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Preliminary Study of the Differential Gene Expression in *Jatropha curcas* L. In Vitro Cultures Exposed to Microgravity

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Jatropha (*Jatropha curcas* L.) has been identified as a suitable species for biofuel production. However, breeding and genetic improvement programs are necessary. Microgravity is a unique environment for the assessment of genetic variation aiming at genetic improvement. The goal of this study was to evaluate the differential gene expression in in vitro jatropha cultures exposed to microgravity. Specific objectives included the evaluation and ranking of RNA isolation methods according to RNA quality and quantity for microarray analysis. In vitro cultures of two jatropha accessions (Brazil and India) were initiated from cotyledon (CO), leaf (L), and stem (ST) sections. Groups of eight petri dishes containing treatments (accession × explant tissue) were arranged in flight hardware and exposed to microgravity for periods varying from 14 to 111 d. Once returned, cultures were processed for RNA isolation and microarray analysis. Gene expression comparisons were performed between ground and orbit samples for the effects of medium and microgravity exposure time. The Plant Reagent protocol gave the highest mean yield in micrograms of RNA and it was almost 30 times greater than the mean concentration given from RNeasy mini kit protocol. The A260/280 ratio mean for the Plant Reagent protocol was higher than 2.0, which is a ratio generally accepted as "pure" for RNA. For all comparisons performed, between 9 and 522 genes were differentially expressed. Over 20% of those genes were expressed at higher levels by more than 2-fold. Differential gene expression was affected by culture medium, with higher levels of expression observed in orbit. Gene expression was also affected by exposure time to microgravity, with periods of 111 d showing higher expression levels. There were 29 genes with high expression levels that were expressed in at least two comparisons. Those genes showing differential gene expression and their importance are discussed. Differential gene expression in microgravity may assist in future jatropha genetic improvement programs.

Jatropha (*Jatropha curcas* L.) is a woody plant belonging to the Euphorbiaceae family and is found in tropical and subtropical countries. This species has been identified as a suitable plant for biofuel production. The oil produced by the seeds is of high quality for biodiesel and jet fuel production (Vendrame and Pinares, 2013). Even though the seeds are highly toxic due to the protein curcin and phorbol esters, almost all parts of this plant have been utilized as insecticides, green manure, soap making, and medicine. However, jatropha is still undomesticated and not considered a crop due to the limited breeding and genetic improvement program (Bhering et al., 2013).

Microgravity is an environment that is defined not only by the intrinsic properties of spaceflight physics, but also by the hardware developed to deliver appropriate growth conditions within orbital habitats (Dutcher et al., 1994; Ferl et al., 2002; Halstead and Dutcher, 1987). Space offers a unique environment and opportunity for studies on plant cell's structural and physiological responses to microgravity (Krikorian, 1996). Molecular genetics tools have revealed apparent stress responses in spaceflight, as well as subtle cell specific gene expression characteristics consistent with signal transduction disruptions in spaceflight-grown plants (Paul et al., 2001).

Gene expression is a highly complex and tightly regulated process that allows a cell to respond dynamically both to environmental stimuli and to its own changing needs. This mechanism acts as both an "on/off" switch to control which genes are expressed in a cell as well as a volume control that increases or decreases the level of expression of particular genes as necessary.

A microarray is a tool for analyzing gene expression that consists of a small membrane or glass slide containing samples of many genes arranged in a regular pattern. It may be used to measure gene expression in many ways, but one of the most popular applications is to compare expression of a set of genes from a cell maintained in a particular condition to the same set of genes from a reference cell maintained under normal conditions (Madan, 2004).

Exposing jatropha cell cultures to microgravity may reveal gene expression changes as a result of this novel physical stimulus. We aim to determine if these changes can potentially result in genetic improvement of the species and therefore contribute to its development as a domesticated crop. The overall objective of this study was to evaluate the differential gene expression in in vitro jatropha cultures exposed to microgravity. Furthermore, understanding that a high quality total RNA is essential for the successful application of many molecular techniques, such as

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reverse transcription polymerase chain reaction (RT-PCR), cDna library construction, and gene expression profiling studies using microarrays (Tattersall et al., 2005); specific objectives included evaluation and ranking of RNA isolation methods according to quality and quantity of RNA obtained and identification of useful RNA samples for microarray analysis.

Material and Methods

Different explant types (cotyledon, leaf and stem sections) from Brazil and India jatropha accessions were selected and in vitro cultures were established in placed in 6 cm (diameter) × 2 cm (height) disposable petri dishes containing Murashige and Skoog (MS) (Murashige and Skoog, 1962) solidified with 7 $g \cdot L^{-1}$ agar and containing 30 $g \cdot L^{-1}$ sucrose, 100 $mg \cdot L^{-1}$ 1 myo-inositol, and 100 $mg \cdot L^{-1}$ casein hydrolysate. The medium pH was adjusted to 5.7 before autoclaving at 121 °C for 15 min at 1.2 $kg \cdot cm^{-2}$. The media was modified with either 4.5 µM thidiazuron (TDZ) (MS1) or 0.75 µM indolebutyric acid (IBA) + 3.6 µM 6 benzyl-aminopurine (6-BA) (MS2).

Groups of eight petri dishes containing treatments (accession x explant tissue) were arranged in a Group Activation Pack (GAP) flight hardware developed by BioServe Space Technologies at the University of Colorado. The hardware has been validated for spaceflight experiments in previous studies (Vendrame and Pinares, 2013). Cultures were exposed to microgravity for periods varying from 14 to 111 d during two separate spaceflights, STS-133 and STS-135. Mission STS-133 had a duration of 111 d in orbit and experiments were performed on board the International Space Station National Laboratory. Mission STS-135 had a duration of 14 d and experiments were performed on board the mid-deck of the Space Shuttle Discovery. Some samples were submitted to 111 d of microgravity during STS-133, followed by subsequent exposure to microgravity for 14 d during STS-135.

Samples from space and ground controls were grouped into six comparisons (Table 1) in order to evaluate the effect of microgravity (Space vs. Ground), culture media (MS1 vs. MS2) and period of exposure to microgravity (14, 111, and 125 d) on differential gene expression in jatropha in vitro cell cultures.

Treatments for period of exposure to microgravity consisted of cultures from space (STS-133) that were transferred to ground (S-G, 111 d in space), cultures from ground that were transferred to ground (G-G, no microgravity exposure), cultures from ground that were transferred to space (STS-135, G-S, 14 d in space), and cultures from space (STS-133) that were transferred to space again (STS-135, S-S, 125 d in space).

Selected samples from space were frozen immediately after return from space. The remaining of the space and ground samples was maintained under room conditions prior to RNA isolation. RNA for microarray analyses was isolated from undifferentiated cell tissues obtained from those explants cultured in space and ground. All glassware for RNA extraction was baked at –180 °C for at least 4 h, whereas plastic ware was either new disposable or treated with 0.1% (v/v) diethylpyrovarbonate (DEPC) water and autoclaved before use. All solutions used in the RNA extraction, except those containing Tris, were treated with 0.1% (v/v) DEPC to destroy potentially damaging RNases. The RNeasy Mini Kit (Qiagen, Velancia, CA) and Plant RNA isolation Reagent (Invitrogen, Carlsbad, CA) protocols were used for RNA isolation. Both protocols were performed according to the manufacturer's instructions for isolation of RNA from plants including a DNAse I treatment (Qiagen).

RNA concentration and purity was determined on a NanoDrop Spectrophotometer (ThermoFisher Scientific Inc). For purity assessment, the absorbance for the A260/280 and A260/230 ratios were evaluated and the samples with more than 1.8 values were considered suitable for the experiment. The integrity of total RNA was determined by running samples on 1% denaturing agarose gel (Qiagen). The gel was run at 7 V/cm and imaged using Doc-it LS Image acquisition software. Also, the RNA integrity number (RIN) and 28S/18S ratio per sample were quantified by Agilent 2100 Bioanalyzer RNA labchip assays following the manufacturer's instructions (Agilent Technologies, Palo Alto, CA).

Flight and ground control samples with high concentration and purity were submitted to the University of Florida's joint Shands Cancer Center/ Interdisciplinary Center for Biotechnology Research (ICBR) Microarray Core Facility (Gainesville, FL) for further analyses. The cDNA was synthesized from 150 ng of total RNA using the Low Input Quick Amp Labeling Kit (Agilent Technologies) according the manufacturer's protocol. The synthesized cDNA was used as a template for in vitro transcription (IVT) in the presence of T7 RNA Polymerase Blend and cyanine labeled CTPs. The amplified, labeled RNA was purified using the RNeasy Mini Kit (Qiagen). For each array, 600 ng of Cy 3 labeled was fragmented and hybridized with rotation at 65 °C for 17 h. The arrays were washed according to the manufacturer's protocol and then scanned on an Agilent G2505B scanner. Data were extracted using Feature Extraction 10.1.1.1 software (Agilent Technologies).

For each gene, estimates of the relative expression differences between the two strains (log ratios) were calculated. The Fs statistics and unadjusted *P*-values were also calculated using the fixed ANOVA. False discovery rates were calculated from the unadjusted *P*-values using q values software (http://faculty.washington.edu/-jstorey/qvalue.R). The significance was determined using the unadjusted *P*-value of the Fs-statistics (*t*-test) and fold change (FC) value. Moderate statically significant gene expression was defined as genes with $0.01 < P\text{-value} \leq 0.05$ and FC > 2. A highly significant gene was defined as genes with $P\text{-value} \leq 0.01$ and FC > 2. Based on this definition, the numbers of statistically significant genes ($P \leq 0.05$), and moderate and highly significant genes were calculated for each comparison.

The number of genes induced (FC > 2) and repressed (FC < 2) to make mRNA, the number of exclusive genes per comparison, and the common genes between comparisons were analyzed. Also, a comparison of number of genes induced (FC > 2) and repressed (FC < 2) between space and ground environments were also determined.

Table 1. Description of comparisons performed to evaluate differential gene expression in *Jatropha curcas* in vitro cell cultures. MS1: MS culture medium with 4.5 µM TDZ; MS2: MS culture medium with 0.75 µM IBA + 3.6 µM 6-BA; G: Ground; S: Space.

Comparison ID	Comparison description
1	Space MS1 vs. MS2
2	Space G-S MS2 vs. space S-S MS2
3	Space G-S MS1 vs. space S-S MS1
4	Ground MS1 vs. MS2
5	Ground G-G MS2 vs. ground S-G MS2
6	Ground G-G MS1 vs. ground S-G MS1

A hierarchical cluster was constructed using the software Cluster 3.0 and TreeView program for the interactive graphical analysis of the results from Cluster (//jtreeview.sourceforge.net/).

Results and Discussion

There were large differences in the amount of RNA extracted per gram of tissue depending on the RNA isolation protocol used. The plant reagent protocol resulted in the highest mean yield in micrograms of RNA, which was almost 30 times higher than the mean concentration yielded by the RNeasy mini kit protocol (Table 2).

The A260/280 ratios indicate the level of protein contamination, based on the principle that nucleic acids display an absorbance optimum at 260 nm, whereas proteins display an absorbance optimum at 280 nm (Winfrey et al., 1997). The plant reagent RNA isolation method provided an A260/280 ratio mean higher than 2.0, which is the ratio generally accepted as "pure" for RNA. An A260/230 mean ratio lower than 1.8 indicates that the total RNA is of low purity, possibly contaminated with polysaccharide compounds. In general, the yield and quality of RNA isolated in this study was higher when using the Plant Reagent protocol, as compared to the RNeasy mini kit.

Most of the RNA samples that could be seen on the agarose gel had a clear band pattern, although many samples had some smearing, which is indicative of RNA degradation. These patterns were reproducible and could be seen more clearly in the gel image and electropherograms from the Agilent 2100 Bioanalyzer.

The RIN and the 28S/18S ratio values were calculated for the RNA samples isolated with the plant reagent protocol. Samples with RIN ≥ 7 and ratio 28S/18S ≥ 2 were considered as having high quality; while samples with RIN 5.6 ≤ X ≤ 7 and ratio 28S/18S 0.8 ≤ X ≤ 2 were borderline; and samples with RIN < 5.6 and ratio 28S/18S < 0.8 were low quality. RNA samples classified as high and borderline were used for microarray analyses. The average ratio and RIN of the ground and space samples were RIN 6.9 ± 1.31, ratio 1.26 ± 0.26, and RIN 7 ± 0.6, ratio 1.26 ± 0.29, respectively.

The number of genes with high expression and the number of exclusive expressed genes were calculated per comparison (Table 3). The number of exclusive genes per comparison shows how the gene expression profile changed under the different conditions to which the samples were exposed.

One hundred and fifty-three genes were altered in their expression under microgravity conditions by more than 2-fold. Of those genes, 65 were induced and 91 were repressed and expressed at high levels. The gene expression profile of samples from the ground showed that 362 genes varied by more than 2-fold and of those genes, 128 were induced and 243 were repressed and expressed at high levels. The reason why the sum of induced and repressed genes in space and ground condition exceeds the numbers of genes expressed in each condition is due to the common genes between comparisons 1 and 3, 4 and 5, 4 and 6, and 5 and 6 (Table 4).

There were 29 genes with high expression levels ($P < 0.01$, FC > 2) that were expressed in at least two comparisons. The highest numbers of common genes were found between comparisons 1 and 5 and between 5 and 6. There were two genes expressed under microgravity and four genes expressed in the ground whose expression profiles changed for different comparisons. The same gene was induced under one condition and repressed under another condition (Table 4). The functions of most common genes are related with catalytic activity, calcium, copper and sugar binding, beta galactoside activity, and hydrolase activity. These genes appear to be involved in important processes, such as cell propagation and plant development. Their function can be related to the different gene expression profiles of the comparisons, in which samples were cultured in different media (MS1 and MS2). This is also supported by the hierarchical cluster generated. Two main groups were formed, each with 3 comparisons. The first group included most of the samples cultured in MS2 media from the comparisons 1, 2, and 5; while the second group included most of the samples cultured in MS1 media from the comparisons 3, 4, and 6.

In this study we demonstrated a feasible method for RNA isolation from jatropha in vitro cultures. The plant reagent protocol returned the highest RNA yield and quality as compared to the RNeasy mini kit. The RIN and 28S/18S ratio values were used to classify the integrity of RNA and the identification of useful RNA samples for microarray analysis.

More than 20% of the genes evaluated using microarray analysis were expressed at higher levels by over 2-fold. Different gene expression profiles were observed and the high number of exclusive genes per comparison demonstrated the differential gene expression under different environmental conditions. Differential gene expression was affected by culture medium, with higher levels of expression observed in orbit. Gene expression was also affected by exposure time to microgravity, with periods of 111 d showing higher expression levels.

Future studies include the analysis of gene ontology to better understand the importance of the gene function for those genes that showed differential gene expression in the present study. Furthermore, the understanding of differential gene expression in jatropha in vitro cultures can provide the foundation for genetic

Table 2. Yield and quality of jatropha RNA isolated using two RNA isolation protocols: plant reagent and RNeasy Mini Kit.

RNA isolation protocols	RNA concn	A260/280	A260/230
Plant reagent	2839.47 ± 2075.78	2.12 ± 0.12	1.73 ± 0.19
RNeasy mini kit	95.04 ± 113.96	1.85 ± 0.23	0.75 ± 0.65

Table 3. Differential gene expression in jatropha in vitro cell cultures under different environmental conditions. MS1: MS culture medium with 4.5 µM TDZ; MS2: MS culture medium with 0.75 µM IBA+ 3.6 µM 6-BA; G: Ground; S: Space.

Environmental conditions	$P<0.05$ FC>2	$P<0.01$ FC>2	Exclusive genes
Space MS1 vs. MS2	229	73	64
Space G-S MS2 vs. Space S-S MS2	9	2	2
Space G-S MS1 vs. Space S-S MS1	270	81	75
Ground MS1 vs. MS2	188	70	62
Ground G-G MS2 vs. Ground S-G MS2	522	239	226
Ground G-G MS1 vs. Ground S-G MS1	191	62	53

Table 4. Common genes from *Jatropha curcas* in vitro cultures that were differentially expressed between comparisons. Non-shaded numbers indicate induced genes and shaded numbers indicate repressed genes. Comparison 2 was not included in the table because it does not have common genes with the other comparisons.

Gene identification	Comparisons				
	1	3	4	5	6
Os07g0529600	2.068	1.197			
MyB11	1.931				
MyB83		1.335			
SCPL 7		1.281			
SCPL 49	1.759				
MATE	1.723		1.541		
PORA	1.024		1.061		
Nodulin Mt N3 family protein	2.068			3.445	
BGAL9 beta galactoside	1.126				
BGAL3 beta galactoside				4.207	
CYC3B	1.089			3.201	
Chromosome associated kinesin, putative	1.641			3.843	
IAA30	1.581				
AKT1	1.252				1.146
RNA binding/protein binding	1.373				1.769
UBX domain containing protein		1.295		2.053	
BAM 2 (beta-amylase 2)		1.057		2.056	
IAA30					2.249
IAA16		1.678			
Peroxidase 50			1.093		
Peroxidase putative				3.598	1.492
PSAT-Phospho 2 Serine, E oxoglutarate aminotransferase			1.036	2.179	
Monosaccharide transporter, putative			1.362		
Polyol transporter, monosaccharide transmembrane transporter				2.424	
ARR30			1.107		1.795
Isoflavone reductase, putative				7.203	1.311
CCR2 (cold, circadian rhythm, and RNA binding 2)				2.078	1.553
ANNAT1 (ATP binding, calcium dependent phospholipid binding				2.517	1.069
ATP binding				2.677	1.291

improvement programs in jatropha aiming at the development of commercial cultivars and the establishment of jatropha as a feasible crop for the biofuel industry.

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