Natural Resources Section

Proc. Fla. State Hort. Soc. 126:xxx–xxx. 2013.

In Vitro Growth of *Jatropha curcas* L. Cell Cultures in Microgravity

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Additional index words. *Jatropha curcas*, micropropagation, space biology

Jatropha is a species identified for biofuel production. Microgravity offers a unique environment for genetic variation studies and in vitro cell cultures are suitable for space-based experiments. The main objective of this study was to compare the in vitro growth of jatropha cell cultures between orbit and ground. The effects of genotype, culture medium, and explant type were evaluated. In vitro cultures were initiated from three jatropha accessions: Brazil, India, and Mexico. Cotyledon, leaf, and stem sections were utilized as explants. A basal Murashige and Skoog (MS) medium was used, modified with either 4.5 µM TDZ (MS1) or 0.75 µM IBA + 3.6 µM 6-BA (MS2). Cultures were maintained in petri dishes, in the dark, at 25 ± 2 °C, arranged in Group Activation Packs (petriGAPs) flight hardware. Control petriGAPs were maintained on the ground. Spaceflight experiments were conducted in the International Space Station for 111 d (STS-133) and in the mid-deck of the space shuttle Atlantis (STS-135) for 14 d. In vitro jatropha cultures showed normal growth in microgravity. The MS1 medium produced structures similar to globular stage somatic embryos, while the MS2 medium produced mostly callus. The genotype had an effect on somatic embryo formation and subsequent shoot regeneration, and cultures from Brazil had the best regeneration capability. Stem sections showed the best capability to form somatic embryos in vitro. Cultures exposed to microgravity showed efficient development of embryogenic cultures. Future efforts should focus on improved root formation and plantlet regeneration.

Jatropha (*Jatropha curcas* L.) is a species in the Euphorbiaceae family that has been identified for biofuel production. The plant is native to Central America and it is cultivated in tropical regions of the world. This shrub can reach up to 15 ft tall. Jatropha is perennial, deciduous, monoecious, and currently still considered an undomesticated crop. It is a wild-growing hardy plant well adapted to various soil and climate conditions (Katwal and Soni, 2003). However, breeding and genetic improvement programs are necessary for the development of commercial cultivars and the establishment of jatropha as a biofuel crop. The seeds contain 27% to 40% oil (average: 34.4%) of high quality, which has been proven suitable for biodiesel and jet fuel mixes. The importance of jatropha oil is that it finds wide usage and it has high economic potential for large-scale industrial use (Raina and Gaikwad, 1987).

Microgravity offers a unique environment for studies of plant cells and space studies have shown that microgravity affects cell growth, including growth and development of in vitro cell cultures (Cogoli and Gmunder, 1991; Krikorian, 1996). Changes induced in microgravity may potentially assist the genetic improvement in jatropha and contribute to its development as a feasible biofuel crop.

The main goal of this work is to assess the in vitro growth and development of jatropha cells cultures in microgravity compared to ground (control) cultures. The effects of genotype (Brazil, India, Mexico), explant type (sections of cotyledon, leaf, and stem) and culture medium on in vitro growth of jatropha cultures in space and in the ground are also evaluated.

The authors would like to thank Vecenergy – The Vecellio Group, Zero Gravity Solutions, Inc., BioServe – University of Colorado, NASA, and the Space Shuttle and International Space Station Crew of STS-133 (Discovery) and STS-135 (Atlantis) for the support provided for this experiment.

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Materials and Methods

Jatropha seeds were collected from three accessions, Brazil, India, and Mexico, located at the University of Florida's Tropical Research and Education Center (TREC) in Homestead, FL. The seeds were sterilized according to the protocol described by Vendrame and Pinares (2013). Briefly, seeds were washed in distilled water and surface sterilized with 1% Alconox solution followed by three distilled water rinses. In a laminar flow hood, seeds were sterilized with 3% sodium hypochlorite for 5 min followed by three 3-min rinses in sterilized distilled water. Sterilized seeds were placed in magenta G-7 vessels containing MS basal culture medium (Murashige and Skoog, 1962) solidified with 7 $g \cdot L^{-1}$ agar for germination. The medium pH was adjusted to 5.7 before autoclaving at 121 °C for 15 min at 1.2 $kg \cdot cm^{-2}$. In vitro seed cultures were maintained at 25 ± 2 °C under a 16-h photoperiod and 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ *PPF*. Two weeks after germination when cotyledons and leaves had expanded, explants were removed and placed in 6-cm (diameter) × 2-cm (height) disposable petri dishes containing MS culture media with 30 $g \cdot L^{-1}$ sucrose, 100 $mg \cdot L^{-1}$ 1 myo-inositol, 100 $mg \cdot L^{-1}$ casein hydrolysate, solidified with 7 $g \cdot L^{-1}$ agar. The media was modified with either 4.5 µM thidiazuron (TDZ) (MS1) or 0.75 µM indolebutyric acid (IBA) + 3.6 µM 6 benzylaminopurine (6-BA) (MS2). The media was prepared as described above.

When seedlings reached about 5 to 6 cm in length, three types of explants were removed and placed in petri dishes containing either MS1 or MS2: 1) cotyledon (CO) sections (1 × 1 cm) (Fig. 1A), 2) leaf (L) sections (1 × 1 cm) (Fig. 1B), and 3) stem (ST) sections (0.5 × 0.5 cm diameter) (Fig. 1C). Due to limited material, no cultures were initiated from Mexico stem sections.

Two experiments were performed on two separate flights,

Fig. 1. Types of explant tissues from *Jatropha curcas* used in the in vitro space studies: (**A**) cotyledon sections, (**B**) leaf sections, and (**C**) stem sections.

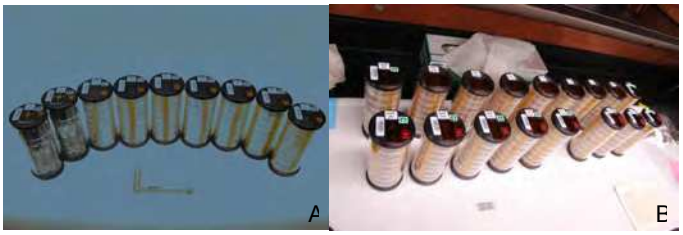

Fig. 2. Group Activation Packs (GAPs) containing petri dishes with *Jatropha curcas* in vitro cultures for spaceflight experiments: (**A**) GAPs prepared for STS-133 included a total of nine GAPS with seven of them containing eight petri dishes for in vitro growth evaluations of jatropha cultures, for a total of 56 samples; and (**B**) GAPs prepared for STS-135 included a total of nine GAPs containing eight petri dishes each for in vitro growth evaluations of jatropha cultures, for a total of 72 samples.

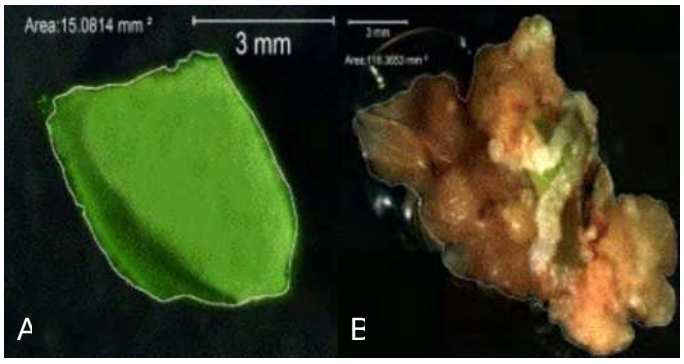

Fig. 3. Pre- and post-flight jatropha tissue area (mm^2) measurements for STS-133: (**A**) jatropha cotyledon section pre-flight; and (**B**) jatropha culture growth measured after 111 d in space flight STS-133.

STS-133 and STS-135. Mission STS-133 had a duration of 111 days 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. Groups of seven petri dishes were arranged into Group Activation Pack (GAP) flight hardware developed by BioServe Space Technologies at the University of Colorado. The hardware has been fitted for eight 6-cm × 2-cm petri dishes (PetriGAP) and has been validated for spaceflight experiments in previous studies (Vendrame and Pinares, 2013). For STS-133, seven GAPs were utilized with a total of 56 samples (Fig. 2A), while for STS-135 nine GAPs were utilized with a total of 72 samples (Fig. 2B). For both experiments, a complete set of replicated GAPs was maintained in the ground as controls.

For both experiments cell growth was evaluated, including the effects of genotype, medium and explant source. The growth of in vitro cell cultures was evaluated using a Leica MZ12s stereo microscope with a SPOT Idea digital camera attached. The SPOT imaging software was utilized for measurement of explant area (mm^2). The initial (pre-flight) and final (post-flight) area of every single explant was measured. The area measurement provided a non-destructive means to estimate the growth of cultures from the beginning to the end of the experiment, and therefore preserve all cultures for subsequent studies, including evaluations of differential gene expression as affected by microgravity and regeneration of cultures into plantlets.

Growth and the effects of genotype, explant type, and culture medium were evaluated using analysis of variance (ANOVA) at $\alpha = 0.05$ using the SAS Software (SAS Institute, Cary, NC). Means were compared using LSD's multiple range test.

Results and Discussion

Cultures showed significant growth for both experiments. However, we are summarizing the results for STS-133 only because results for STS-135 were similar. Growth was observed as a significant percentage increase in area for the in vitro cultures (Fig. 3). Best results were observed for MS1 with the formation of pre-embryogenic masses and subsequent shoot formation, while MS2 produced mostly callus. No contamination was observed. Genotype and explant type had a direct effect on the growth of cultures. Jatropha in vitro cultures from Brazil and India accessions had greater growth (percent area increase) than cultures from Mexico in both space and ground (Fig. 4). Stem explants showed greater growth as compared to cotyledon and leaf explants. Differences in in vitro cell growth for jatropha were previously reported in preliminary pre-flight studies (Vendrame and Pinares, 2013).

Stem explants provided the greatest growth for both space and ground environments (Fig. 4). In contrast, in preliminary studies, stem and cotyledon explants provided the greatest growth of in vitro jatropha cell cultures (Vendrame and Pinares, 2013). No significant differences were observed in growth for all accessions when using leaf explants in both space and ground (Fig. 4).

When comparing growth between space and ground samples, no significant differences were observed, except for a greater growth for cotyledon explants of Brazil accession cultures in the ground (Fig. 4).

The effects of genotype and explant type for in vitro growth of cultures in a space environment provides some valuable information that can be applied for the selection of plant material that is more suitable for space experiments in the future. It also provides some germplasm diversity for potential induction of genetic changes. Genetic variability in jatropha can assist in jatropha breeding and genetic improvement programs (Bhering et al., 2013).

Plant regeneration from in vitro cultures is affected by the type of cells formed, resulting in the need for evaluating different explant types and their vitro growth. While all explants showed some undifferentiated type of growth (callus), subsequent shoot initiation occurred (Fig.5A) with shoot formation observed (Fig. 5B) from cotyledon and leaf explants. For stem explants, structures similar to pre-embryogenic masses (PEMs) were observed (Fig. 5C), which differentiate into somatic embryos. Somatic embryogenesis is a desirable in vitro culture system for jatropha, as it

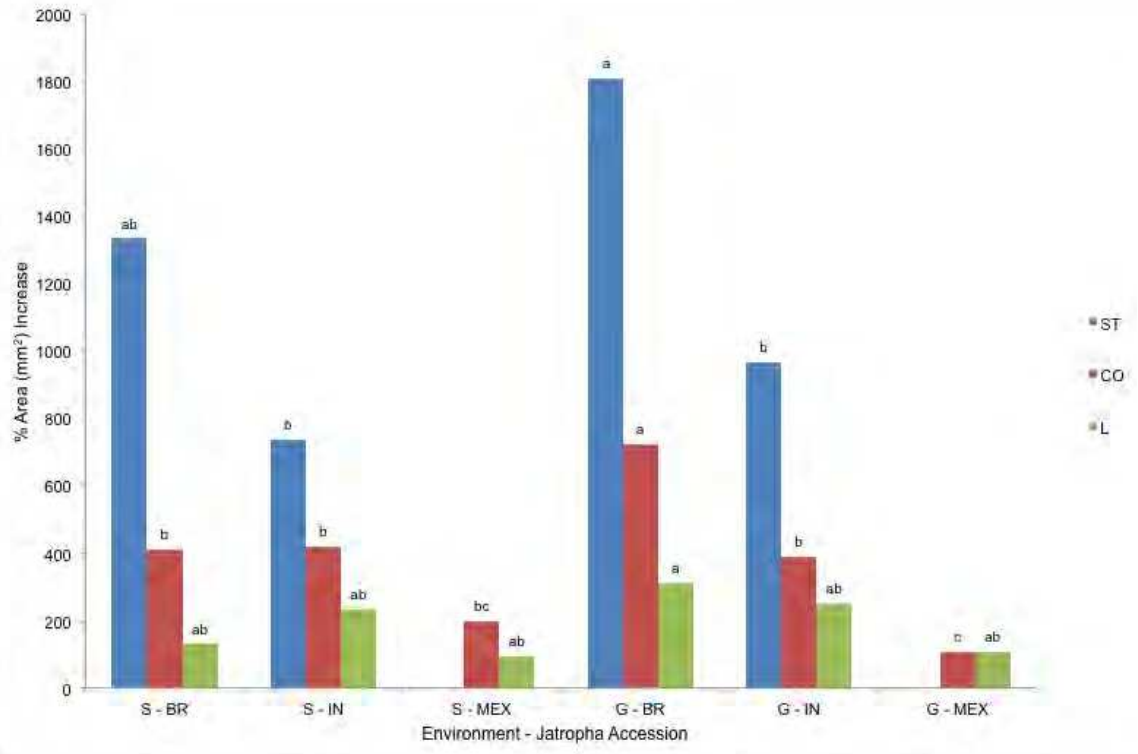

Fig. 4. Percentage area (mm²) increase of *Jatropha curcas* in vitro cell cultures submitted to microgravity (S) as compared to ground controls (G) during mission STS-133. Three genotypes were evaluated: Brazil (BR), India (IN), and Mexico (MEX), as well as three explant types: stem (ST), cotyledon (CO), and leaf (L) sections. Same letters indicate no significant differences by LSD evaluation at $\alpha = 0.05$.

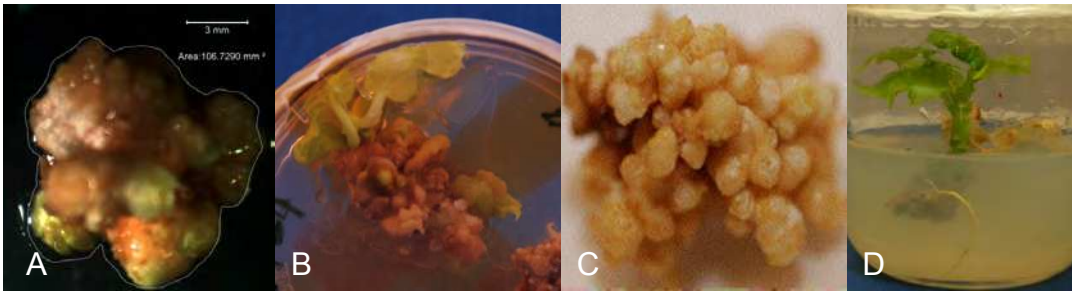

Fig. 5. Jatropha in vitro cultures from STS-133 after spaceflight experiment: (**A**) shoot initiation, (**B**) shoot formation, (**C**) pro-embryogenic masses, and (**D**) plantlet regeneration.

allows high multiplication rates and efficient plant regeneration (Vendrame et al., 1999, 2001). In this study, cultures subsequently regenerated into plantlets (Fig. 5D).

The present study demonstrated the successful growth and development of jatropha in vitro cell cultures plant cells in microgravity. Cultures can be grown in microgravity from 14 (STS-135) to 111 (STS-133) days without any abnormalities. Culture medium MS1 containing 4.5 µM TDZ showed to be the most suitable for growth of in vitro jatropha cultures and allowed the formation of PEMs from stem explants. Cotyledon and leaf sections showed the best capability to induce and form shoots. Genotype had a significant effect on the growth of cultures. The proper selection of genotype, explant type, and culture medium combined with exposure to microgravity provides unique tools for assisting in the breeding and genetic improvement of jatropha. Such tools can have a significant impact towards the development of jatropha commercial cultivars and the establishment of jatropha as a feasible biofuel crop.

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