plant length, leaf area, root length and stem diameter. Values are the means

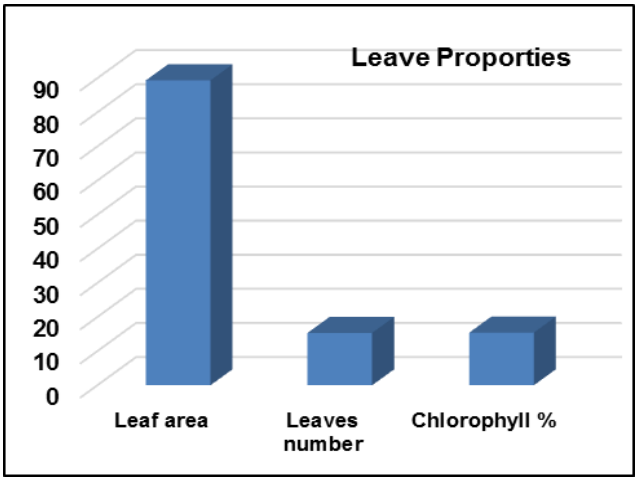

Fig. 4: Morphological analysis of the leave properties of Jatropha seedling plant. Values are the means of the two seasons.

B- Chemical composition

The approximated analysis of samples tested for different plant organs are presented in Tables (1 and 2). The highest CP content was found in leaves (21.56%) followed by seeds (13.35%). The lowest content of CP was found with stems and roots (4.91% and 5.90%, respectively) (Table 1). The samples tested contained fluctuation values of CF which ranged between 25.18–54.78percent. The highest levels of CF were found with stems and seeds (54.06% and 54.78%, respectively), while leaves contained the lowest value of CF (25.18%). The different parts of plant contain moderate values of EE, except seeds which showed extremely high value (22.69%). The other parts of plant tested showed fluctuating values of EE which ranged between (1.33% - 3.61%) (Table 1). The high EE contents of seeds could be attributed to the high ratio of waxes and oils to true fats present in these parts of plants.

The seeds, roots and stems showed lowest values of ash content being 5.65%, 10.35% and 13.12%, respectively. Meanwhile, leaves showed the highest ash content (22.32%). This high value of ash content might be due to their high content of sand contamination which could be the plant grow in desert area (Table 2). The highest content of NDF found with seeds and stems (64.91% and 62.77%, respectively), followed by roots (59.76%). The lowest content were recorded with leaves (45.13%) (Table 2). These results are in agreement with those obtained by [28] found that *J. curcas* seeds have 4.2% ash and 25% crud protein. [26] mentioned that *J. curcas* leaves have crud protein have 6.37% [29].

Table 1: Chemical composition for different parts of *Jatropha* plant as DM basis.

Seedlings organs	CP	CF	EE	NFE
Leaves	21.56 a ±0.49	25.18 b ±0.37	3.61 b ±0.623	27.33 b ±1.21
Stems	4.91 c ±0.53	54.06 a ±6.75	1.33 c ±0.20	26.82 b ±3.77
Seeds	13.35 b ±2.054	54.78 a ±3.04	22.69 a ±2.03	4.86 c ±2.64
Roots	5.90 c ±1.02	46.94 a ±2.69	1.99 bc ±0.48	34.81 a ±1.76

Means within the same column and treatment followed by the same letter are not significantly different according to Duncan ($P \leq 0.05$).

CP: Crude protein CF: Crude fiber EE: Ether extracts NFE: Nitrogen free extract

Table 2: Chemical composition for different parts of *Jatropha* plant as DM basis.

Seedlings organs	DM	OM	Ash	NDF
Leaves	9.19 a ±0.32	77.68 c ±0.59	22.32 a ±0.59	45.13 ±0.44
Stems	8.1 0 b ±0.45	86.88 b ±2.58	13.12 b ±2.58	62.77 ±1.82
Seeds	8.69 ab ±0.35	94.35 a ±0.31	5.65 c ±0.31	64.91 ±1.99
Roots	6.66 c ±0.25	89.65 b ±1.49	10.35 b ±1.49	59.76 ±1.77

Means within the same column and treatment followed by the same letter are not significantly different according to Duncan ($P \leq 0.05$).

DM: Dry matter OM: Organic matter

NDF: Neutral detergent fiber was calculated according to Cheek (1987) using the following equation: NDF= 28.924 + 0,657 (CF %).

C- ISSR analysis:

Our results showed that ten primers ISSR successfully amplified genomic DNA isolated of *J. curcas* L plant. The ISSR banding patterns of the ten primers were illustrated in Fig. (5) and Table (3). The ISSR profiles gave a total number of 124 reproducible bands ranging from 100 to 2000 bp scored using ten primers. The maximum number of amplified fragments was observed with primer ISSR-2 (18), followed by primer ISSR-21 (16). However, the minimum number of ampilcons was observed with primers ISSR-8 and ISSR-23 (nine) (Table 3). On the other hand, (ISSR-4, ISSR-12 and ISSR-24) and (ISSR-7 and ISSR-22)

were equally in bands number (12 and 13), respectively (Fig. 5 and Table 3). [30, 31, 32] observed that ISSRs analysis are arbitrary multiloci marker involve PCR amplification by microsatellite primer. The ISSR primers have been proven to supply fast, reproducible, no prior genomic information is needed for their use and cost efficient technique to evaluate the genetic variability and identify closely related varieties of several species. It is the least technically demanding and offers a rapid assay for supplying information of a big number of allele, especially in species where no study has previously been undertaken.

D- DAMD profiles:

Four primers DAMD amplified genomic DNA extracted of *J. curcas* L leaves. A total of 33 bands were obtained using four primers DAMD (Fig. 6 and Table 4). The number of amplified product varied from 185-1300 bp depending on the minisatellite primers used. The highest number of loci was recorded using primer M13 (ten), followed primer HBV (nine). However, the lowest number of amplicons was observed using primers 33.6 and HVR (seven) (Table 4). In this study, we observed that microsatellites primers gave a large number of alleles compared with minisatellite primers. [33]found that ISSR primers to generate a large number of polymorphic loci [35,19] mentioned that minisatellites are tandem repeated DNA sequences, which exhibit high levels of allelic length difference ascribable variations in the number of repeated nucleotide sequences. Minisatellite loci are highly informative genetic markers that have been applied extensively in several plant species involving fungi and yeasts [34]. Minisatellite loci could be specifically amplified using DAMD-PCR assay. DAMD-PCR profile, polymorphism on the use of single primer flanking the minisatellite units; repeat unit differences could be observed. On the other hand, annealing temperature, primers, type and quality of PCR materials applied in PCR can affect presence or absence of minisatellite in DAMD-PCR technique.[35]

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Table 3: The nucleotide sequences of ISSR primers and sizes of amplified fragments each primer.

Primer Name	Sequence (5′ to 3′)	Size range of the scorable bands (bp)	No. of amplified fragments
ISSR-2	$(AG)_8T$	100-950	18
ISSR-4	$(GA)_8T$	250-2000	12
ISSR-7	$(CT)_8T$	190-900	13
ISSR-8	$(AC)_8T$	100-1600	9
ISSR-12	$(TG)_8A$	120-980	12
ISSR-21	$(AG)_8YT$	160-1300	16
ISSR-22	$(GA)_8YT$	170-1500	13
ISSR-23	$(CT)_8RA$	230-1060	9
ISSR-24	$(GA)_9C$	200-1500	12
UBC-841	$(GA)_8YC$	300-1400	10
		100-2000	**Total number of bands =124**

Table 4: The nucleotide sequences of DAMD Primers and sizes of amplified fragments each primer.

Primer Name	Sequence (5′ to 3′)	Size range of the scorable bands (bp)	No. of amplified fragments
33.6	GGTGTAGAGAGGGGT	185-700	7
HBV	CCTCCTCCCTCCT	210-910	9
HVR	GGAGGTTTTCA	245-710	7
M13	GAGGGTGGCGGTTCT	210-1300	10
		185-1300	**Total number of bands= 33**

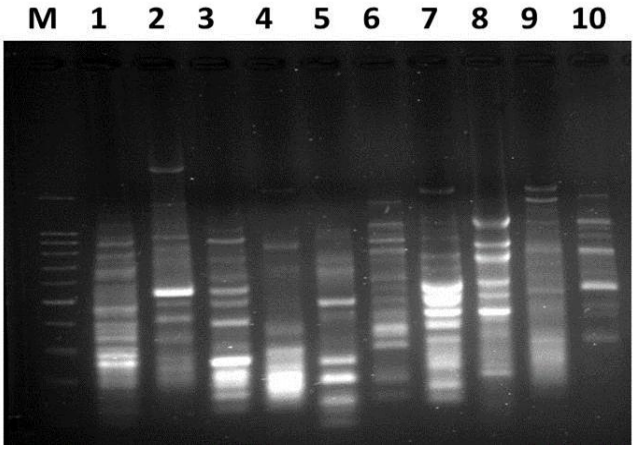

Fig. 5: ISSR-PCR analysis of *Jatropha curcus* plant using ten primers. Lane 1: Primer ISSR-2, lane 2: Primer ISSR-4, lane 3: Primer ISSR-7, and lane 4: Primer ISSR-8, lane 5: Primer ISSR-12, lane 6: Primer ISSR-21, lane 7: Primer ISSR-22, lane 8: Primer ISSR-23, lane 9: Primer ISSR-24 and lane 10: UBC-841. Lane M = 100 bp DNA ladder.

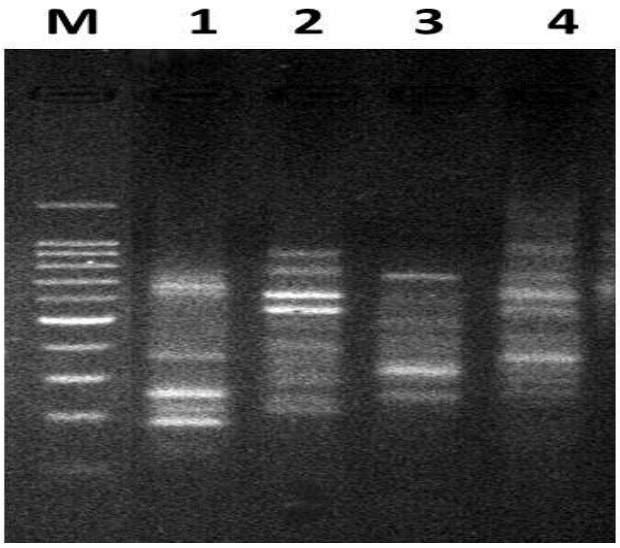

Fig. 6: DAMD analysis of *J. curcus* plant using four primers. Lane 1: Primer 33.6, lane 2: Primer HBV, lane 3: Primer HVR and lane 4: Primer M13. Lane M = 100 bp DNA ladder.

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