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TISSUE CULTURE AND TRANSFORMATION STUDIES OF *JATROPHA CURCAS*, A SECOND GENERATION BIOFUEL CROP

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ABSTRACT

Jatropha curcas L. (Euphorbiaceae) is an important second generation biofuel crop. In a time when energy needs are coming to the forefront of our nation's concerns, development and improvement of alternate, sustainable biofuel crops are critical. *J. curcas* is especially useful because it is a drought-tolerant, non-food crop that can be grown on marginal lands, thus not competing for the fertile agriculture land used for food production. *Jatropha* seeds contain approximately 40% oil that can be used as a feedstock for biodiesel production. However, one of the limitations of *J.* curcas is that it is adapted to warm, tropical and subtropical climate and it is susceptible to cold stress. Combining the tools of biotechnology such as tissue culture and genetic engineering, value added traits (e. g. tolerance to biotic and abiotic stress) can incorporated to *J. curcas* leading to accelerated production of superior cultivars. We report here the development of an efficient protocol for *in vitro* regeneration of *J. curcas* using various genotypes and explants (leaf, nodal segment, and embryo). Likewise, a protocol for genetic engineering of *J. curcas* via *Agrobacterium*-mediated transformation was also optimized to improve its qualities such as tolerance to abiotic stress and oil yield.

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CHAPTER 1

INTRODUCTION

1. *Jatropha curcas* L.: A biofuel crop with great potential

1.1. Classification and Distribution

Jatropha curcas is a large shrub/small tree that grows in primarily tropical areas such as Central America, South America, Southeast Asia, and South Asia (Sujatha & Mukta, 1996). It is classified in the Kingdom Plantae, Phylum Magnoliophyta, Class Magnoliopsida, and Order Euphorbiales (Accessed through GBIF Data Portal, data.gbif.org, 2011-4-17). It belongs to the Euphorbiaceae family, more commonly known as the Spurge family, and also goes by the name "Physic nut". Among the approximately 8,000 species in the family, other economically beneficial species are Cassava, Castor, and Rubber tree (Schultes, 1987), along with ornamental plants like Poinsettia. *Jatropha curcas* is believed to be the most primitive member of the family (Dehgan & Webster, 1979).

The genus is *Jatropha* and species is *curcas*. Other species in the genus *Jatropha* include *J. cuneata, J. integerrima* (Spicy *Jatropha*), and *J. podagrica* (Buddha Belly). The genus contains approximately 175 species of plants, shrubs, and trees, many of which are highly toxic. They generally prefer to grow in warm, dry climates around the world (Dehgan & Webster, 1979). *J. curcas* is believed to have originated in tropical South America (Carels, 2009).

1.2. Applications and Potential Benefits of *Jatropha curcas* L.

Jatropha is a plant of great economic potential; most notably, its seed oil can be used as a source for biodiesel, as approximately 30 to 40% of its seed content is oil (Giibitz, Mittelbach, & Trabi, 1999). This high seed oil content has been shown to give a higher yield

of biofuel than other crops: over ten times as much as maize, four times as much as soybean, and almost twice as much as rapeseed from the life cycle perspective (Chisti, 2007). *Jatropha* oil can be easily transesterified to biodiesel and it has a favorable pour point to use as a biofuel. Glycerol is an industrial by-product of biodiesel production from *Jatropha* oil that can be used to manufacture soap (Kumar & Sharma, 2008). Oil cake, a by-product of oil extraction is a valuable biofertilizer, which can also be used as a solid fuel for heating purpose. *J. curcas* is also an important medicinal plant in the Traditional Indian Medicine, with numerous therapeutic applications (Oksoueian et al., 2011; Thomas, Sah, & Sharma, 2008).

The potential economic benefits of *Jatropha* as a biofuel crop are bolstered by a number of advantages the plant has. Unlike other biofuel crops such as corn and soybean, *Jatropha* is not a food crop. In the past, concerns have been raised about using food crops for energy because the perception is that they would compete with and cut down the food supply and raise food prices. This issue has been especially brought up in the public forum in national media outlets like *USA Today* (Weise, 2011), CNN Money (Goldman, 2008), and *The New York Times* (Wald, 2006). Furthermore, *Jatropha* is a fairly drought-resistant plant that can also be grown in less than ideal conditions for crop production. For example, not only can it grow in arid conditions, but it can also grow on marginal lands that many cereal and other food crops cannot grow on (Openshaw, 2000). This is important because another concern that some have about biofuel crops are that they would compete for land with food crops, which could also lead to an increase in food prices. In Pennsylvania, this could be especially important as we have hundreds of thousands of acres of abandoned mine lands; most crops are unable to grow on these lands due to poor fertility and high acidity of the soils (Chhetri, Tango, Budge, Watts, & Islam, 2008). *J. curcus* yields 194-394 gallons of oil per acre, which is higher than most biodiesel crops (Table 1.1).

Table 1.1: Oil Yields of Selected Biodiesel Crops (Modified from Tickell, 2003 & Currie, 2007)

Plant	Gal Oil/Acre
Palm Oil	508
Coconut	276
Jatropha	**194-394**
Castor	145
Rapeseed	122
Peanut	109
Cocoa	105
Camelina	60
Soybean	46-60
Hemp	37

Table 1.1 compares oil yields (gallons/acre) of certain biodiesel crops.

1.3. Limitations of *Jatropha curcas* L.

Despite the advantages, one of the limitations is to grow *Jatropha* in Pennsylvania or other temperate climatic zones. *Jatropha* is not a naturally cold tolerant plant, and its natural range distribution, is more tropical climates like Central America, South America, Southeast Asia, and South Asia. This drawback could be addressed using a combination of biotechnology tools such as plant tissue culture and genetic engineering leading to the development of cold/freeze tolerant *Jatropha*. Another limitation is very little research has been done on the breeding aspects of *Jatropha*, so very little is known about diseases, fertilizer, and nutrient requirements for producing high yielding *Jatropha* with advantages like biotic and abiotic stress tolerance (Achten, 2010).

1.4. Classification of Biofuels

Biofuels are classified into first through fourth generation (DOE, 2007). First generation biofuel is derived from food crops like corn (starch based ethanol) and soybean. Second generation biofuel is produced from non-food crops like *Jatropha, Camelina*, switchgrass, and miscanthus. Third generation biofuels come from algae. Fourth generation biofuels are generated through chemical processes such as biobutanol.

2. Plant Tissue Culture

Plant tissue culture is the field of study that can enable one to use a defined growth medium under sterile conditions to rapidly generate genetically similar plants. In other words, it produces large numbers of cloned plants in a short period of time.

Plant tissue culture can be used to create a quick and efficient way to regenerate many plants from a single piece of a "mother" plant. An explant is a piece of living tissue that is transferred to an artificial growth medium. The explant could come from numerous parts of the plant, including the leaf, stem, root, flower or seed (Radzan, 2003). The foundation of plant tissue culture is the principle of totipotentcy, which means that any single cell can differentiate into all other cell types, and thus, regenerate a whole plant. This makes it possible to regenerate many genetically similar plants (clones) from various explants in a short time, in a small laboratory space and without being affected by the seasons. There are several reports on tissue culture of *J. curcas* describing different ways to regenerate plants from different explants (Cartagena, Shibagaki, & Fukui, 2011; Deore & Johnson, 2008; Kumar, 2010a, b, c; Sujatha, Makkar, & Becker, 2005, Sujatha & Mukta, 1996).

Plants can be regenerated *in vitro* either through organogenesis or somatic embryogenesis. Organogenesis is the process by which an explant is used to form different organs (shoots, roots) (Loyola-Vargas, De-la-Peña, Galaz-Ávalos, & Quiroz-Figueroa, 2008), either directly or indirectly. The indirect pathway produces a callus, or undifferentiated mass of cells (Phillips, 2004). In somatic embryogenesis, non-sexual embryos are formed from the explants directly of from the callus (Quiroz-Figueroa, Rojas-Herrera, Galaz-Avalos, & Loyola-Vargas, 2006).

2.1. Brief History of Plant Tissue Culture

The foundations of plant tissue culture were laid in 1902 by the German scientist Gottleib Haberlandt, known as “Father of Plant Tissue Culture”, who first proposed the concept of totipotentcy. Simon in 1908 successfully regenerated callus, buds, and roots from poplar In 1922, Kotte and Robbins were the first to suggest using meristematic cells for culture using maize and pea (Radzan, 2003). Many of the early plant tissue culture experiments were that of root cultures of tomato and other crops (Robbins, 1922). In the 1930s, Frenchman Gautherat was the first to successfully use isolated cambium plant tissues of carrot and tobacco after having less success with woody trees (first cultures with sycamore maple) for *in vitro* culture. In 1934, Kögl et al. discovered the auxin indole-3-acetic acid (Monnier, 1995), which turned out to be a crucial discovery that opened many doors. White, Gautherat, and Nobécourt separately found in 1939 that unlimited culture was possible with media supplemented with auxins (Radzan, 2003).

Throughout the mid to late 20th century, many advances were made in the field of plant tissue culture. Miller, Skoog, Van Saltza, and Strong (1955) were the first to isolate the cytokinin kinetin from yeast. Two years later, Skoog & Miller (1957), through experiments done with tobacco plants, proposed that the balance of cytokinin and auxin had a significant effect on the formation of organs (root, shoot, etc.) in culture. There were also important advances made in the nutrient media being used. The first bipolar somatic embryos in carrot cultures were reported by Steward and Reinhart independently of one another in 1958 (Thorpe, 2007). One of the most notable breakthroughs in this area occurred in 1962 when Murishige and Skoog developed a new media, with a notably high amount of nitrogen in it, for tobacco cultures (Murashige & Skoog, 1962). Finally, Vasil & Hildebrandt (1965) demonstrated the principle of totipotentcy through regeneration of a whole tobacco plant. More recently, Thorpe (1990) has classified five areas of applications of plant tissue culture

since the 1960s: cell behavior, plant modification and improvement, pathogen free plants and germplasm storage, clonal propagation, and product formation.

2.2. Plant Tissue Culture Media

Tissue culture medium is composed of macronutrients (Ca, K, Mg, N, P and S) micronutrients (B, Fe, Mn, Zn, Mo, Cu), vitamins, a carbon source (usually sucrose), plant growth regulators (PGRs), and sometimes with other additives (e. g. coconut milk, casein hydrolysate. Different classes, amounts, and combinations of PGRs have different functions. The three classes of PGRs used commonly in tissue culture are cytokinins, auxins, and gibberellins. Cytokinins are commonly used for cell division, shoot induction, and bud multiplication (Davies, 2010). Cytokinins include compounds such as 6-Benzylaminopurine (BAP), Thidiazuron (TDZ), and Kinetin (Kn). Auxins are commonly used for root induction, and cell division (especially in combination with a cytokinin) (Davies, 2010). Examples of commonly used auxins are Indole-3-butyric acid (IBA), α-Naphthalene acetic acid (NAA), and 2, 4-Dichlorophenoxyacetic acid (2, 4-D). Gibberellic acid (Gibberellins (GAs)) is important for seed germination, breaking seed dormancy and shoot elongation (Davies, 2010). The most commonly used gibberellin in plant tissue culture is $GA_{3.}$

2.3. Literature Review of Tissue Culture of *J. curcas*

There have been many articles using various tissue culture methods on various explants of *J. curcas.* Sujatha & Mukta (1996) found good callusing on media with different combinations of BAP and IBA (90%) from 3rd position leaves, leading to a protocol for successful indirect regeneration of *J. curcas*. They also reported direct adventitious shoot regeneration of *J. curcas* and used hypocotyl, leaf, and petiole explants. Sujatha et al. (2005) nodal explants for axillary shoot proliferation in addition to leaf explants for adventitious shoot regeneration. Jha & Mukherjee (2007) reported a successful protocol for somatic embryogenesis using

leaves from 7 month old *J. curcas* plants. Li, Li, Pan, & Wu (2008) reported an indirect regeneration protocol from *J. curcas* leaf explants. Deore and Johnson (2008) reported 80% rooting using full strength MS medium with 0.5 mg/L IBA and developed a protocol for direct shoot regeneration from leaf explants. Kumar et al. (2010a, b, c) has direct regeneration from leaf, petiole and cotyledonary petiole explants. Recently, Khemkladngoen, Cartagena, Shibagaki, & Fukui (2011) reported protocol for direct adventitious shoot regeneration from cotyledons.

3. Transformation

Genetic transformation is a change in the genetic make-up of a target organism through the introduction of foreign DNA into that organism. The two important prerequisites for developing an efficient transformation system are a) totipotent/meristematic cells capable of uptake of foreign DNA and b) regenerate the cells under *in vitro* conditions. A good *in vitro* regeneration protocol and a way to deliver the gene into the target species with high degree of integration of foreign DNA with that of the recipient organism are essential to generating transgenic plants efficiently. The two most common ways to genetically transform plant cell are *Agrobacterium*-mediated transformation (Schell, 1987) and particle bombardment (also known as Biolistics) (Klein et al., 1987). The focus of genetic engineering is to incorporate value-added traits like insect, pest, and disease resistance into crop plants (Thorpe, 2007).

3.1. *Agrobacterium*-mediated transformation

Agrobacterium-mediated transformation uses bacteria that are naturally present in soil to transfer a plasmid carrying the gene into the target plant (Schell, 1987). In nature, the bacteria cause crown gall tumors to form on wounded tissue of plants. The transferred DNA (T-DNA) is located on the tumor-inducing plasmid (Ti-plasmid), which is extrachromosomal (Stewart, 2008). However, scientists have disarmed the *Agrobacterium* by modifying and removing the

T-DNA from the Ti-plasmid, and having the modified T-DNA minus hormone and opine biosynthesis genes, which cause the tumors, and with the gene of interest and right and left borders, located on a second binary plasmid (Stewart, 2008). Numerous proteins are involved in the process of the Ti- plasmid going from the *Agrobacterium* to the nucleus of the plant, and eventually integrating into the DNA (Gelvin, 2010).

Agrobacterium in nature normally causes galls or tumors (Stewart, 2008). When the host plant is inoculated with the *Agrobacterium*, wounded tissues typically produce phenolic compounds similar to a synthetic compound known as Acetosyringone, which can also be added externally. This compound activates the *virulence (vir)* genes on the Ti-plasmid that are necessary for transferring the T-DNA from the bacteria to the nucleus of the plant cell and into its chromosomal DNA (Stewart, 2008). Tzfira & Citovsky (2006) describe the transformation as a 10 step process. First, the bacterium attaches to the plant cell, then next the activation of the *vir* genes. The VirD1/2 protein complex then produces the single stranded T-DNA that is to be sent to the host via the VirB/D4 type IV secretion system with other Vir proteins. Once it reaches the host, the T-DNA is coated with VirE2 proteins for protection and maintaining structure while making its way to the nucleus; at this point, it is called a mature T-complex. It is then escorted into the nucleus, de-coated, and integrated into the host's chromosomal DNA.

Agrobacterium-mediated transformation is the preferred method over Biolistic method because of its high efficiency and, unlike particle bombardment, it can integrate a single copy of the gene into a cell (Pan, Fu, & Zheng-Fu, 2010). Single copy transgenics are preferred to multiple copy transgenics since multiple copies of the introduced DNA in a cell could lead to issues like gene silencing (Muskens, Vissers, Mol, &, Kooter, 2000). In addition to transformation of nuclear DNA, some researchers have worked with chloroplast

DNA transformation using *Agrobacterium*-mediated transformation (Daniel, Khan, & Allison, 2008; Maliga, 2004).

3.2. Particle bombardment (Biolistics)

Particle bombardment is a physical method of genetic transformation. Gold or tungsten particles are coated with DNA, then shot at a high velocity towards target explants to penetrate the cell wall, allowing the DNA to enter then nucleus and integrate itself into the target cell's DNA (Klein, 1987; Stewart, 2008). The gene gun, the primary instrument in this process, was invented by Cornell University Professor John C. Sanford and is essentially a high pressure air gun connected to a vacuum pump. Once the plasmid-coated particles are loaded into the gun, a plate with the explants (callus, embryos, leaf) placed in the center of the plate is loaded into the gene gun chamber. The chamber then is vacuumed and the DNA with gold particles is shot on to the plant material.

Several parameters are tested to optimize and efficiently deliver DNA to plant material. These include pressure, distance, and concentration of the DNA. In addition, pretreatment of the explants on high glucose/mannitol (plasmolysis) medium could help the efficiency of transformation. A benefit of this method is that it is more tissue-independent than other methods since cells are being hit by separate plasmid-covered particles (Lazzeri, Alwine, & Horst, 1992). However, it is also more likely than *Agrobacterium*-mediated transformation to have multiple copies of the gene inserted into the cells, which could lead to gene silencing.

3.3. Protoplast

A protoplast is a plant cell whose cell wall has been removed or neutralized making the cell much easier to breach than a normal plant cell. Protoplasts are usually formed by either electroporation (high current pulses) or polyethylene glycol (PEG) treatment to create pores

in the cell wall (Jahne, Becker, & Lorz, 1995). Protoplasts can be manipulated through microinjection or *Agrobacterium*-mediated transformation (Stewart, 2008).

3.4. Electroporation

Electroporation of plant cells with intact cell wells is another physical method of transformation. D'Halluin, Bonne, Bossut, De Beuckeleer, & Leemans (1992) have successfully developed transgenic maize with this method.

3.5. Silicon Carbide Whiskers

Silicon Carbide Whisker mediated transformation is another less common method. In this process, very small whiskers are vortexed with plants cells and DNA. A drawback to this method is that it does not have a high efficiency (Stewart, 2008)

3.6. Transformation of *J. curcas*

Transformation of *J. curcas* has been reported in a number of recent publications. Both *Agrobacterium*-mediated transformation (Li et al., 2008) and transformation through particle bombardment (Purkayastha et al., 2010) have been reported. Trivedi et al. (2009) reported successful transformation using cotyledons and *in vitro* leaves using hygromycin selection. Mazumar, Basu**,** Paul**,** Mahanta, & Sahoo (2010) reported a significant different in success of *Agrobacterium*-mediated transformation of leaf explants based on age and orientation of the explants. Pan, Fu, & Xu (2010) reported *Agrobacterium*-mediated transformation regeneration of cotyledons using kanamycin selection.

4. Scorable and Screenable Markers

A scorable marker gene results in modification in the plant tissue that can easily be detected (fluorescence, staining, etc.). The scorable marker gene *GUS* (Jefferson, 1987) is used in the

transformation alongside a gene of interest (e.g. *CBF3*) to make it possible to see if the transformation worked. The *GUS* gene codes for the enzyme beta-glucuronidase that causes a blue staining on the transformed explants when exposed to a substrate (5-bromo-4-chloro-3-indolyl glucuronide, or X-Gluc, in this case)(Jefferson, 1987).

A screenable marker gene gives the transformed cell the ability to survive a specific environment that would kill untransformed cells. Examples of this would be genes that code for antibiotic or herbicide resistance. Both the scorable gene and gene of interest have a screenable marker gene on the plasmid so the bacteria with this plasmid can be selected for over bacteria in the culture that don't have the desired plasmid. In this case the *NPTII* selectable marker was used, which codes for resistance to the antibiotic kanamycin. As a result, when kanamycin is added to the culture medium it will select for the bacteria containing the desired plasmid with the gene of interest or scorable gene.

5. Molecular Analyses of Putative Transgenics

Performing molecular analysis on putative transgenics is the final gold standard for confirming the integration of the gene. In addition to selectable markers and reporter genes, molecular analyses of the putative transgenics also need to be done to prove that the gene is actually integrated in the genome. Two tests that are commonly used are polymerase chain reaction (PCR) and Southern blot. PCR is meant to amplify DNA. The PCR product then has a gel electrophoresis run on it to see if the gene of interest's band shows up on the gel. A Southern blot is essential in any molecular analyses of potential transgenics. It shows not only the presence of the gene in the DNA sequence, but also the number of copies of the gene present in the target organism. Therefore, running a Southern blot will confirm whether or not a desired, single copy of transgenic is present. A recently developed analytical tool, reverse transcriptase PCR (RT-PCR) can also be used to analyze putative transgenic plants.

Reverse transcriptase requires only a small amount of RNA to generate cDNA, which can then be amplified through PCR making it possible to measure the amount of gene expression from those small amounts of DNA (Freeman, Walker, & Vrana, 1999).

6. Goal and Objectives

The goal of this work is to develop an efficient method of regeneration and optimization of transformation system for *J. curcas*. Two objectives were set to achieve the goal: (1) Develop an efficient protocol for *in vitro* regeneration of *J. curcas* from various explants and (2) Optimize *Agrobacterium*-mediated transformation for *J. curcas*.

CHAPTER 2

MATERIALS AND METHODS

1. Plant material

Plant material used in this study was *Jatropha curcas* L. young, fully developed leaves and shoot apices as well as tender nodal segments collected from (two to three year old) greenhouse grown plants. *In vitro* grown *Jatropha* seedlings and *Jatropha* embryos were also used for some experiments. We received seeds from Nandan Biomatrix (Hyderabad, India), Freedom Energy, and Chennai Research Foundation (Chennai, India).

2.1. Surface Sterilization of explants

Leaves (fully opened young leaves, first to third position from the tip) and young shoot tips were washed with detergent (Tween20®) for 10 minutes, rinsed with purified water (RO pure), and transferred to a sterile flask in the laminar flow hood. Further treatments were carried out in the hood. The cleaned plant materials were treated with 70% ethanol for one minute, rinsed with sterile water three to four times, and further soaked in 0.1% (w/v) $HgCl_2$ for five minutes and rinsed with sterile water five to six times. Approximately one cm^2 leaf segments and stem segments containing one to two nodes (two to three cm) were excised and used as explants for tissue culture.

For surface sterilization of the seeds, the seed coat was first removed with a nutcracker, and then the seeds were washed with Tween20® for 10 minutes and rinsed with RO pure water before transferring to the laminar flow hood. The clean seeds were treated with 10% (v/v) Chlorox® bleach containing 6% of sodium hypochlorite for five minutes, and rinsed with sterile water three times. To complete the surface sterilization process, the seeds

were soaked in 0.1% (w/v) $HgCl_2$ for five minutes and rinsed five times with sterile water. After sterilization, the soft outer seed coat was removed.

2. Chemicals and supplies

Tissue culture media (e.g. MS Medium with vitamins, MS medium with B5 vitamins) and plant hormones (e. g. Kinetin, Zeatin) were purchased from the Phytotechnology Inc., Overland Park, KS, USA. Plastic ware and other chemicals (analytical grade) were obtained from VWR Scientific, Radnor, PA, USA.

3. Tissue Culture

Surface sterilized leaf explants (one cm^2 leaf segments) were placed on *Jatropha* callus medium (JCM), which composed of full strength (4.43 g/L) Murishige and Skoog medium (Murashige and Skoog, 1962) with vitamins, supplemented with 1.5 mg/L BAP, 0.05 mg/L IBA, 30 g/L sucrose, and 7 g/L agar, adjusted to a pH of 5.8 (Li, Li, Pan, & Wu, 2008). The leaves were moved to *Jatropha* shoot regeneration media (JSR) after calli were developed (usually 4-6 weeks). JSR medium was comprised of full strength MS w/ vitamins supplemented with 1.5 mg/L BAP, 0.05 mg/L IBA, 0.5 mg/L GA_3 (filter sterilized and added after autoclaving), 30 g/L sucrose, and pH was adjusted to 5.8 before adding 7 g/ L agar.

Seed germination was achieved on filter paper soaked in sterile water or on nutrient medium. The sterilized seeds were cut in half and the embryo was isolated from the rest of the seed. Embryos were put on various media, known as *Jatropha* Embryo Germination (JEG) media, with ½ strength MS with vitamins, 20 g/L sucrose, pH of 5.8 and 6.5 g/L of agar, alone or in combination with Kinetin (Kn), GA_3, or IBA (Table 2.1).

Table 2.1: Composition Tissue Culture Media

Media	Components	Purpose
JCM	MS medium w/ 1.5 mg/L BAP, 0.05 mg/L IBA, 30 g/L Sucrose	Callus Induction
JEG1	½ MS medium w/ 20 g/L Sucrose	Embryo Germination
JEG2	½ MS medium w/ 0.1 mg/L Kn, 20 g/L Sucrose	Embryo Germination
JEG3	½ MS medium w/ 0.5 mg/L Kn, 20 g/L Sucrose	Embryo Germination
JEG4	½ MS medium w/ 0.5 mg/L Kn, 0.5 mg/L GA_3, 20 g/L Sucrose	Embryo Germination
JEG5	½ MS medium w/ 0.5 mg/L Kn, 0.1 mg/L IBA, 20 g/L Sucrose	Embryo Germination
JTC	MS medium w/ 5.0 mg/L BAP, 0.5 mg/L, 30 g/L Sucrose	Shoot Induction
JSM1	MS medium w/ 1.0 mg/L BAP, 0.5 mg/L Kn, 30 g/L Sucrose	Shoot Proliferation
JSM2	MS medium w/ 2.0 mg/L BAP, 0.5 mg/L Kn, 30 g/L Sucrose	Shoot Proliferation
JSE3	MS medium w/ 0.5 mg/L BAP, 0.5 mg/L Kn, 2 mg/L GA_3, 30 g/L Sucrose	Shoot Elongation
JSE4	MS medium w/ 1.0 mg/L BAP, 0.5 mg/L Kn, 2 mg/L GA_3, 30 g/L Sucrose	Shoot Elongation
JRM1	½ MS medium w/ 1X B5 vitamins, 0.1 mg/L NAA, 0.1 mg/L IBA, 20 g/L Sucrose	Rooting
JRM2	½ MS medium w/ 1X B5 vitamins, 0.1 mg/L NAA, 0.2 mg/L IBA, 20 g/L Sucrose	Rooting
JRM3	½ MS medium w/ 1X B5 vitamins, 0.1 mg/L NAA, 0.3 mg/L IBA, 20 g/L Sucrose	Rooting
JRM4	½ MS medium w/ 1X B5 vitamins, 0.1 mg/L NAA, 0.5 mg/L IBA, 20 g/L Sucrose	Rooting

Above are the compositions and functions of the different tissue culture media.

Once germinated, the young shoots were transferred to *Jatropha* shoot multiplication media (JSM 1 and JSM 2) or *Jatropha* tissue culture media (JTC). To attain shoot elongation, excised shoots were transferred to *Jatropha* shoot elongation (JSE) medium.

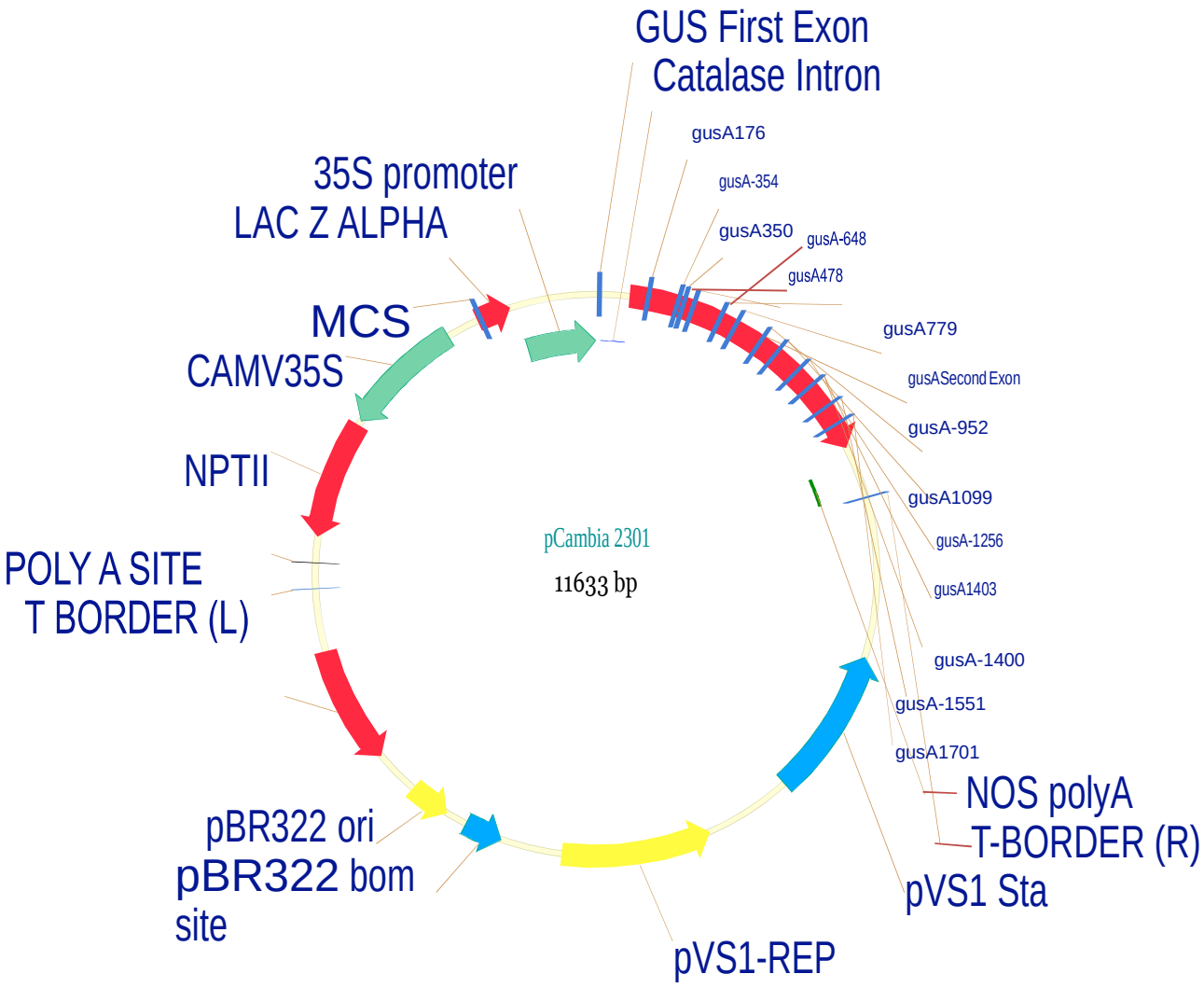
Elongated shoots were rooted in *Jatropha* rooting media (JRM). The rooted plants were transplanted into four inch pots filled with peat moss and moved to a climate controlled greenhouse (≥ 90%, 27±3°C and 25% shading) for acclimatization. The pots were covered with clear plastic bag for 7-10 days to keep high levels of humidity (≥ 90%) during the acclimation period.

4. *Agrobacterium*-mediated transformation

The *Agrobacterium* strain GV3101 containing plasmid pCambia 2301 carrying the *GUS* gene (Figure 2.1) was grown in LB medium (Bertini, 1951) supplemented with 50 mg/L Kanamycin overnight to achieve an optical density of 0.6-0.8. Once ready (reached OD of 0.6-0.8) the desired amount of culture was centrifuged at 4000 rpm for 15 minutes at 25°C

and the supernatant was removed. The pellet was suspended in simplified induction media (SIM), containing MS w/ vitamins + 30 g/L sucrose+ 20 mM sodium citrate (filter sterilized, added after autoclave) + 200 µM Acetosyringone (filter sterilized, added after autoclave) at a pH of 5.5 (Alt-Morbe et al, 1989), to an optical density of 0.6 to 0.7. The culture was then put in a shaker at 250 rpm at 28°C for 4 hours. After the incubation period is over, the *J. curcas* seeds were cut in half to expose the embryo as well as the cotyledons and submerged in the *Agrobacterium* solution for 20-30 minutes. The explants were blot dried on sterile filter paper to remove excess *Agrobacterium.* The *Agrobacterium* treated explants were inoculated on to co-cultivation medium (MS w/ vitamins+ 2.0 mg/L TDZ+ 2.0 mg/L IBA+ 40 mg/L sucrose+ 8.0 g/L of agar+ 100 µM Acetosyringone (filter sterilized, added to autoclaved medium maintained at 50-60℃, pH 5.7)) (Yancheva et al., 2005). The inoculated Petri dishes were incubated for 3 days in the dark at 25°C.

Figure 2.1: pCambia 2301 *GUS* Plasmid



The pCambia 2301 is a plasmid contains the *GUS* reporter gene with the *NPTII* selectable marker and a constitutive 35S promoter.

After three days, one to three explants from each type of explant (embryo, cotyledon, etc.) were transformed with *GUS* and moved to *GUS* staining solution after 72 hrs. The *GUS* staining solution was composed of the substrate X-gluc. The explants were incubated in the X-gluc solution in the dark at 37°C for 24 hours before they were microscopically examined for *GUS* expression. The remainder of the explants (embryo, leaf and cotyledon) putatively transformed with *GUS* gene were moved to JEG5D medium (½ MS w/ vitamins+ 0.5 Kn+ 0.1 IBA+ 20 g/L sucrose+ 6.5 g/L agar) supplemented with 500 mg/L Cefotaxime and 250 mg/L Carbencillin to kill excess *Agrobacterium* and maintained in cool white light for a 16 hour photoperiod. The remainder of the cotyledonary leaves and cotyledons were moved to Jatropha Callus Induction (JCM) containing 500 mg/L Cefotaxime and 250 mg/L Carbencillin and incubated in the dark at 25°C. All explants moved to JCM or JEG5D media were first washed with liquid MS w/ vitamins+ 500 mg/L Cefotaxime and blot dried to remove excess *Agrobacterium*.

These cultures were maintained on respective media to regenerate shoots directly or indirectly through a callus phase. The cultures were monitored every two days for *Agrobacterium* growth. Excessive growth of *Agrobacterium* was controlled by washing the cultures with liquid MS medium containing 500 mg/L Cefotaxime and 250 mg/L Carbencillin, followed by blot drying before transferring to fresh media. Once there was no regrowth of excess *Agrobacterium*, the cultures were removed from the Carbencillin/Cefotaxime medium.

5. Molecular Analysis

Molecular analysis is performed to prove the integration of the gene of interest in *Jatropha*. A PCR assay was performed to confirm the presence of the gene on new leaves and shoots from the putative transgenic plants.

6. Analysis of data

Raw data was tabulated and standard deviation was calculated to compare data to make scientific conclusions. Single factor ANOVA and two-tailed T-tests with a confidence interval of 95% were run to compare data as well.

Chapter 3

RESULTS AND DISCUSSION

1. Tissue Culture

1.1. Embryo Germination

Isolated mature embryos cultured on media JEG1-5 germinated at higher rates (≥90%) than embryos incubated on moist filter paper (JEG6). In the greenhouse experiments seeds planted after removing the seed coat (JEG7) germinated at higher rates than entire seeds (JEG8) planted in soil. There was no significant difference in the germination rate when the embryos were cultured in media JEG1-4 (Table 3.1 and 3.2). However, embryos planted in JEG1-5 media germinated at significantly higher rates compared to the seeds planted on moist filter paper or in the soil in greenhouse. There was a significant difference in germination between the different treatments $F(7, 402) = 34.87$, $p = 5.97\text{x}10^{-38}$ using the ANOVA single factor test. These results indicate that (1) the hard seed coat is a barrier for germination of *Jatropha* seeds (2) the enriched medium (JEG1-5) assisted seed germination and (3) added cytokinins in the medium did not have a significant effect on seed germination (Figure 3.1).

Table 3.1: Seed Germination Rates of *J. curcas* NBM

Treatment	# of Seeds	# Seeds Germinated	% Germination
JEG1	68	67	$98.53 \pm 6.25^{a,b}$
JEG2	68	68	100.00 ± 0.00^{a}
JEG3	68	67	$98.53 \pm 6.06^{a,b}$
JEG4	68	67	$98.53 \pm 6.06^{a,b}$
JEG5	68	63	92.65 ± 11.74^{b}
JEG6	30	20	66.67^{c}
JEG 7	20	10	$50.00^{c,d}$
JEG8	20	6	30.00^{d}

Table 2 shows the germination rates of *J. curcas* variety NBM planted in different media (JEG1-5) with SD, on moist filter paper (JEG6), seeds planted in soil after removing the seed coat (JEG7) and entire seeds planted in soil (JEG8). ANOVA and 2-tailed student's T-test were used to determine significance.

Table 3.2: Seed Germination Rates of *J. curcas* variety MC

Treatment	# of Seeds	# Seeds Germinated	% Germination
JEG1	50	44	88.00 ± 1.30^{a}
JEG2	50	46	92.00 ± 0.45^{a}
JEG3	50	43	86.00 ± 2.17^{a}
JEG4	50	47	94.00 ± 0.55^{a}
JEG5	50	46	92.00 ± 0.84^{a}
JEG6	50	25	50.00^{b}
JEG7	281	168	59.79^{b}
JEG8	20	2	10.00^{c}

Table 3 shows the seed germination rates of *J. curcas* variety MC on media JEG1-5, on moist filter paper (JEG6), seeds planted in soil after removing the seed coat (JEG7) and entire seeds planted in soil (JEG8). ANOVA and 2-tailed student's T-test were used to determine significance.

Figure 3.1: Comparative rates of seed Germination for *J. curcas* varieties NBM and MC

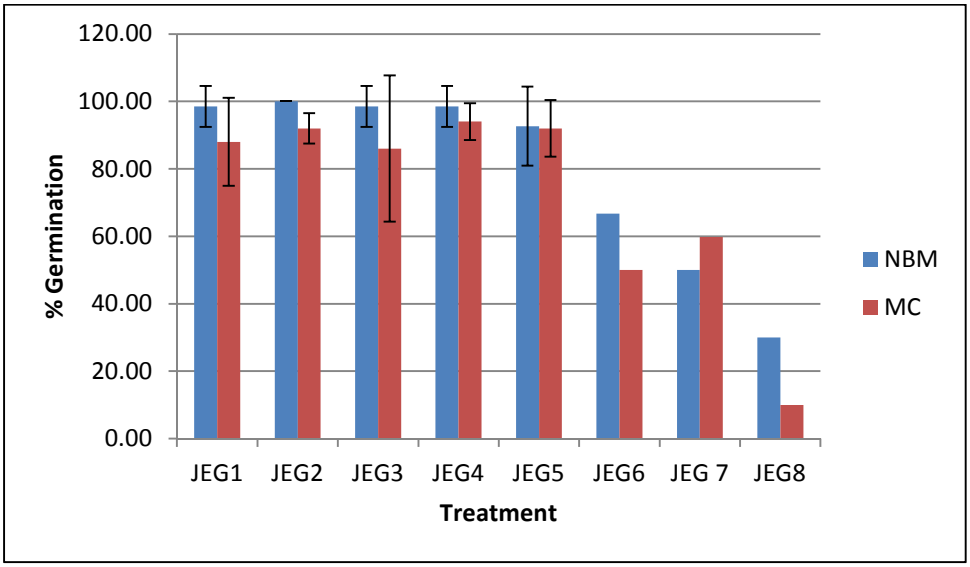


Figure 3.1 compares the effect of different media on germination rates of *J. curcas* variety NBM and MC.

Islam, Anuar, & Yaakob (2009) investigated three different pretreatments and how they affect germination rate and speed of germination in 20 genotypes of *J. curcas*. They found that keeping seeds moistened under sand stone resulted in higher and faster germination rate (95.85%) than keeping seeds on moistened filter paper in a petri dish (84.24%) or directly planting the seeds in the soil (77.17%) for germination. They also found that seeds directly planted in soil germinated at a slower pace than the seeds incubated on moist filter paper.

The objective of this study was to increase the rate and speed of seed germination to generate an efficient and commercially-viable way to rapidly produce *J. curcas* plants in a relatively short time frame. Traditional planting of *J. curcas* seeds results is a wide variation in germination rates due to seed dormancy. As a result, it is necessary to devise physical and chemicals treatments, and to use plant tissue culture techniques, to raise those germination rates (Islam et al., 2009). The embryos could also be genetically transformed and quickly regenerated. The application from this study is if one does not have plant tissue culture media or other biotechnology means at ones disposal then seed germination could be enhanced simply taking

off the hard seed coat before planting in soil or by soaking seeds in water before incubating on moist filter paper until the seed starts to germinate. Another factor that played a key role in the success of the study was the quality of the seed material. Initially seeds from Freedom Energy were older and, as a result, few of the seeds germinated. Moreover, over 90% of the seeds placed on media would become badly contaminated with fungus, even with applications of the fungicide Bavastin. However, upon receiving fresh seeds from Nandan Biomatrix (NBM) and the Chennai Research Foundation (MC) germination rates as shown above were very high in the growth media (Figure 3.2).

Figure 3.2: *J. curcas* Embryo Germination *in vitro*

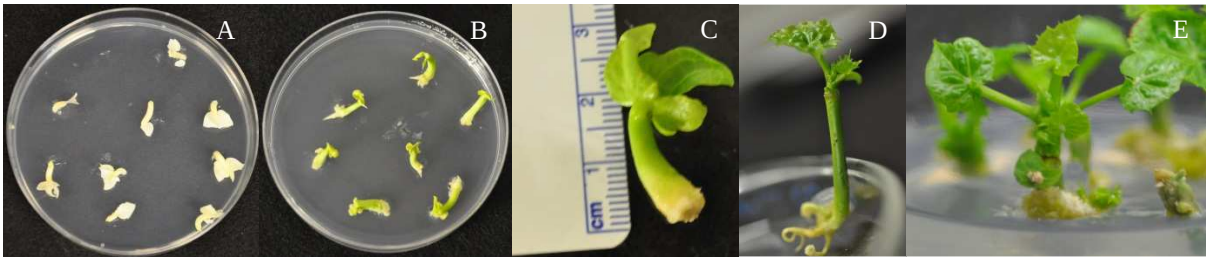


Figure3.2 shows developmental stages of *J. curcas* var. NBM embryos cultured *in vitro*. A. Freshly isolated embryos cultured on the JEG5 medium (Day 0) (B) germinated and greening embryos (Day 5) (C) small shoot and cotyledonary leaf development (Day 15) (D) shoot elongation and primary leaf development (Day 24) (E) Further growth and development of shoot in JEG medium (Day 40).

1.2.Callus culture

Young leaves from 10 genotypes of *J. curcas* were used to induce calli and study the effect of different *Jatropha* genotypes on callus induction (Figure 3.4). Ten genotypes were examined with one genotype representing one *J. curcas* tree in our greenhouse at Penn State Harrisburg. Callus induction rates seemed to vary significantly ranging from 32% (genotype 8) to over 96% (genotype 2). Genotypes 2, 7, and 9 all have average induction rates of 90% or higher; 2 and 7 are the most likely to have a very high callus response due to their relatively small standard deviation in this set (Table 3.3 and Figure 3.3). The other fairly safe conclusion

that can be made from this study is that genotype 8 has the lowest rates of callusing response and the response was inconsistent.

Table 3.3: Comparison of Callus Induction Rates by *J. curcas* Genotypes

Genotype #	# of Explants	# Explants callusing	% Explants callusing
1	24	16	66.67 ± 43.78
2	28	27	96.43 ± 10.21
3	32	21	65.63 ± 48.07
4	32	26	81.25 ± 36.44
5	36	32	88.89 ± 17.85
6	20	13	65.00 ± 37.91
7	16	15	93.75 ± 12.50
8	25	8	32.00 ± 32.51
9	20	18	90.00 ± 22.36
10	31	25	80.65 ± 22.44

Table 3.3 shows callus induction rates for leaf explants of 10 selected genotypes of *J. curcas* grown in the greenhouse at Penn State Harrisburg. Data presented are average ± SD collected after 4 weeks incubation in CI1.

Figure 3.3: Callus Regeneration for 10 Selected *J. curcas* Genotypes

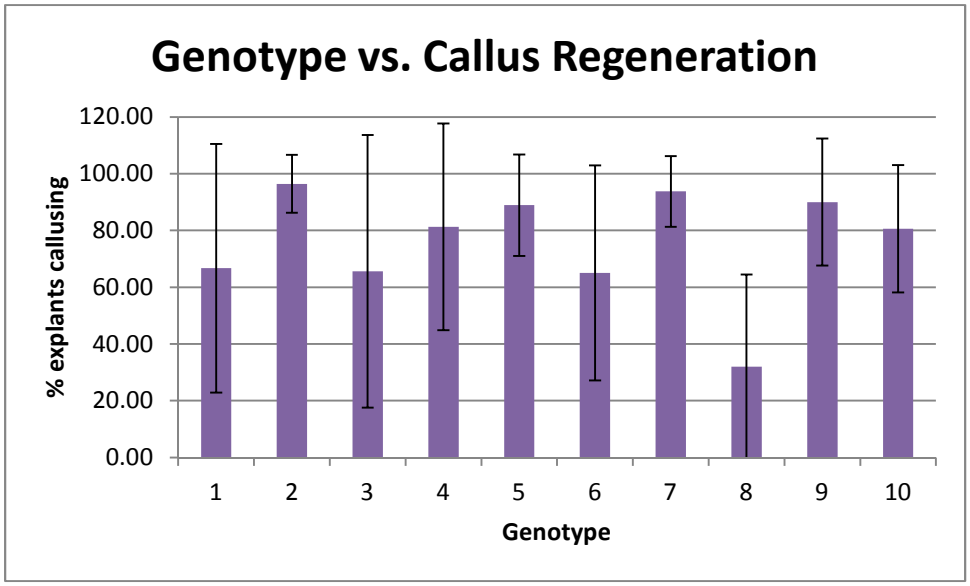


Figure 3.3 shows the callusing responses of leaf explants from 10 *J. curcas* genotypes after four weeks of incubation on CI1 medium. Data presented are average ± SD after 4 weeks in culture.

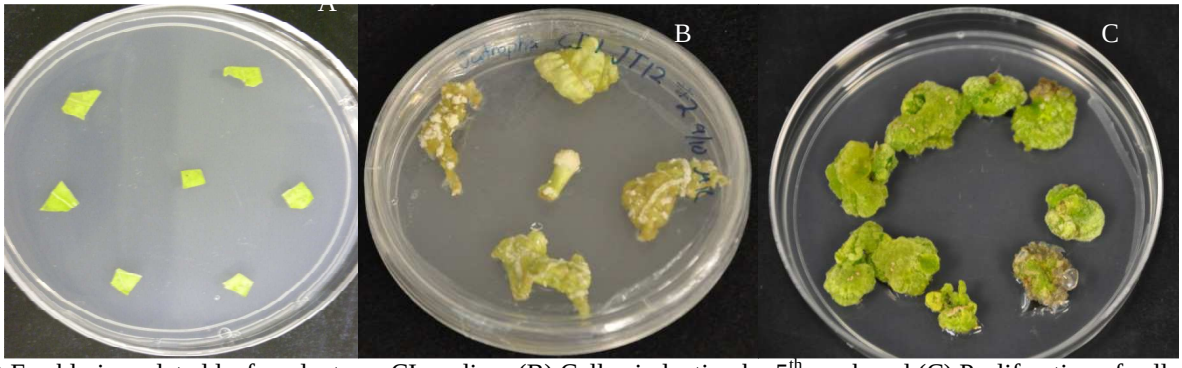
There have been other studies on callus induction in *J. curcas* from different explants. Rajore and Batra (2007) described the morphological characteristics of calli formed by certain plant growth regulators. They claimed NAA produced better, regenerable callus than either IAA or IBA, and that the best media for callus induction contains 1.0 mg/L NAA and 5 mg/L BAP. However Li et al. (2008) claimed that 1.5 mg/L BAP and 0.5 mg/L IBA was the optimal combination for callus induction. Sujatha and Mukta (1995) investigated various methods of regeneration from hypocotyls, leaf, and petiole explants using different media. For callus induction, they found a high response of callus induction from various combinations of BAP and IBA.

Studying genotypic differences in certain species is a very important parameter that needs to be taken into account when working in tissue culture and transformation. While often overlooked, it is always important to remember that just because two individuals are part of the same species, variety, or even population, they are not genetically completely identical. Different plants of the same species will respond differently to a given environment or treatment. Knowing which plants are particularly easily regenerated or transformed can allow us to focus on those genotypes, making the transformation and propagation processes more efficient. Different genotypes may also have different traits from one another that may or may not be desirable, such as oil content, growth, germination, and toxicity (Ginwal 2005; Kaushik 2007; Rao 2008). Genotype variation is commonly known in tissue culture and especially so with *J. curcas* since very little breeding studies have been done.

While this study led to the above mentioned conclusions, it could definitely be improved and extended upon. More genotypes could be included; also a tissue culture study like this could be combined with transformation studies to see not only which genotype regenerates the best, but

also which genotype is the most responsive to genetic transformation. This information could lead to finding a genotype that regenerates and transforms relatively easily, increasing the chances of getting a transgenic plant. Another expansion of this work would be to increase the number of replicates, which could neutralize the high variability in response as indicated by the standard deviation in the data presented. Lastly, more media could be tested for the rate of callus induction and the most regenerable callus formed.

Figure 3.4: Callus Induction from leaf explants of *J. curcas*



(A) Freshly inoculated leaf explants on CI medium (B) Callus induction by 5th week and (C) Proliferation of callus by 7th week.

1.3. Shoot Induction, Proliferation, and Elongation

Shoot induction response of cultured nodal explants from 10 different genotypes of *J. curcas* was compared after two weeks incubation on culture medium. It was observed that, regardless of the genotype, shoot induction took place within two weeks (Figure 3.6). Genotype 7 exhibited low percentage shoot induction (40%) but more than 80% of the explants of other nine genotypes showed shoot induction within two weeks. All the explants belonging to genotypes 2, 3, 4, 9 and 10 showed shoot induction within two weeks of culturing (Table 3.4 and Figure 3.5). The lack of variability of the data set could also be an indicator that this particular media and its response are not significantly affected by the genotype.

Table 3.4: Comparison of Shoot Induction Rates by Genotype

Genotype	# of Explants	# Explants shooting	% Shooting
1	10	8	80 ± 0
2	10	10	100 ± 0
3	10	10	100 ± 0
4	10	10	100 ± 0
5	10	9	90 ± 14.14
6	9	9	100 ± 0
7	10	4	40 ± 28.28
8	10	9	90 ± 14.14
9	10	10	100 ± 0
10	10	10	100 ± 0

Table 5 shows rate of shoot induction from nodal segments of 10 genotypes of *J. curcas*. Data presented is the average ± SD. Data was collected after two weeks incubation on JTC medium.

Figure 3.5: Shoot Induction Response of 10 Selected *J. curcas* Genotypes

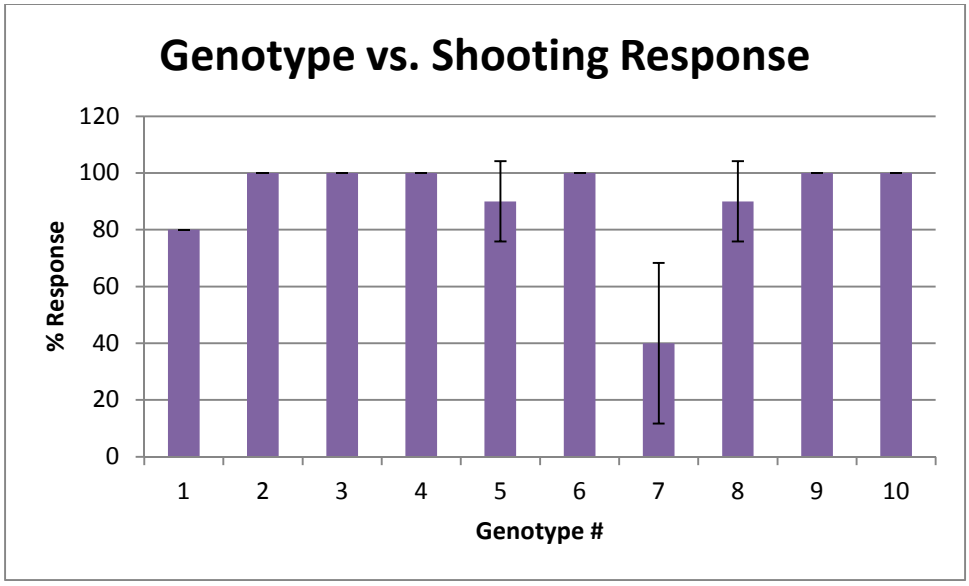


Figure 3.5 shows the shoot induction response by nodal segments of ten genotypes of *J. Curcas* incubated on JTC media for two weeks. Data presented is the average ± SD.

There have been several reports of shoot induction using different explants. Deore and Johnson (2008) reported an optimal direct shoot bud induction from leaf explants using MS medium with 2.27 µM TDZ, 2.22 µM BAP, and 0.49 IBAµM of 53.5%. Kumar et al. (2010 a, b, c) investigated direct shoot induction of cotyledonary leaf and petiole explants and the effect of several variables on shoot induction. They claim that the optimal medium for shoot induction

with petiole and leaf explants is MS medium with 2.27 µM TDZ, where they report induction rates of 59.11% for cotyledonary petiole explants and 58.35% for petiole explants from *in vitro* grown plants. They also reported significant differences in shoot induction rates based on orientation of the explant, explant source, and plant genotype. Recently, Khemkladngoen, Cartagena, Shibagaki, & Fukui (2011) have reported a protocol for direct adventitious shoot regeneration from cotyledons and the effect of TDZ on shoot regeneration. They found the best direct shoot regeneration among media with TDZ from MS basal medium with 1.0 mg/L BAP, 0.1 mg/L IBA, 0.5 mg/L TDZ (78.42%) and the best without TDZ from MS basal medium with 3.0 mg/L BAP and 0.1 mg/L IBA (77.63%); these values were not significantly different. Another observation they made was that shoots from media without TDZ elongated much better than media with TDZ. In fact, they report that explants from media with high TDZ or BAP do not elongate well.

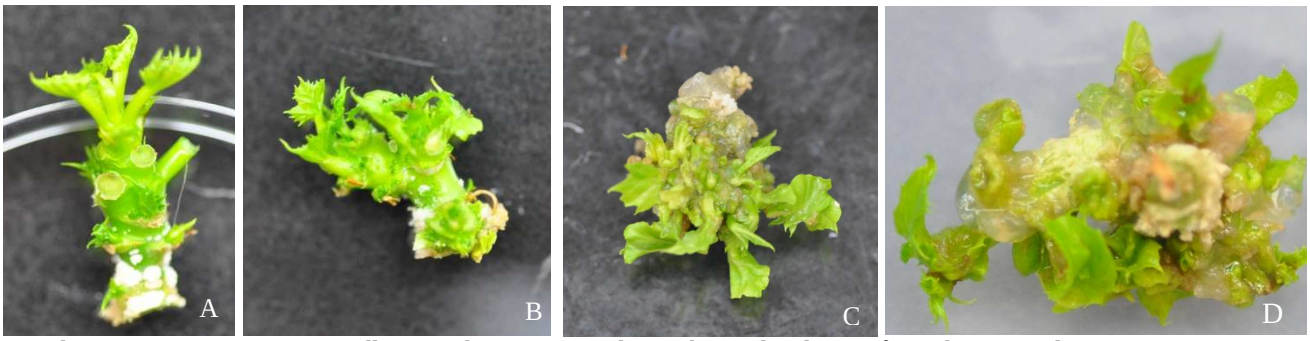
J. curcas nodal segment explants from different genotypes also behaved differently in direct shoot induction experiments. The objective of this study was to study the response of genotype to the nodal explants. Nodal explants allows for rapidly developing an efficient, genetically stable process for developing many genetically similar plants in a short time that could potentially lead to commercial viability (Sujatha et al., 2005). Future work will include expanding the number of genotypes and including accessions from different locations around the world. The sample size and replicates could also be increased to gain more certainty about how the genotypes compare. These changes would help further explore any genotypic differences in regeneration from genotype to genotype.

Figure 3.6: Shoot induction from cultured shoot explants from Mature *J. curcas* trees

(A) Cultured shoot apex (Day 0), (B) Budbreak and formation of multiple shoots (C) Further development of the shoot and formation of many axillary shoots.

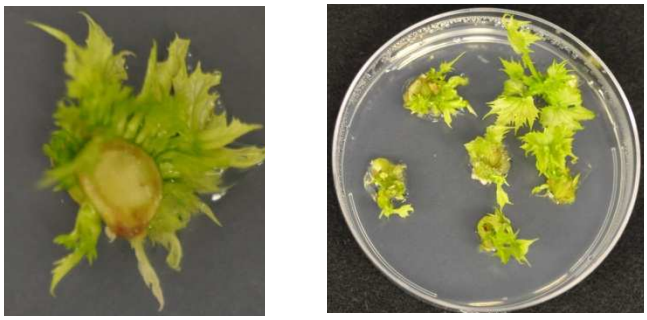
High shoot proliferation, both from the epicotyl and hypocotyl regions *in vitro J. curcas* seedlings was observed after 6-8 weeks in JSM2 media (Figure 3.7). Responding explants averaged 10.93±5.43 shoots per explant. High levels of shoot elongation have been observed on both JSE3 and JSE4 media (Figure 3.8) over two weeks. Sujatha et al. (2005) observed the number of shoots per explants initially on media with different cytokinins after 12 weeks of culture of axillary buds. They reported that buds initially on Kinetin had 4 shoots per explant, buds initially on BAP had 10 shoots per explant, and buds initially on TDZ had 24 shoot per explant.

Figure 3.7: Multiple Shoot Proliferation from Epicotyl and Hypocotyl



A and B are *in vitro J. curcas* seedlings with approximately 10 shoots developing from the epicotyl region, particularly near the nodes of the explants after 45 days. C and D are explants from the hypocotyl of *in vitro* seedlings after 6 weeks with 10-15 shoots developing.

Figure 3.8: Shoot Elongation



Shoot elongation after 2 weeks on JSE3 and JSE4

1.4. Rooting and Acclimatization

Explants with established shoots were moved to the four different rooting media, and their responses are represented in Table 3.5 and Figures 3.9, 3.10, and 3.11. Two parameters studied in this experiment were (1) percent of explants that were rooting and (2) average number of roots on those explants. Medium JRM1 was found to have the highest percentage of explants rooting; however, it produced least number of roots per shoot. An interesting trend noticed here is that the percent rooting and number of roots produced per shoot are inversely correlated. Furthermore, the two media with lower concentration of IBA (JRM1 and 2) caused higher percent of number of shoots rooting and less number of roots per explant, while the two medias with higher concentration of IBA regenerated more roots per shoot, but a lower percent of the shoots rooted in these media. This correlation is quite evident from JRM1 to JRM2 and JRM2 to JRM3, but not evident at all from JRM3 to JRM4 potentially suggesting that a slight change in the amount of IBA at low levels (at or below 0.3 mg/L) can affect the rooting response of *J. curcas* shoots.

Table 3.5: Rooting Responses of Different Media

Media	# of shoots	# shoots rooting	% Rooting	Total # roots	Mean # of Roots/shoot
JRM1	15	14	93.33±0.58	51	3.64±2.10
JRM2	**46**	**37**	**80.43±1.14**	**164**	**4.43±2.64**
JRM3	48	33	68.75±0.98	160	4.85±2.93
JRM4	13	9	69.23±0.58	43	4.78±2.67

Table 3.6 describes rooting response of *J. curcas* cultured on different rooting media for one month.

Figure 3.9: Effect of media on *in vitro* rooting of *J. curcas* shoots

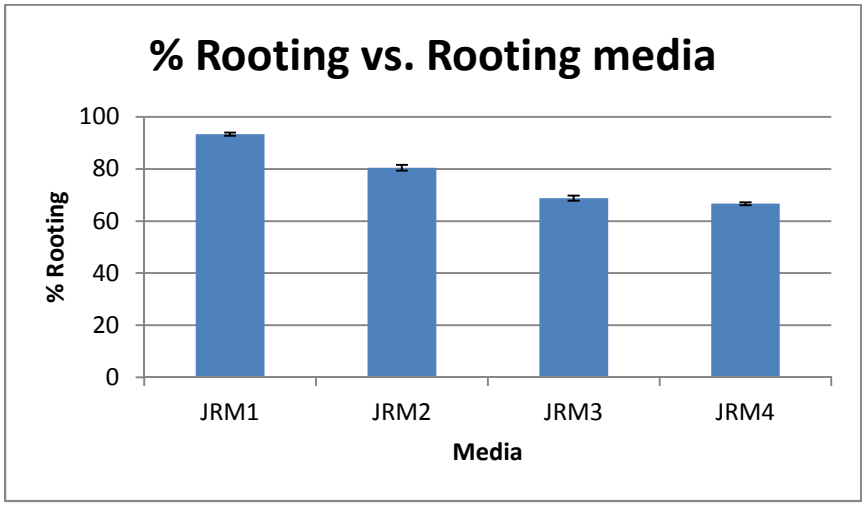


Figure 3.7 is a graphic representation of the percent of explants that were successfully rooted *in vitro* on different media after one month incubation in rooting media.

Figure 3.10: Roots/Explant Rates

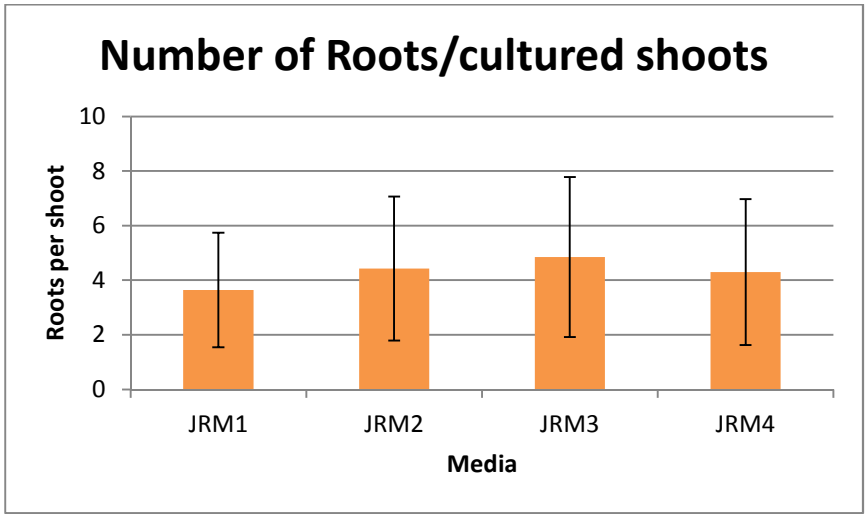


Figure 3.8 shows average number of roots produced per cultured shoots on different rooting media. Data presented is the mean number of roots per shoot.

Earlier acclimatization experiments took place in an off-campus greenhouse with no controls for humidity, light, and other conditions that could possibly have an adverse effect on the experiment. The best percentage of plants surviving acclimatization achieved in experiment was 38% (8 of 21 plantlets). Recently, a new on campus greenhouse facility became available with all the necessary environmental controls to assure a better environment for acclimatizing plants. In a recent acclimatizing experiment, we also used seed starter mixture (Miracle Gro®) instead of peat moss and received a better survival rate of 70% (7 of 10 plantlets) was achieved.

Sujatha and Mukta (1996) found that full strength hormone-free MS medium was best for rooting for *J. curcas* (88% in 8-10 days). For acclimatization, they claimed over 80% success using vermiculite and watering with IBA solution. Deore and Johnson (2008) reported 80% rooting using full strength MS medium with 0.5 mg/L IBA, 80% survival rate for acclimatization following (Bavastin wash and transfer to 1:1 cocopeat: garden soil mixture, covered by a bag for three to four weeks). Li et al. (2008) reported 86% rooting in half strength MS medium supplemented with 0.3 mg/L IBA.

This experiment could be expanded upon by including more media with different concentrations and combinations of auxins to try to increase the percentage of explants rooting. Acclimatization could be attempted using different soil mixes instead of just using one soil. Different conditions in the greenhouse (potting mixes, temperature, humidity, light quality and quantity and photoperiod) could also be tested during acclimatization.

Figure 3.11: Rooting and Acclimatization

A and B. Rooted shoots in medium JRM3 C. Rooted plants transferred to pots filled with peatmoss for acclimation D. 21 days old acclimated plant.

2. Optimization of Transformation

2.1. *GUS* Expression

J. curcas embryos, young leaves, and cotyledonary leaves were treated with the *Agrobacterium* strain GV3101 carrying the *GUS* containing pCambia 2301 plasmid with a 35S constitutive promoter and *NPTII* screenable marker gene coding for kanamycin resistance. *Agrobacterium*-mediated transformation experiments using *GUS* as marker gene was tested in a variety of different explants including embryos, cotyledon/cotyledonary leaves, and young leaf tissue (Figure 3.12). Of the 16 experiments with young leaves, 13 experiments had at least one *GUS* positive explant (Table 3.6). All 19 experiments testing embryos had at least one *GUS* positive explant (Table 3.7). Eight experiments that included cotyledon and cotyledonary leaf explants showed *GUS* positive explants.

Table 3.7: *GUS* Expression of Putatively Transformed Leaf Explants

Experiment	# of Explants	Plasmid	Explant type	Percent w/ GUS		Avg .GUS spots
				# w/ GUS	%	
JAT-1	4	26	leaf	0	0.00	0.00
JAT-1	2	26	leaf	0	0.00	0.00
JAT-2	3	26	leaf	2	66.67	2.00
JAT-3	3	26	leaf	2	66.67	19.00
JAT-4	3	26	leaf	3	100.00	23.33
JAT-5	3	26	leaf	1	33.33	40.00
JAT-6	3	26	leaf	0	0.00	0.00
JAT-7	3	26	leaf	2	66.67	7.67
JAT-8	3	26	leaf	0	0.00	0.00
JAT-24	1	26	leaf	1	100.00	210.00
JAT-25	2	26	leaf	0	0.00	0.00
JAT-26	2	26	leaf	2	100.00	12.50
JAT-27	2	26	leaf	1	50.00	8.00
JAT-28	2	26	leaf	2	100.00	40.50
JAT-29	2	26	leaf	2	100.00	11.00
JAT 9/12	15	26-2	leaf	9	60.00	39.33
JAT 9/12	15	26-1	leaf	11	73.33	82.55
JAT-32	2	26	leaf	2	100.00	33.50

Table 3.7 shows % explants with *GUS* expression and average number of *GUS* spots in transformation experiments with leaves.

Table 3.8: *GUS* Expression of Putatively Transgenic Embryos

Experiment	# of Explants	Plasmid	Explant	Percent w/ GUS	
JAT-8	3	26	embryo	3	100.00
JAT-10	2	26	embryo	2	100.00
JAT-11	3	26	embryo	3	100.00
JAT-12	2	26	embryo	2	100.00
JAT-13	3	26	embryo	2	66.67
JAT-14	2	26	embryo	2	100.00
JAT-15	1	26	embryo	1	100.00
JAT-16	1	26	embryo	1	100.00
JAT-17	1	26	embryo	1	100.00
JAT-18	1	26	embryo	1	100.00
JAT-19	1	26	embryo	1	100.00
JAT-20	1	26	embryo	1	100.00
JAT-21	1	26	embryo	1	100.00
JAT-22	1	26	embryo	1	100.00
JAT-23	2	26	embryo	2	100.00
JAT-24	2	26	embryo	2	100.00
JAT-28	2	26	embryo	1	50.00
JAT-29	1	26	embryo	1	100.00

Table 3.8 shows % explants with *GUS* expression transformation experiments done with embryos.

Because it is a crucial tool that can be used to optimize the transformation protocol, *GUS* expression is preliminary proof of a marker gene. Therefore, high *GUS* expression is an indication of the protocol likely being optimized. Pan et al. (2010) reported that 37 of 120 putative *GUS* transformants generated were transgenic plants and *GUS* positive using a *GUS* staining of leaves from regenerated transgenic plants.

An optimized transformation protocol can be expanded to a gene of interest that has a value added trait which could be expressed. Examples of this include genes that code for disease resistance, enhanced nutrition, increased oil content, and abiotic stress tolerance (e.g. cold, drought). For *J. curcas*, this work will be expanded into the gene *CBF3*, which codes for cold, drought, and salinity tolerance. Overexpression of this gene could allow it to be grown and cultivated in a temperate climate like Pennsylvania, making it possible for the State to be able to take advantage of its great energy and economic potential.

Figure 3.12: *GUS* Expression in Different Explants

GUS expression has been demonstrated on several explants: (A) young leaf, (B) embryo, (C) cotyledonary leaf.

3. Molecular Analysis

3.1. Polymerase Chain Reaction (PCR)

PCR screening assay was performed on *J. curcas* young *in vitro* leaves from germinated embryos transformed with *GUS*. In the PCR, two out of the five plants tested showed a positive band for the *GUS* gene. Figure 3.13 shows the clear band around the same area as the band of the positive control

Figure 3.13: PCR of Putative Transgenic *J. curcas*

Lane #	1	2	3	4
Description	-ve Control	+ve Control	*Jatropha* Plant 1	*Jatropha* Plant 2
DNA Intensity	0	54.56	0	69.70

Lane #	5	6	7	8
Description	*Jatropha* Plant 3	*Jatropha* Plant 4	*Jatropha* Plant 5	1 Kb Ladder
DNA Intensity	0	0	72.55	-

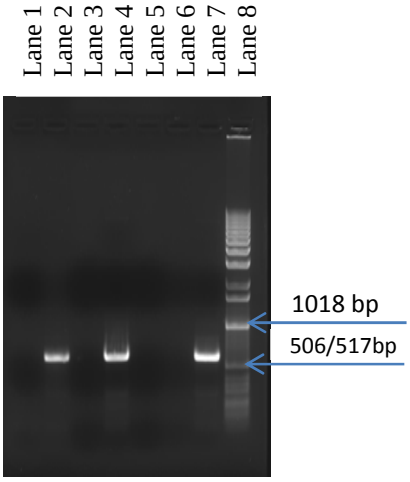


Figure 3.13 shows the gel of PCR products from five different putative transgenic *J. curcas*. Two of the five plants test positive for the presence of the *GUS* gene.

4. Conclusion

4.1. Summary of Findings

Tissue culture was used to study different methods and explants to regenerate the important second-generation biofuel crop *J. curcas* using a variety of explants. Embryos were germinated at high rates using a number of different media and methods that were then compared. *Ex vitro* young leaves were used for indirect shoot regeneration and *ex vitro* shoot buds were used for direct shoot induction. Callus and shoot induction from different plants (genotypes) was studied to understand the effect of genotype on *in vitro* regeneration of *J. curcas*, and it was found that there is more variance between genotypes in callus induction than shoot induction. Different

rooting media were also tested to optimize a medium for *in vitro* rooting of *J. curcas* to see which one gave the best percentage of explants rooting and which gave the best number of roots per explant. As a result, JRM1 recorded the best rooting percentage, but produced least number of roots per explant; JRM3 had the lowest rooting percentage, but most number of roots per explant. Acclimatization percentage has increased since a new environment controlled greenhouse has been made available and since seed starter was used as potting mix instead of peat moss. Finally, an optimized *Agrobacterium*-transformation protocol has been generated using the *GUS* reporter system, which can be used to genetically transform *J. curcas* with genes with value-added traits like *CBF3* to impart cold tolerance.

3.2 Future Studies

Future studies will include molecular screening of putative transgenic material. This will be done using the techniques of Southern Hybridization and RT-PCR on the new growth leaves of these plants. Experiments are already underway with transforming *J. curcas* genes with value-added traits such as increased abiotic stress tolerance using the *CBF3* gene (cold, drought, and salinity), oil yield, and disease and pest resistance. These traits will then be tested in the greenhouse and field through physiological evaluation of the transgenics. Lastly, *in vitro* flowering and seed set can be examined to further allow us to rapidly regenerate screen transgenic plants under *in vitro* conditions and to advance the breeding studies.

Reference List

Achten W M J, Nielsen L R, Aerts R, Lengkeek A G, Kjær E D, Trabucco A... ,Muys B. (2010). Towards domestication of *Jatropha curcas*. *Biofuels* 1(1): 91–107.

Al-Abed D, Madaswamy P, Reddy T, Goldman S, Rudrabhatla S. (2007). Genetic Engineering of Maize with the Arabidopsis DREB1A/CBF3 Gene Using Split-seed Explants. *Crop Science* 47 (6): 2390-2402.

Alt-Moerbe, J., H. Kühlmann and J. Schröder. (1989). Differences in induction of Ti plasmid virulence genes *virG* and *virD*, and continued control of *virD* expression by four external factors. *Molecular Plant–Microbe Interactions* 2: 301–308.

Bertini G. (1951). Studies on lysogenesis. I. The mode of phage liberation by lysogenic Escherichia coli. *Journal of Bacteriology* 62: 293-300.

Bieniawska Z, Espinoza C, Schlereth A, Sulpice R, Hincha D K, Hannah,M A. (2008). Disruption of the Arabidopsis circadian clock is responsible Plant Cold Acclimation for extensive variation in the cold-responsive transcriptome. *Plant Physiology* 147: 263–279

Carels, N. (2009). Jatropha: A Review. *Advances in Botanical Research* 50: 39-86.

Chhetri A B, Tango M S, Budge S M, Watts K C, Islam, M R. (2008). Non-Edible Plant Oils as New Sources for Biodiesel Production. *International Journal of Molecular Sciences* 9: 169-180.

Chisti, Y. (2007). Biodiesel from microalgae. *Biotechnology* 25: 294-306.

Currie, Jeff. (2007). Food, Feed and Fuels: An outlook on the agriculture, livestock and biofuel markets [Power Point Slides]. Retrieved from http://www.gceholdings.com/pdf/GoldmanReportFoodFeedFuel.pdf

Daniel H, Khan M S, Allison L. (2008). Milestones in chloroplast genetic engineering: an environmentally friendly era in biotechnology. *Trends in Plant Scienc*e 7: 84-91.

Davies P J. The Plant Hormones: Their Nature, Occurances, and Functions. In *Plant Hormones* by P. J. Davies, 1-15. New York: Springer, 2010.

Dehgan B, Webster G L. *Morphology and infrageneric relationships of the genus Jatropha (Euphorbiaceae).* Los Angeles: University of California Press, 1979.

Deore A C, Johnson T S. (2008). High-frequency plant regeneration from leaf-disc cultures of *Jatropha curcas* L.: an important biodiesel plant. *Plant Biotechnol*ogy *Reports* 2: 7–11.

D'Halluin K, Bonne E, Bossut M, De Beuckeleer M, Leemans J. (1992) . Transgenic maize plants by tissue electroporation. *Plant Cell* 4: 1495-1505.

Dhillon T, Pearce S P, Stockinger E J, Distelfeld A, Li C X, Knox A K, Vashegyi I, Vágújfalvi A, Galiba G, Dubcovsky J. (2010). Regulation of Freezing Tolerance and Flowering in Temperate Cereals: The *VRN-1* Connection. *Plant Physiology* 153: 1846–1858.

Freeman W M, Walker S J, Vrana K E. (1999). Quantitative RT-PCR: Pitfalls and Potential. *Biotechniques* 26: 112-125.

Gelvin S B. (2010). Finding a way to the nucleus. *Current Opinion in Microbiology* 13(1): 53-58.

Giibitz G M, Mittelbach M, and Trabi M. (1999). Exploitation of the tropical oil seed plant Jatropha curcas L. *Bioresource Technology* 73-82.

Ginwal H S, Phartyal, S S Rawat P S, Srivastava R L. (2005). Seed Source Variation in Morphology, Germination, and Seedling Growth of *Jatropha curcas* Linn. in Central India. *Silvae Genetica* 54(2): 76-80.

Goldman D. (2008, June 27). Food price spike: Is ethanol to blame? *CNN Money*. Retrieved from http://money.cnn.com/2008/06/27/news/economy/ethanol_food_prices/

Jahne A, Becker D, & Lorz, H. (1995). Genetic engineering of cereal crop plants: a review. *Euphytica* 85: 35-44.

Jefferson R A. (1987). Assaying chimeric genes in plants: The GUS gene fusion system. *Plant Molecular Biology Reporter* 387-405.

Islam A K M A, Anuar A, Yaakob Z. (2009). Effect of Genotypes and Pre-Sowing Treatments on Seed Germination Behavior of *Jatropha*. *Asian Journal of Plant Sciences* 8(6): 433-439.

Klein T M, Wolf B D, Wu R, Sanford J C. (1987). High-velocity microprojectiles for delivering nucleic acids into living cells. *Nature* 327: 70–73.

Kasuga M, Miura S, Shinozaki K, Yamaguchi-Shinozaki K. (2004). A combination of the *Arabidopsis* DREB1A gene and stress-inducible *rd29A* promoter improved drought- and low-temperature stress tolerance in tobacco by gene transfer. *Plant Cell Physiology* 45:346–350.

Kaushik N, Kumar K, Kumar S, Kaushik N. (2007). Genetic variability and divergence studies in seed traits and oil content of *Jatropha* (*Jatropha curcas* L.) accessions. *Biomass and Bioenergy* 31 (7): 497-502.

Khemkladngoen N, Cartagena J, Shibagaki N, Fukui K, (2011). Adventitious shoot regeneration from juvenile cotyledons of a biodiesel producing Plant, Jatropha curcas L. *Journal of Bioscience and Bioengineering* 111 (1): 67-70.

Kumar A, Sharma S. (2008). An evaluation of multipurpose oil seed crop for industrial uses (*Jatropha curcas* L.): A review. *Industrial Crops and Products* 28: 1-10.

Kumar N, Reddy M P. (2010a). Plant regeneration through the direct induction of shoot buds from petiole explants of *Jatropha curcas*: a biofuel plant. *Annals of Applied Biology* 156: 367-375.

Kumar N, Anand V K G, Reddy M P. (2010b). *In vitro* plant regeneration of non-toxic Jatropha curcas L.: Direct shoot organogenesis from cotyledonary petiole explants. *Journal of Crop Science and Biotechnology* 13(3): 189-194.

Kumar N, Anand V K G, Reddy M P. (2010c). Shoot regeneration from cotyledonary leaf explants of Jatropha curcas: a biodiesel plant. *Acta Physiologia Plantarum* 32 (5): 917-924.

Lazzeri P A, Alwine J, and Horst L. "Culture, Regeneration, and Transformation of Barley Protoplasts." In *Plant tissue culture manual: fundamentals and applications*, by Keith Lindsey, B10:1-11. Dordrecht, The Netherlands: Kluwer Academic Publishers, 1992.

Li M R, Li H Q, Pan X, and Wu G J. (2008). Establishment of *an Agrobacterium*-mediated cotyledon disc transformation method for *Jatropha curcas*. *Plant Cell Tissue Organ and Culture* 92 (2):173-181.

Loyola-Vargas V M, De-la-Peña C., Galaz-Ávalos R M, and Quiroz-Figueroa F R. "Plant Tissue Culture." In *Molecular Biomethods Handbook* , by John M. Walker and Ralph Rapley, 875-904. Totown, New Jersey: Humana Press, 2008.

Maliga P. (2004). Plastid Transformation in Higher Plants. *Annual Review of Plant Biology*, 55: 289–313.

Mazumdar P, Basu A, Paul A, Mahanta C, Sahoo L. (2010). Age and orientation of the cotyledonary leaf explants determine the efficiency of *de novo* plant regeneration and *Agrobacterium tumefaciens*-mediated transformation in *Jatropha curcas* L. *South African Journal of Botany*. 76(2) 337-344.

Miller C O, Skoog F, Von Saltza M H, Strong F M. (1955). Kinetin, a cell division factor from deoxyribonucleic acid. *Journal of the American Chemical Society*, 77(5): 1392.

Monnier M. "Culture of zygotic embryos." In *In Vitro embryogenesis in plants*, by T. A. Thorpe, 117–153. Dordrecht: Kluwer Academic, 1995.

Murashige T, and Skoog F. (1962) "A revised medium for rapid growth and bioassays with tobacco tissue cultures." *Physiologia Plantarum* 15: 473–497.

Muskens M W, Vissers A P, Mol J N, Kooter J M. (2000) Role of inverted DNA repeats in transcriptional and post-transcriptional gene silencing. *Plant Molecular Biology* 43:243-260.

Openshaw K. (2000). A review of *Jatropha curcas*: an oil plant of unfulfilled promise. *Biomass Bioenergy* 19: 1-15.

Oskoueian E, Abdullah N, Saad W Z, Omar A R, Ahmad S, Kuan W B, Zolkfli N A, Hendra R, Ho Y W. Antioxidant, anti-inflammatory, and anticancer activities of methanolic extracts from *Jatropha curcas* Linn. *Journal of Medicinal Plants Research* 5(1): 49-57.

Pan J, Fu Q, Zheng-Fu X. (2010). *Agrobacterium tumefaciens*-mediated transformation of biofuels plant Jatropha curcas using kanamycin selection. *African Journal of Biotechnology* 9 (39): 6477-6481.

Phillips G C. (2004) *In vitro* Morphogenesis in Plants: Recent Advances. *In Vitro Cellular & Developmental Biology* 40: 342-345.

Purkayastha J, Sugla T, Paul A, Solleti S K, Mazumdar P Basu A, Mohommad A, Ahmed Z, Sahoo L. (2010) Efficient *in vitro* plant regeneration from shoot apices and gene transfer by particle bombardment in *Jatropha curcas*. *Biologia Plantarium* 54: 13-20.

Quiroz-Figueroa F R., Rojas-Herrera R, Galaz-Avalos R M, Loyola-Vargas V M. (2006) Embryo production through somatic embryogenesis can be used to study. *Plant Cell Tissue Organ Culture* 86: 285–301.

Radzan M K. (2003). *Introduction to Plant Tissue Culture* (2nd ed.). Enfield, NH: Science Publishers, Inc.

Rajore S and Batra A. (2007). An alternative source for organogenic callus culture in *Jatropha curcas* L. *Indian Journal of Biotechnology* 6: 545-548.

Rao G R, Korwar G R, Shanker A K, and Ramakrishna Y S. (2008). Genetic associations, variability, and diversity in seed characters, growth, reproductive phenology and yield in *Jatropha curcas* (L.) accessions. *Trees- Structure and Function* 22(5): 697-709.

Robbins W J. (1922). Cultivation of excised root tips and stem. *Botannical Gazette* 73: 376-390.

Sairam R V, Al-Abed D, Johnson J, Muzynski M, Raab M, Reddy T V, Goldman S. (2008). Maize. *In*: Kole C, Hall TC (eds.) *A Compendium of Transgenic Crop Plants* 49-82.

Skoog F, Miller C O. (1957). Chemical regulation of growth and organ formation in plant tissue cultures *in vitro*. *Symposia of the Society for Experimental Biology*, 11: 118–131.

Schell J S. (1987). Transgenic plants as tools to study the molecular organization of plant genes. *Science* 237: 1176–1183.

Schultes R E. (1987). Members of Euphorbiaceae in primitive and advanced societes. *Botanical Journal of the Linnean Society* 94: 79-95.

Stewart C N. (2008). *Plant Biotechnology and Genetics*. Hoboken, NJ: Wiley.

Sujatha M, Makkar H P S, Becker K. (2005) Shoot bud proliferation from axillary nodes and leaf sections of non-toxic *Jatropha curcas* L. *Plant Growth Regulation*. 47: 83-90.

Sujatha M, Mukta N. (1996). Morphogenesis and plant regeneration from tissue cultures of *Jatropha curcas*. *Plant Cell Tissue and Organ Culture* 44:135–141.

Tabatabai B, Reitzel M, Breidigan C, Funk A, Gaud S, Curtis K, Vaidya K, Veledar N, Patel P, Rudrabhatla S, Potlakayala S D, Josekutty P C. (2010). *In vitro* Propagation and Genetic Transformation of *Jatropha curcas*. Poster. Presented at 2010 SIVB/IAPB Conference: St. Louis.

Thomas R, Sah N K, Sharma P B. (2008). Therapeutic Biology of *Jatropha curcas*: A Mini Review. *Current Pharmaceutical Biotechnology* 9: 315-324.

Thomashow M F. (2010). Molecular Basis of Plant Cold Acclimation: Insights Gained from Studying the CBF Cold Response Pathway. *Plant Physiology* 154:571-577.

Thorpe T A. (1990). The current status of plant tissue culture. *In* S. S. Bhojwani (Ed.*), Plant tissue culture: Applications and limitations* 1–33.

Thorpe T A. (2007). History of plant tissue culture . *Molecular Biotechnology* 37: 169-180.

Tickell, Joshua. (2003). From Fryer to Fuel Tank: The Complete Guide to Using Vegetable Oil as an Alternative Fuel. Hollywood, CA: Tickell Energy.

Tzfira T, Citovsky V. (2006). Agrobacterium-mediated genetic transformation of plants:biology and biotechnology. *Current Opinion in Biotechnology* 17(2): 147-154.

Varshney A, Johnson T S. Efficient plant regeneration from immature embryo cultures of Jatropha curcas, a biodiesel plant. *Plant Biotechnology Reports* 4: 139-148.

Vasil V, Hildebrandt A C. (1965). Differentiation of tobacco plants from single, isolated cells in micro cultures. *Science* 150: 889–892.

Wald M L. (2006, January 16). Corn Farmers Smile as Ethanol Prices Rise, but Experts on Food Supplies Worry. *The New York Times*. Retrieved from http://www.nytimes.com/2006/01/16/national/16ethanol.html.

Weise E. (2011, February 9). Ethanol pumping up food prices. *USA Today*. Retrieved from http://www.usatoday.com/money/industries/food/2011-02-09-corn-low_N.htm

Zheng Y L. (2009). Different photosynthetic responses to night chilling among twelve populations of *Jatropha curcas*. *Photosynthetica* 47(4): 559-566