5-2016

Jatropha curcas protoplast isolation and inhibitory effects of culture

Christopher S. Lukaszewski
The University of Texas Rio Grande Valley

JATROPHA CURCAS PROTOPLAST ISOLATION

AND INHIBITORY EFFECTS

OF CULTURE

A Thesis

by

CHRISTOPHER S. LUKASZEWSKI

Submitted to the Graduate College of
The University of Texas Rio Grande Valley
In partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2016

Major Subject: Biology

JATROPHA CURCAS PROTOPLAST ISOLATION

AND INHIBITORY EFFECTS

OF CULTURE

A Thesis
by
CHRISTOPHER S. LUKASZEWSKI

COMMITTEE MEMBERS

Dr. Frederic Zaidan
Chair of Committee

Dr. Robert Edwards
Committee Member

Dr. Matthew Terry
Committee Member

May 2016

ABSTRACT

Lukaszewski, Christopher S., Jatropha Curcas Protoplast Isolation and Inhibitory Effects Of Culture. Master of Science (MS), May, 2016, 40 pp., 1 table, 7 figures, references, 76 titles.

Improvement on the sub-tropical plant *Jatropha curcas* may increase the use of this drought tolerant oilseed crop for the production of biodiesel. One of the methods for enhancing a species is through genetic manipulation of the protoplast. Isolating protoplasts is challenging and culturing has many obstacles similar to those seen in tissue culture, such as oxidative browning. Successful isolation of *J. curcas* leaf protoplasts resulted in an average of 3.23×10^6 viable protoplasts/mL. However, protoplasts diminished within 2 days after culturing. A review of the potential solutions used for overcoming the inhibitory effects of culture may reveal further insight on the culturing of *J. curcas* protoplasts.

DEDICATION

This thesis would not have been easy without the women in my life. Including, but not limited to my Grandmother, Mother, Wife, and volunteers that have given me nothing but positive reinforcement throughout the entire process of this academic endeavor.

ACKNOWLEDGMENTS

The Biology Department faculty and staff has been invaluable to performing research. Without the many facilities provided, such as the specialized work hoods in the research labs, the equipment in HHMI and corridor labs, and the growth chamber. Special thanks to the secretary of the department for completing supply orders and maintenance staff for repairing growth room malfunctions as well. In addition to the university, help from the Texas A&M Citrus Center in Weslaco was very supportive with protoplast isolation and pest management.

TABLE OF CONTENTS

Page

ABSTRACT……. iii

DEDICATION……..……. iv

ACKNOWLEDGMENTS…….……...…… v

TABLE OF CONTENTS……..……. vi

LIST OF FIGURES……...….. viii

CHAPTER I. INTRODUCTION……... 1

Importance of *Jatropha curcas*…….. 1

Description……. 1

Biodiesel…… 2

Other Uses……. 4

Biotechnology…… 4

Hybridization……. 5

Propagation…… 5

Genetic Manipulation……. 6

Obstacles in Culturing Protoplasts……. 6

Browning…… 6

Hypothesis……..... 8

CHAPTER II. MATERIALS AND METHODS…… 10

Protoplast Isolation, Purification, and Culture……..... 10

Tissue Culture…… 13

CHAPTER III. RESULTS AND DISCUSSION…… 15

Protoplast Isolation, Purification, and Culture……...……... 15

Tissue Culture……...…… 22
Potential Use of Antioxidants…….…….. 24
Further Research…… 29
CHAPTER IV. CONCLUSION……... 32
REFERENCES……. 33
BIOGRAPHICAL SKETCH…… 40

LIST OF FIGURES

Page

Figure 1: Enzyme Combinations……..……..…..16

Figure 2: Individual Enzyme Activity……..……...….17

Figure 3: MES Buffer…….....17

Figure 4: pH of MES Buffer……...….18

Figure 5: Mannitol Osmoticum……...…18

Figure 6: Flotation Purification……..20

Figure 7: Total Isolation Data…….....21

CHAPTER I

INTRODUCTION

Importance of *Jatropha curcas*

Description

Jatropha curcas is a member of the Euphorbiaceae family with the genus name, Jatropha, derived from the Greek words *íatrós* (physician) and *trophé* (nutrition). (Heller, 1996) The origin of the species name *curcas* is unknown, however it was first documented by a Portuguese doctor named Garchia de Orta more than 400 years ago. (Parmar, et al., 2014) Its common names include Physic Nut, Yu-lu-tzu (Chinese), Bagbherenda (Hindi), and Piñoncillo (Mexico). *Jatropha curcas* grows as a large shrub or small tree reaching heights of 3-5m and forms a flowering inflorescence yielding up to 10 fruits with three seeds per fruit. (Heller, 1996; Kumar and Sharma, 2008) The seeds contain high amounts of oil which are analogous to those found in diesel fuel. For this reason, it has been cultivated in many parts of the world as a potential oilseed crop for the production of biodiesel.

Over a century of widespread cultivation has lead to some disagreement over the origin of *J. curcas*, however it is believed to stem from Mexico and Central America. Reports of *J. curcas* cultivation are found as early as 1810 and subsequent exportation by 1836 to the Cape Verde Islands. (Heller, 1996) It is known as a drought tolerant species and has been introduced to seasonally dry areas of the world; such as grassland savannas, thorn scrub forests, and desert regions. (Heller, 1996) It grows in locations receiving as little as 250mm of rainfall annually.

(Kumar and Sharma, 2008) Records have even indicated its ability to survive three consecutive years of drought. (Frazão LA et al., 2010) Its succulent stem, hardy taproot system, and leaf senescence play roles in balancing water loss during times of drought. (Maes et al., 2009; Kumar and Sharma, 2008) *Jatropha curcas* is also known for its capability to grow on poor and marginal soils with a low nutrient content. (Kumar and Sharma, 2008) In fact, it enriches surrounding soil from shed leaves that form mulch thus increases earthworm activity around the base of the plant. (Kumar and Sharma, 2008) Other features include it being a perennial species; therefore does not require replanting every season. After a short gestation period it bears fruit as early as 2-4 years and produces fruit for 40 to 50 years. (Sujatha et al., 2005) Also, pruning increases the number of fruiting branches and maintains a suitable height for harvesting. Lastly, it is known to withstand a light frost even though it grows best in warmer climates. (Kumar and Sharma, 2008) For these many reasons, it is being cultivated as a biodiesel crop in various regions of the world such as Brazil, Fiji, Honduras, India, Jamaica, Nicaragua, Panama, Puerto Rico, Mexico, El Salvador, Thailand, and many African countries. (Pahl, 2005; Kohli et al., 2009)

Biodiesel

Presently, the use of up to 5% biodiesel is approved for blending with conventional diesel fuel for vehicles in the U.S. and in some cases up to 20% is allowed depending on the industry. Reports show that *J. curcas* oil has lower tailpipe emissions than diesel. (Kohli et al., 2009) Its oil can even be used directly as fuel in some diesel engines after minute filtration. Such is the case in Mali, West Africa, during WWII. (Heller, 1996) Over the year of 2014 the amount of oil almost reached $110 per barrel and then fell just below $50 a barrel at the beginning of 2015 according to NASDAQ. The most current pricing of *J. curcas* oil is from June 2008 reported by

Peter Pae from the L.A. Times at $43 a barrel. Preparation of *J. curcas* oil includes harvesting, extraction, and transesterification into biodiesel. The process becomes more economical on a larger scale. For example, it can achieve an energy input to output ratio as high as 1 to 5.2 under good conditions. (Heller, 1996) Additionally, it may be possible to use the oil to power the very equipment used to harvest and extract it.

Jatropha curcas oil is considered non-edible oil due to its toxicity. Therefore, it serves as an alternative to edible oilseed crops that are currently used to produce biodiesel. The amount of oil derived from *J. curcas* seeds varies, with yields ranging from 20-50% and a high of 60%. (Sujatha et al., 2005; Jha et al., 2007; Deore and Johnson, 2008; Kumar and Sharma, 2008; Kohli et al., 2009; Misra et al., 2010; Sujatha and Mukta, 1996; Rajore and Batra, 2007; He et al., 2009) This variability of oil is due to heterozygous seeds not breeding true to trait; thus proving a need for elite varieties.

Many members of the Euphorbiaceae family have seeds that are rich in reduced hydrocarbon material and are subsequently used in the production of oils. (Shrivastava and Banerjee, 2008) However, most of them also contain toxic compounds. After extraction the oil and remaining seedcake contain phorbol esters and curcin. Phorbol esters are considered cocarcinogenic and curcin is an extremely lethal protein comparable to ricin. Due to its toxicity the seedcake must be handled with caution and cannot be used as feed for livestock. A biodiesel plant owner in South Africa once stated that *J. curcas* would benefit the farmers in the area since the seed crop is so toxic it would not be stolen by people nor consumed by animals. (Pahl, 2005) China also prefers *Jatropha* based biodiesel due to its lower water usage and its status as a non-food crop. (Scheffran, 2010) Recently, studies have been undertaken to remove the toxicity of the seedcake through heat treatment, ionizing radiation, and chemicals. (Kumar and Sharma

2008) In addition, there are non-toxic varieties from Mexico that are being considered for cultivation. (Openshaw, 2000; Wilson and Hildebrand, 2010)

Other Uses

Jatropha curcas is known as a multipurpose plant with the advantage of supplying many byproducts and co-products. Traditionally the seeds are used medicinally as a purgative, although more recently its pharmacological array of activity includes, analgesic, antibacterial, anticonvulsant, antifungal, antifertility, anti-inflammatory, antitumor, anticancer, antiviral, anthelmintic, and diuretic. (Sujatha and Mukta, 1996; Ross IA, 1999; Kumar and Sharma, 2008; Baldi, et al., 2007) The seedcake would serve as an excellent fertilizer because it has a higher N:P:K than cow manure, or the seedcake could be used as an animal feed due to its high amount of protein and outstanding levels of amino acids. (Kumar and Sharma, 2008; Sujatha et al., 2005) Fermented seed cake may also be used as a low cost production of industrial enzymes or as a form of biogas production. (Kumar and Sharma, 2008) Also, its oil can be used in making pesticides; soaps, candles, paints, and lubricants. (Kumar and Sharma, 2008; Sujatha and Mukta, 1996) Its bark contains tannins that can be used for treating leather or for coloring dye. (Kumar and Sharma, 2008) Trials have been conducted for the efficacy of using *J. curcas* leaves for silk worm rearing. (Kumar and Elangovan, 2010) Traditionally, the plant as a whole is used as a hedgerow and can be used to prevent soil erosion. Lastly, the attraction of bees to flowers may give the potential for honey production while aiding in pollination. (Kumar and Sharma, 2008)

Biotechnology

Since the beginning of agriculture farmers have been altering the genetic makeup of plants by simply selecting seeds from plants with desired traits, like higher seed sets, larger fruit size, and disease resistance. This was the only method of plant breeding until farmers started

cross-pollinating selected plants by hand to produce hybrids with desired traits. Some hybrids arise naturally and in fact several of the world's major food crops are naturally formed hybrids; such as bananas, coffee, peanuts, wheat, etc.

Hybridization

Jatropha tanjorensis is found to be a naturally occurring interspecific hybrid between *Jatropha curcas* and *Jatropha gossypifolia*. (Prabakaran and Sujatha, 1999) However, when attempts were made to hybridize the two species artificially they bore no fruit. (Prabakaran and Sujatha, 1999) In another study, *J. curcas* was artificially hybridized with all other *Jatropha* species, except *J. podagrica*, which revealed the compatibility among species for potential hybridization. (Basha and Sujatha, 2009)

Propagation

Jatropha curcas is propagated directly from seeds, cuttings, or by tissue culture. The downfall of propagation from seeds is that they have a low germination rate and may transmit undesired traits. On the other hand, some genetic variability amongst plants from seeds may reduce widespread disease of an entire crop. Propagation of seeds in containers is more promising because only healthy plants can be transferred to soil, although their taproots become diminished. Seeds can also be propagated *in vitro*, which increases the germination rate and time. Propagation from cuttings promotes genetic stability because they are essentially clones of the mother plant. However, cuttings are more susceptible to disease and have shown weaker tap root systems. (Heller, 1996) Propagation through tissue culture also produces clones, but takes longer to produce fully grown plants. The other advantage of tissue culture is the ability to alter their development through the use of hormones and to capacity to genetically manipulate tissues with the insertion of genes.

Genetic Manipulation

Jatropha curcas has already undergone genetic manipulation through the insertion of plasmid particles via soaking and bombardment of plant tissue. (Li et al., 2008; Joshi et al., 2010) Another form of genetic manipulation is the insertion of genes into protoplasts. Research has already been conducted on producing high yields of *J. curcas* protoplasts. (Tudses et al., 2014) Protoplasts offer another method of genetic manipulation to improve a plant species. Protoplasts can also be fused; thus doubling all internal organelles. Fused protoplasts may become stable hybrids only if fusion of the nuclei occurs and subsequent coordination of chromosome division for mitosis undergoes successfully. The chances of a stable hybrid forming are increased when the fused protoplasts result in an even number of chromosomes.

Obstacles in Culturing Protoplasts

The pathway to transforming protoplasts to fully grown plants has many difficulties along the way. Isolation requires the removal of cell walls via digestive enzymes. This action disrupts the entirety of cell function including the plasma membrane, cytoskeleton, and microtubules, thus resulting in the inhibition of mitosis and low viability yields. (Cutler et al., 1991) The removal of the cell wall may also elicit a wound response similar to what is observed in tissue culture browning. (Beyl, 2005)

Browning

Browning is especially significant when establishing cultures because it affects new growth tissue and therefore inhibits all other plant organ development. It may eventually lead to necrosis of explants and is predominantly seen in the tissue culture of woody species from the tropics. (Malabadi and Van Staden, 2005; Ahmad, et al., 2013) Since *J. curcas* is a semi-woody

subtropical plant, overcoming browning is of great importance in respect to performing tissue culture on this species.

This occurrence of browning is due in part to phenolic compounds released after cutting the explant. Phenolic compounds produced by plants may be water soluble or soluble by organic solvents and include tannins and flavonoids that contain carboxylic acids and glycosides. (Jha TB & Ghosh B, 2005) When released from the explant these compounds leach into the media and spread to nearby healthy explants, thus when multiple explants are cultured together it causes a chain reaction. Many plants contain phenolic compounds and when they become oxidized it releases phytotoxic products. (Çördük and Aki, 2011) The appearance of brown color is due to the formation of quinine substances from the oxidation of phenolic compounds. (Ahmad, et al., 2013) These quinine substances elicit a lethal response by repressing the activity of enzymes that eventually spreads through the single explant tissue and onto other explants. (Feng-jie, et al., 2007)

Other causing factors of browning are the oxidation of enzymes and reactive oxygen species (ROS). These include, but are not limited to, polyphenoloxidase (PPO), peroxidase (POD), and hydrogen peroxide (H_2O_2). (Yinghui, 2008) Each of these play a unique role in the browning process and often interact with each other in what can result in the loss of calli during tissue culture. PPO is a copper-containing enzyme that catalyzes the oxidation of polyphenols. These phenolic compounds may activate PPO to oxidize tissues and form the brown colored quinine. (Feng-jie, et al., 2007) The enzyme POD found in the peroxisomes of plant cells that are involved in the polymerization of the precursors of lignin. During the browning process the activities of PPO and POD often become interconnected. For example, phenolic compounds may activate PPO and in turn can promote POD to oxidize phenolic compounds. (Feng-jie, et

al., 2007; Ahmad, et al., 2013) POD is responsible for catalyzing the oxidation of ROS and in doing so form substances like H_2O_2. In plants, H_2O_2 acts as a stress-signaling compound, but is considered a lesser type of ROS. (Yinghui, 2008; Cassells and Gahan, 2006) However, POD is also capable of scavenging H_2O_2 and reducing it to water and oxygen. H_2O_2 still causes molecular damage to living cells and may even be converted into more reactive species. (Yinghui, 2008) Many ROS, like H_2O_2, are also known to react with biomolecules, proteins, lipids, and DNA. (Yinghui, 2008) Therefore, large amounts of PPO, POD, and ROS might create oxidative stress resulting in the loss of cell function and eventually cell necrosis.

Any alterations of the plant cell membrane results in a decrease of the antioxidant defense mechanisms of the cell; resulting in additional oxidative stress. (Cutler et al., 1991) It has been observed that the levels of many antioxidant enzymes, such as glutathione reductase and ascorbate peroxidase, begin to decrease over time and in effect an increase of ROS occurs. (Kapur et al., 1993; Papadakis et al., 2001; Kennedy and Filippis, 2003) Lipid peroxidation and a gradual decline in chlorophyll content have also been observed during the stress of isolation. (Kennedy and Filippis, 2003) Furthermore, research has shown that the increase in ROS is linked to the repression of totipotency amongst protoplasts. (Papadakis et al., 2001)

Hypothesis

The purpose of isolating *J. curcas* protoplasts is to manipulate them through genetic means, but it is futile if they cannot be cultured and regenerated into fully mature plants. Thus far, protoplasts of this species may be isolated and plants are developed through tissue culture. Therefore, the underlying obstacle to overcome is the culturing of protoplasts. There are multiple approaches to isolating plant protoplasts in existence. These include varying means of digestion, altering purification methods, and many additional additives to protoplast solutions.

All of which are implemented with the hopes of increasing viability for culture. Reports have shown there to be a correlation between poor regeneration of protoplasts and oxidative browning. (Kennedy and Filippis, 2003) This may be overcome by implementing the use of antioxidants during the isolation and culture of protoplasts. There is very little information on the use of antioxidants with protoplasts, yet many instances where they are used in tissue culture. A review of the most beneficial antioxidants used in tissue culture must be performed before implementing those to future experiments in protoplast isolation and culture. *Jatropha curcas* protoplasts are a prime candidate for trials on the use of antioxidants to combat oxidative stress that occurs during their isolation and culture. This may improve the regeneration of protoplasts by repairing the antioxidant defense mechanisms that are disturbed during isolation for further use in culture.

CHAPTER II

MATERIALS AND METHODS

Protoplast Isolation, Purification, and Culture

Leaf explants were selected from *J. curcas* plants in a growth chamber at 26 $\pm$ 0.5º C, under a 16-hour photoperiod, with a humidity level of ~60%. Only third generation leaves were selected for culture according to previous research stating them to have a higher callus frequency. (Sujatha and Mukta, 1996) Leaves were sterilized by first rinsing them in deionized H_2O, soaking for 4 minutes in a solution containing 10% bleach with 0.1% Triton-X, followed by a quick submersion in 70% ethanol, and finally triple rinsed in autoclaved deionized H_2O. Interveinal leaf tissue was excised and sliced into <1mm strips for use in enzymatic digestion.

A range of enzymes were investigated in order to determine the optimum enzyme concentration and combinations that released the highest number of protoplasts. These included solutions of cellulase at 1.0%, 2.0%, and 2.5%; pectinase at 0.5%, 0.75%, and 1.0%; and hemicellulase at 0.2%, 0.35%, and 0.5%. (Evans et al., 1983; Chawla, 2003; Evans and Bravo, 1983) These ranges were mixed to form 27 combinations. Sliced leaf tissue was placed into microcentrifuge tubes containing each enzyme combination and vortexed for 5 seconds. Leaf tissue was allowed to incubate in the dark for 8 hours at room temperature. The number of protoplasts released was recorded and the best enzyme combination was used to test the following step. Three MES buffer concentrations were tested in order determine which maintained a pH of 5.8 $\pm$ 0.1 while releasing the most protoplasts. These consisted of 5mM,

10mM, and 15mM. The number of protoplasts was recorded, the pH was tested, and the best concentration was used for the next step. Ten mannitol concentrations were tested in order to determine which retained osmotic stability of protoplasts while giving the highest yield of viability. These ranged from 11%, 12%, 13%, 14%, 15%, 16%, 17%, and 18%. (Dodds and Roberts, 1985) All counts were made using a hemocytometer and viability was tested using 0.01% fluorescein diacetate (FDA) under a fluorescent microscope. (Bengochea and Dodds, 1986; Chawla, 2003; Murch and Saxena, 2005, Davey et al., 2006) After an optimum isolation solution was found it was then used for further studies. Lastly, each concentration and combination studied was repeated three times.

Immediately after the isolation solution finished incubating the purification process was started by gently agitating the isolation solution by hand to further release protoplasts. (Chawla, 2003) The isolation solution was then centrifuged at 1,000 RPM for 5 minutes at room temperature, then the supernatant was removed and replaced with a wash solution. The wash solution was similar to isolation solution, minus the enzymes. Several methods of purification were attempted. These included washing, filtering, and flotation of the protoplasts. Washing the protoplasts involved centrifuging the solution at 1,000 RPM for 10 minutes, removing the supernatant, and repeating this three times. Filtering was attempted either by pouring the solution through a 200µm stainless steel screen embedded in a cut off syringe or a by vacuum filtration through 20µm nylon mesh screen. Flotation purification was done by slowly pipetting the first washed protoplast solution containing mannitol atop a solution containing sucrose and centrifuging at 1,000 RPM for 5 minutes at room temperature. (Bengochea and Dodds, 1986; Veilleux, 2005) The interface layer between the two solutions was removed and then washed three times by centrifuging at 1,000 RPM for 10 minutes each. Additional purification studies

were performed using a different concentrations of mannitol and sucrose solutions. Each purification study was repeated three times. Viability of protoplasts was tested using 0.01% FDA and the plating density was recorded using a hemocytometer under a fluorescent microscope. (Bengochea and Dodds, 1986; Chawla, 2003; Murch and Saxena, 2005; Davey et al., 2006) In addition, the optimum plating density to achieve for culture ranges from $5 \times 10^3 - 1 \times 10^6$. (Bengochea and Dodds, 1986; Veilleux, 2005; Murch and Saxena, 2005) Lastly, protoplast diameter was observed.

Purified protoplasts were centrifuged again at 1,000 RPM for 5 minutes at room temperature and the supernatant was replaced with an equal amount of culture solution consisting of 10% mannitol, 1.5% sucrose, MS salts, and 5mM MES buffer adjusted to a pH of 5.8 ± 0.1. This was supplemented with 2.22µM BA and 4.9µM IBA. (Sujatha and Mukta, 1996) All cultures were placed in microcentrifuge tubes and incubated in the dark at room temperature. Viability was tested every 24 hours using 0.01% FDA, as well as 0.1% calcofluor white for cell wall synthesis under a fluorescent microscope. (Bengochea and Dodds, 1986; Chawla, 2003; Butcher and Ingram, 1976; Neumann, et al., 2009)

In order to increase the yield of viability several factors were added to the isolation protocol. Pre-plasmolysis treatment of leaves by submersion in a mixture similar to that of the isolation solution reduces protoplast damage by sealing plasmodesmata between cells, therefore decreasing the uptake of enzymes during the incubation period. (Murch and Saxena, 2005; Evans and Bravo, 1983) Sterile leaves were submerged in a pre-plasmolysis solution containing MS salts, 13% mannitol, and 5mM MES buffer adjusted to a pH of 5.8 ± 0.1. Leaf tissue was then cut in the pre-plasmolysis solution and allowed to incubate for one hour in the dark. In addition, the isolation solution was purified by centrifuging at 2,500 RPM for 20min at 10 ºC and

the remaining supernatant was used for enzymatic digestion. Also, an antibiotic antimycotic solution formulated to contain 10,000 units/mL penicillin, 10 mg/mL streptomycin, and 25 µg/mL amphotericin B was added all solutions in order to prevent contamination. All solutions were also supplemented with 50µL/mL nystatin. MS salts were diluted by a factor of two so as to reduce the amount of nitrogen in solution, which increases yields and improves regeneration, and 5mM $CaCl_2$ and 5mM $MgSO_4$ was supplemented to the isolation solution to stabilize protoplast membrane permeability. (Fukunaga and King, 1977; Tremblay and Tremblay, 1994; Bengochea and Dodds, 1986; Neumann, et al., 2009; Torres, 1989; Evans and Bravo, 1983)

Tissue Culture

To achieve rapid callus growth ten *J. curcas* hormone protocols were tested and observed. These included: 1) 2.22µM BA and 4.9µM IBA, 2) 4.5µM TDZ, 3) 23.2µM Kn, 4) 8.9µM BA, 5) 9.3µM Kn, 6) 2.27µM TDZ, 2.22µM BA, and 0.49µM IBA, 7) 5.0mg/L BA and 1.0mg/L NAA, 8) 3.0mg/L BA and 1.0mg/L IBA, 9) 1.0mg/L BA and 0.5mg/L IBA, 10) 0.5mg/L BA and 0.5mg/L NAA. (Sujatha and Mukta, 1996; Sujatha et al., 2005; Jha et al., 2007; Deore and Johnson, 2008; Rajore and Batra, 2007; Shrivastava and Banerjee, 2008; Varshney and Johnson, 2010; Nassar et al., 2013) Media consisted of 0.8% agar, 3% sucrose, MS salts, adjusted to a pH of 5.8 $\pm$ 0.1. (Nassar et al., 2013) Leaf discs were cut from third generation leaves and cultured on media in slant tubes. Cultures were grown at 26 $\pm$ 0.5 ºC, under a 16hr photoperiod at 3000 Lux, with a humidity level of ~60% for two weeks. Each hormone protocol was repeated 5 times.

The hormone protocol with the fastest growing calli was chosen to test environmental factors of culturing. These included cutting explants exposed in air, submersed in water, or submersed in a nutrient solution, then culturing in the dark versus under light conditions. The

nutrient solution consisted of 3% sucrose and MS salts adjusted to a pH of 5.8 $\pm$ 0.1. Observations of the cultures were made by counting the number of calli exhibiting symptoms of browning after two weeks according to the following categories: green, yellow-green, yellow-brown, brown, and dark brown. (He et al., 2009) A percentage was calculated by dividing the number of symptomatic calli with the total number of calli in each treatment. Lastly, the fresh weight of 45 calli were taken after two weeks of culture to determine a difference in relation to the environmental factors.

CHAPTER III

RESULTS AND DISCUSSION

Protoplast Isolation, Purification, and Culture

The optimum enzyme combination for protoplast isolation that released the highest number of protoplasts was found to consist of 1% cellulase, 1% pectinase, and 0.2% hemicellulase resulting in an average of 3.07×10^6 protoplasts/mL (Fig. 1). Even though this combination produced the highest number of protoplasts there was no significant difference at $P < 0.05$ using the Kruskal-Wallis test amongst the three types of enzymes in 27 combinations. When individual enzymes were analyzed it appeared that the increase in concentration of pectinase resulted in an increase in protoplasts/mL and as the concentrations of cellulase and hemicellulase increased, the number of protoplasts/mL decreased (Fig. 2). The concentration of buffer that released the most protoplasts and maintained a pH closest 5.8 appeared to be 5mM MES giving an average of 2.89×10^6 protoplasts/mL (Fig. 3 and 4). The concentration of sugar which maintained osmotic stability thus giving the highest number of viable protoplasts was 16% mannitol releasing an average of 5.33×10^4 viable protoplasts/mL (Fig. 5). In addition, both the concentrations of MES buffers and the concentrations of mannitol showed no significant difference at $P < 0.05$ using the Kruskal-Wallis test. However, the combinations with the highest number of viable protoplasts was chosen to continue on with purification studies.

Still the average number of viable protoplasts remained low. This was overcome by the purification of the enzyme solution via centrifugation and the addition of 5mM $CaCl_2$ along with

5mM $MgSO_4$. Other factors included the addition of antibiotic antimycotic solutions and the dilution of MS salts used. These modifications increased the number of viable protoplasts significantly after purification. It was also found that placing tissue and enzyme solution under a vacuum for a brief period before incubation or on a shaker for the duration of incubation reduced the viability of protoplasts.

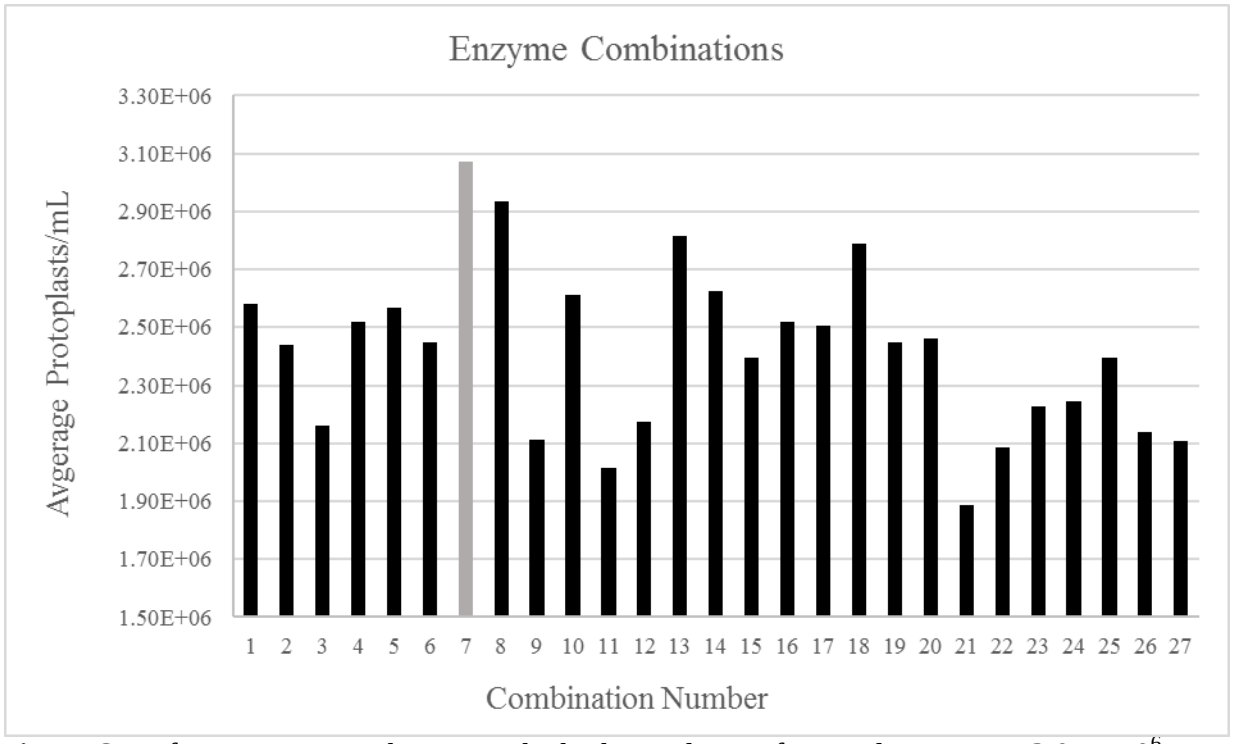

Fig. 1: Out of 27 enzyme combinations the highest release of protoplasts giving 3.07 x 10^6 protoplasts/mL and was achieved using combination number 7 consisting of 1% cellulase, 1% pectinase, and 0.2% hemicellulase. Although this combination was not statistically significant it was used to perform all other tests. All combinations were tested three times.

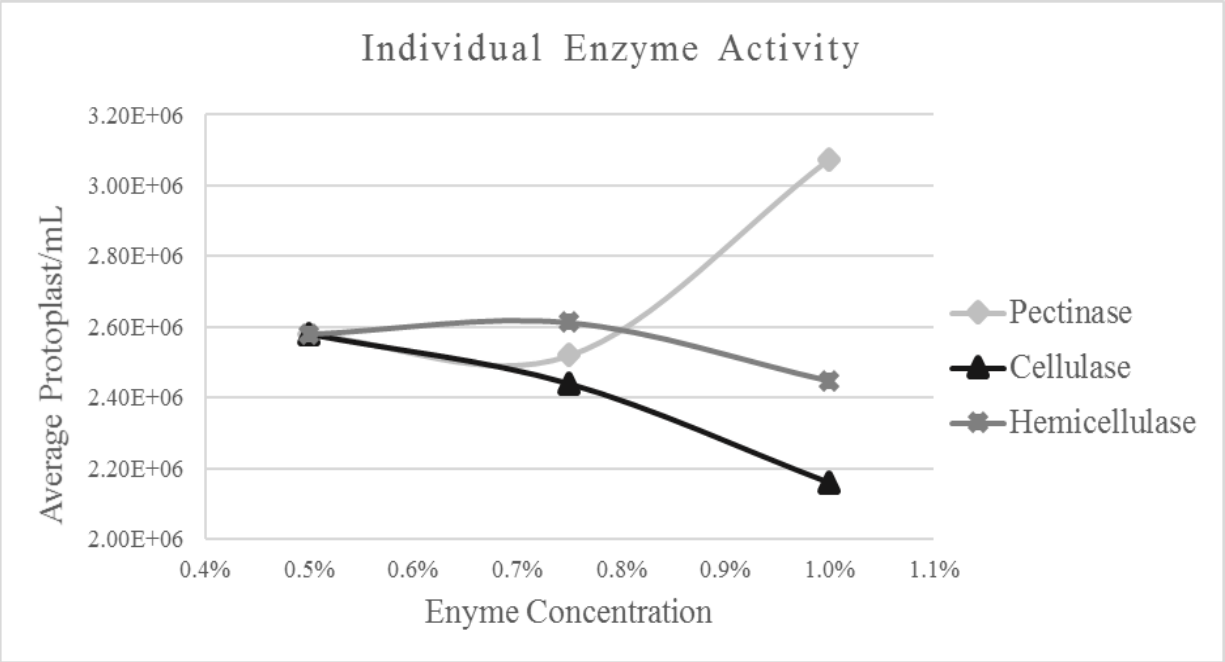

Fig. 2: Although the number of protoplasts increased as the concentration of pectinase increased, the opposite effect was seen as the concentration of cellulase and hemicellulase increased.

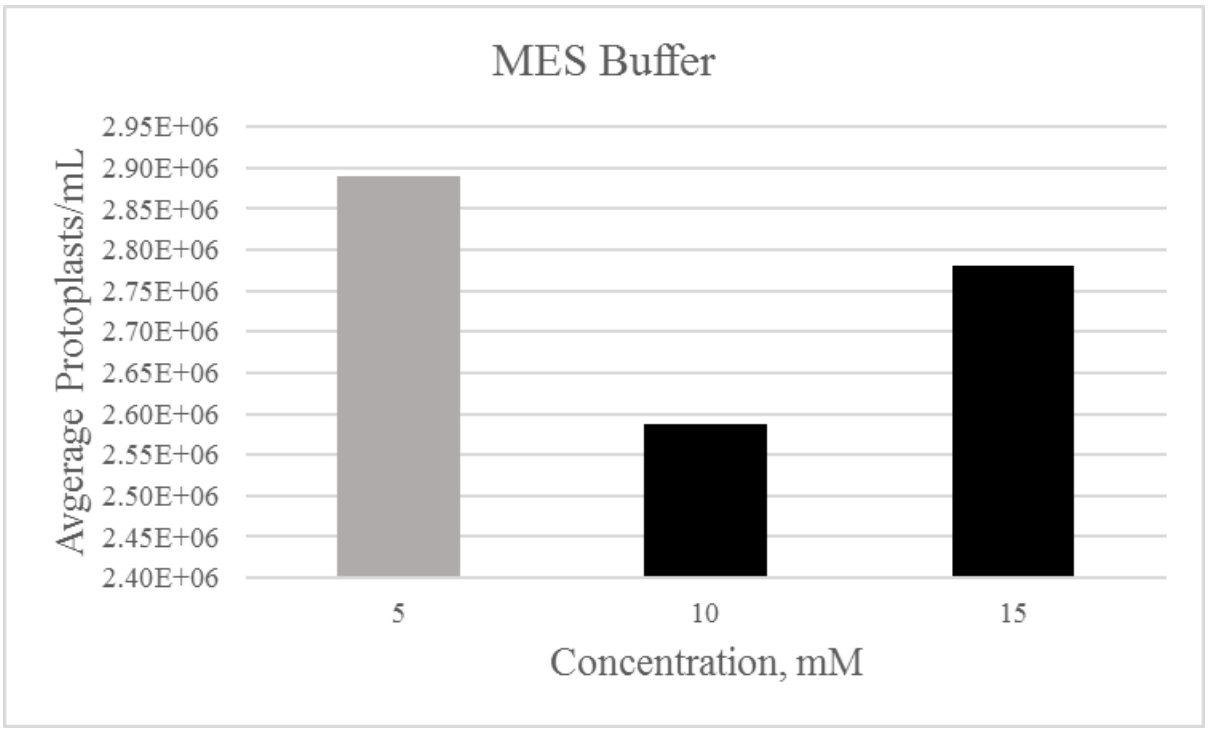

Fig. 3: The concentration of buffer that released the most protoplasts/mL was 5mM. All concentrations were tested three times.

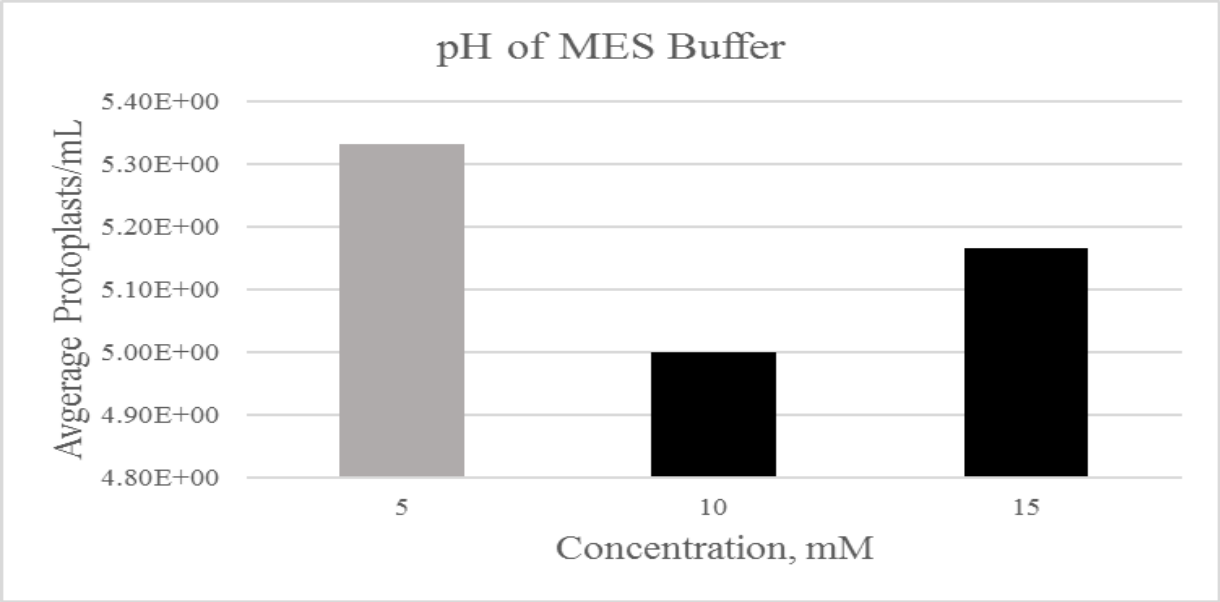

Fig. 4: The concentration of buffer that maintained a pH closer to 5.8 was also 5mM. All concentrations were tested three times.

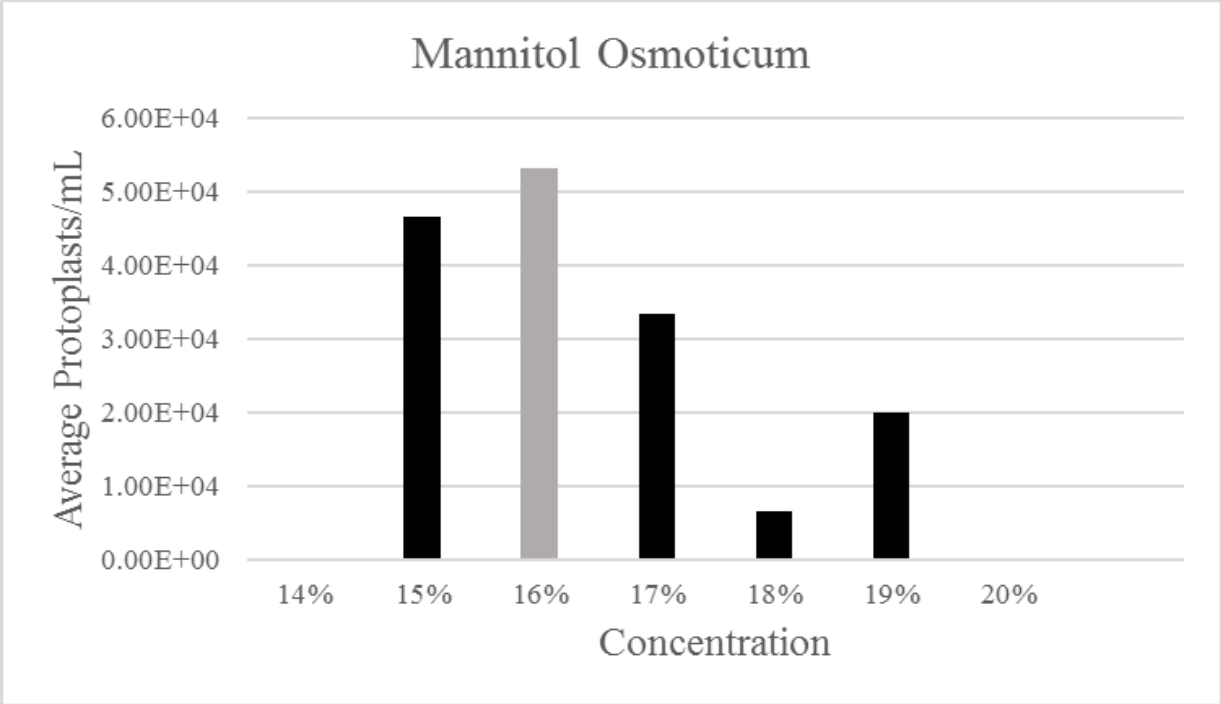

Fig: 5: The concentration of osmoticum that released the most protoplasts/mL was 16%. All concentrations were tested three times.

Several methods of purification were attempted; including washing, filtering, and flotation. Washing left a large amount of debris and filtering showed a low viability of protoplasts; perhaps due to the screens or vacuum pressure damaging the plasma membrane. Therefore, it reasoned that the flotation method of purification would allow for the most viable protoplasts with the least amount of debris. Using this method gave an average of 1.79×10^6 viable protoplasts/mL when the protoplast solution containing 16% mannitol was placed atop a 32% sucrose solution. Unexpectedly, adjusting the mannitol in the protoplast solution to 13% atop a 25% sucrose solution resulted in an average of 3.23×10^6 viable protoplasts/mL bringing the total of viable protoplasts above the initial isolated protoplasts (Fig. 6 and 7). Another anomaly occurred when the average viable protoplast diameter was found to be 1-3µm, whereas other species of plants exhibited protoplasts with diameters ranging from 20-60µm. (Azad, et al., 2006; Balestri, et al., 2001; González-Vallejo, 2000; Kennedy and De Filippis, 2004; Pan, et al., 2005; Rao and Prakash, 1995; Shiba and Mii, 2005) This was also very small compared to reports of *J. curcas* leaf protoplasts, which were 15-25µm in diameter. (Tudses, et al., 2014)

The average protoplast density achieved was well near the high end of the optimum range between $5 \times 10^3 - 1 \times 10^6$. (Bengochea and Dodds, 1986; Veilleux, 2005; Murch and Saxena, 2005) The high average found may be reduced 2 fold and may help to increase viability. Viable protoplasts were then transferred to microcentrifuge tubes in culture solution. After 24 hours the viability of protoplasts decreased significantly and had diminished completely after 2 days. Also, cell wall synthesis was tested using 0.1% calcofluor white under a fluorescent microscope. (Bengochea and Dodds, 1986; Chawla, 2003; Butcher and Ingram, 1976; Neumann, et al., 2009)

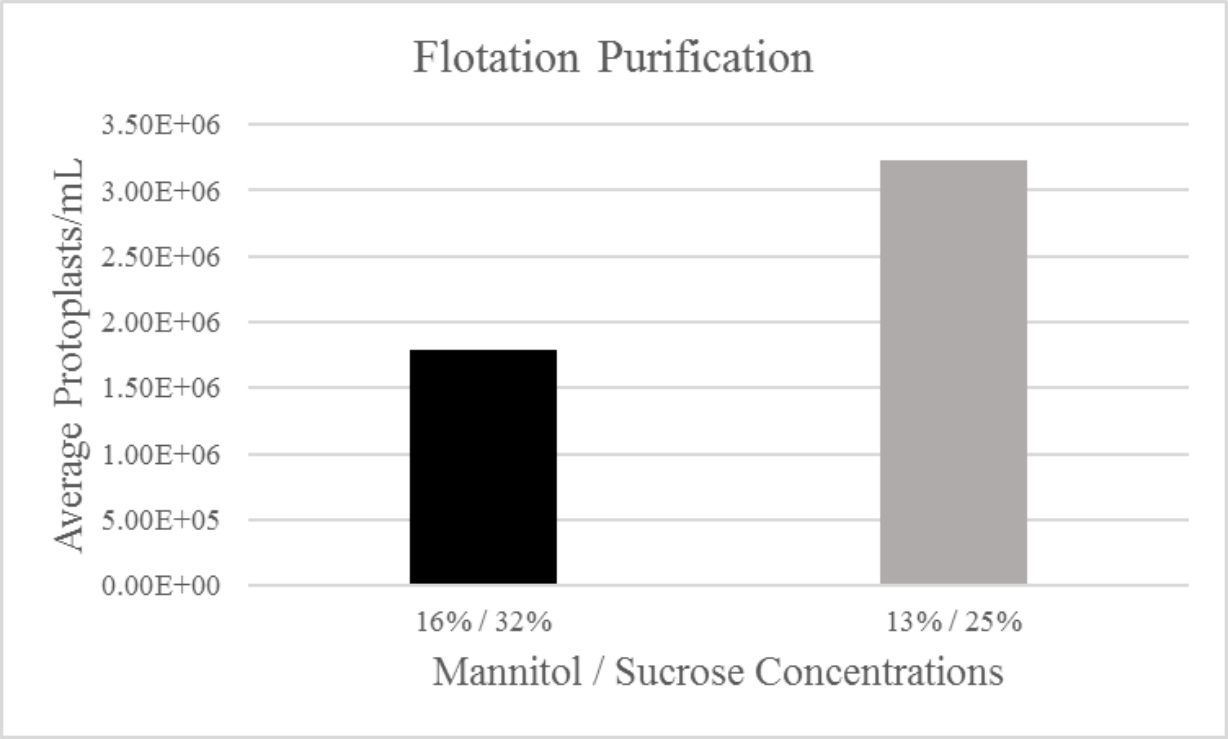

Fig. 6: Protoplasts were isolated and purified in a 13% mannitol solution then laid atop a 25% sucrose solution that resulted in 3.23 x 10^6 viable protoplasts/mL versus 1.79 x 10^6 viable protoplasts/mL when isolating and purifying in a 16% mannitol solution laid atop a 32% sucrose solution.

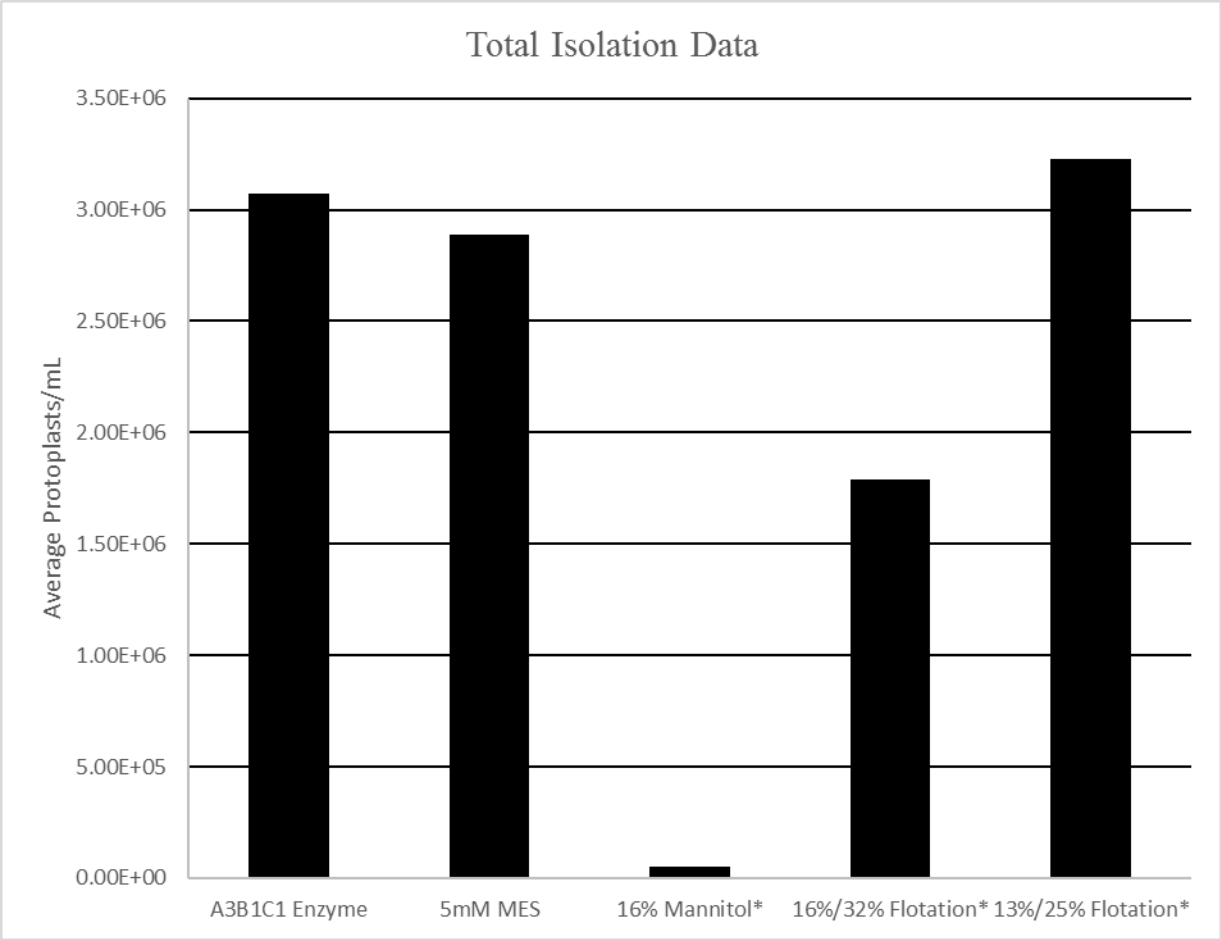

Fig. 7: The increase of protoplasts/mL was visible over the progression of isolation. *indicates when viability was tested.

Cell wall formation was observed briefly, but diminished as well and may have been a remnant of digestion. Under optimum conditions cell division occurs within 2 to 10 days, then colonies form in the following 2 to 3 weeks, microcalli form in another 4 weeks, then shoots arise within a month. (Veilleux, et al., 2005; Neuman, et al., 2009)

Other culture methods were attempted, which included hanging drops from petri dish lids, micro-chambers built out of slides, and glass test tubes. Each system tried had a purpose and a limitation. The hanging drop method was attempted first in order to observe protoplasts in culture under light microscope without disturbing culture conditions. This proved difficult due to volume of culture being low (in the 25-50uL range) and the displacement of drops during

observation. Micro-chambers were built by using deep cavity slides and coverslips sealed with petroleum jelly. However, these micro-chambers became contaminated rapidly and cultures deceased. Glass test tubes were used for cultures to analyze chlorophyll fluorescence using a fluorometer. Chlorophyll fluorescence may comparable to viable yields of protoplasts, which may eventually determine growth rate without disturbing culture. However, this method was terminated due to the requirement of large volumes and the excessive amount of enzymes to achieve this. Observations on cultures in 9 various hormonal concentrations revealed a parallel correlation between a decrease in protoplasts and chlorophyll fluorescence over a span of two days until cultures diminished.

One factor recognized as being detrimental to protoplast viability may be the use of antiobiotics and/or antimycotics. These may have placed high amounts of stress on newly exposed plasma membranes. Even the solvents in which these were dissolved in may be harmful. For example, the nystatin suspension used in isolation and culture required dimethyl sulfoxide (DMSO) to dissolve the powder. Recently, this has been reported to inhibit peroxidase activity in *J. curcas*. (Cai, et al., 2012) Lastly, one factor that may have been helpful to include in the culturing process is placing protoplast solutions on a shaker as done when preparing a cell suspension. However, this would require a large volume of protoplasts.

Tissue Culture

Out of the ten hormone protocols tested the fastest growing callus observed was found using 0.5mg/L BA and 0.5mg/L NAA with callus growth in 15 days and 45 discs were cultured for further observations. (Nassar et al., 2013) This hormone combination is different to all previous tissue cultures with *J. curcas* done in our lab before using 2.22µM BA and 4.9µM IBA. (Sujatha and Mukta, 1996) All following explants were either cut under exposure to air, under

submersed in water, or submersed in a nutrient solution, then cultured in the dark versus under light conditions. Observations were made using the categories green, yellow-green, yellow-brown, brown, and dark brown. (He et al., 2009) The following results were recorded after 2 weeks of culturing under light conditions: 65% remained green when cut exposed to air with the remaining 35% turning yellow-green, % were yellow-green when cut submersed under water with 91% turning brown, and 51% remained green when cut submersed under nutrient solution with the remaining 49% turning yellow-green (Table 1).

Light	Air	Water	Nutrient Solution
Green	65%	-------	51%
Yellow-Green	35%	9%	49%
Brown	------	91%	----------
Dark	Air	Water	Nutrient Solution
Green	71%	18%	27%
Yellow-Green	29%	82%	73%
Brown	------	------	----------

Table 1: Percentage of calli exhibiting conditions of browning under various excision and environmental conditions.

Under dark conditions the following resulted: 71% remained green when cut exposed to air with 29% turning yellow-green, 18% remained green when cut submersed under water with 82% turning yellow-green, and 27% remained green when cut submersed under nutrient solution with the remaining 73% turning yellow-green. In both light and dark conditions those cut exposed to air had a higher percentage of green calli. The most negative effects were seen from cutting while submerged in water under both light and dark conditions. The negative effects from cutting submerged in water may be a result of hyperhydricity seen in the lysis of cells on the outer edge of cut explants. The viability of calli from explants cut under submersion in nutrient solution was expected to increase, whereas it remained fairly neutral under light conditions and slightly detrimental under dark conditions. Therefore, the normal protocol for cutting exposed to air under light conditions remained the most beneficial. However, the measurements of total fresh weight gained after two weeks in culture under the different environmental factors

concluded no varying differences between light and dark conditions giving an average increase of 3.51×10^{-2}g and 3.28×10^{-2}g amongst 45 discs cultured, respectively. The highest average increase of total fresh weight observed was 5.96×10^{-2}g when explants were cut submerged in a nutrient solution and cultured under dark conditions. The combination of the nutrient solution explant preparation and culturing in the dark was expected to increase weight because of the following: a) exposure of the cut tissue to air would be limited in a solution, b) cut tissue supplemented by nutrient solution would ease the transition to media, and c) culturing in the dark would omit the negative effects of photooxidation.

Potential Use of Antioxidants

Antioxidants indirectly aid in stopping ROS by blocking the formation of its precursors such as H_2O_2. (Yinghui, 2008) They also inhibit phenolic compounds by lowering their redox potential and may curb the effects of the oxidative stress in plant tissue cultures such as browning. The use of antioxidants on *J. curcas* tissue cultures could lead to their application in protoplast isolation and culture. There are many antioxidants used in present day tissue culture. Some are traditional, such as citric acid and ascorbic acid; while others are contemporary such as cysteine, glutathione, and dithiothreitol (DTT). Below is a description of each type of antioxidant mentioned, along with several others, that have been reported to alleviate browning in tissue culture.

Citric acid is one of many organic acids found in the Krebs' cycle that have been investigated for nutrient requirements in plant tissue culture media. Studies have shown that the addition of citric acid to media promotes the most growth stimulation out of any other organic acid involved in the Krebs cycle. (Thorpe, et al., 2008) It has been reported to decrease browning and increase organogenesis along with somatic embryogenesis and enhance shoot

growth in a number of plant species. (Yinghui, 2008) Studies of its use in the culturing of *Citrus* sp. cultures showed an increase in overall growth of calli. (Thorpe, et al., 2008) Citric acid is often used in the pretreatment of explant material and is commonly used in combination with ascorbic acid. (He, et al., 2009) For example, *Adonis amurensis* explants that were pretreated with this combination displayed an increase in survival rate of about two times higher than untreated control groups. (Yinghui, 2008) Citric acid has been used in media in ranges of 50mg/l up to 1.5g/L. (Bonga, 1981; Ahmad, et al., 2013; Çördük and Aki, 2011; Chawla, 2003; Buendia-Gonzalez, et al., 2007; Yinghui, 2008; Narayanaswamy, 1994; He, et al., 2009) One downside of using citric acid is that it increases the pH of media after being autoclaved.

Ascorbic acid is found in plant cells at relatively high concentrations. It acts as a catalyst in photosynthetic phosphorylation and serves in electron transfer from NADPH to oxygen through cyclic oxidation-reduction. (Torres, 1989) It acts as a reductant by converting into dehydroascorbic acid after it becomes oxidized and is reconverted back to ascorbic acid by the enzyme dehydroascorbate reductase that inadvertently oxidizes glutathione; another antioxidant discussed later. (George and Davies, 2008) Also, it protects cells against oxidative injury by scavenging free radicals, like H_2O_2. (George and Davies, 2008) Studies have shown ascorbic acid to be involved in the initiation of cell division and elongation in tobacco cells, as well as an increase in the cell number of carrot tissue grown *in vitro*. (Thorpe, 2008; Torres, 1989) Ascorbic acid has been reported to stop the formation of melanin thereby improving the color and increase the yields of calli of tobacco. (Torres, 1989) It also been known to prevent browning by decreasing oxidized substrates at target sites, like the leaves of shoots, through absorption and translocation by the plantlet. (Ahmad, et al., 2013) As mentioned previously, ascorbic acid has been used in combination with citric acid in the pretreatment of explants and

one example of this is seen in the decrease of browning in *Pinus patula* explants, although the percentage of surviving cultures decreased. (Malabadi and Van Staden, 2005) In another example, the pretreatment of *Protea cynaroides* explants in a combination of ascorbic acid and citric acid had a 100% survival rate while only 20% survived without pretreatment. (Yihghui, 2008) Ascorbic acid has been used in media in ranges of 1mg/L up to 1g/L. (Beyl, 2005; Thorpe, et al., 1998; Ahmad, et al., 2013; Çördük and Aki, 2011; Chawla, 2003; Buendia-Gonzalez, et al., 2007; Yinghui, 2008; Narayanaswamy, 1994; Agrawal and Purohit, 2012) In cultures of *J. curcas*, the addition of 10mg/L of ascorbic acid in combination with 25mg/L of glutathione proved beneficial for proliferating shoots and increasing antioxidant enzymes, like superoxide dismutase, while control shoots exhibited higher H_2O_2 and phenolic compounds. (Misra, et al., 2010)

Cysteine is an amino acid containing sulfur used by plants during lipid synthesis and protein structure regulation of the S-S bridges. (George and de Klerk, 2008) As an antioxidant it has been shown to enhance shoot and root growth in multiple species. (Yinghui, 2008) Cysteine has also been used in pretreatment of explants to manage phenolic oxidation. (Malabadi and Van Staden, 2005; Narayanaswamy, 1994) However, negative effects of cysteine have also been observed as seen in the increase of browning was reported when added to cultures of bamboo. (Ahmad, et al., 2013) Cysteine is used in media at ranges of 10mg/L up to 100mg/L. (Bonga, 1981; Narayanaswamy, 1994; He, et al., 2009) In addition, it showed no effects on the browning of *J. curcas* tissue cultures when used at low concentrations. (Misra, et al., 2010) Although, it has been reported to increase protoplast yield in some species; perhaps by aiding during the initial wound response. (Neumann, et al., 2009)

Glutathione is also a sulfur containing compound, like cysteine, and plays a role in the detoxification of oxygen radicals. (George and de Klerk, 2008) It is known to react quickly with free radicals formed when DNA is attacked by OH' groups. (Yingui, 2008) When implemented in tissue culture at high levels it is thought to keep enough NADPH available for use as an electron carrier during photosynthesis. (George and de Klerk, 2008) Glutathione has been reported to suppress hyperhydricity of calli and prevented browning of shoot tips when pretreated in glutathione. (Yingui, 2008) It is also known to maintain protein synthesis, cell elongation, and cell division. (George and de Klerk, 2008) As an antioxidant it reacts with phenols after cutting of explants and in turn restores enzyme activity of the plant cells. (Narayanaswamy, 1994) There are several conflicting reports on the actions of glutathione as an antioxidant. For example, one report states that it performs as a reducing agent that may promote callus growth by inhibiting embryogenesis while another states it promotes embryogenesis when used at low concentrations. (George and Davies, 2008; Agrawal and Purohit, 2012) A different study revealed that the pretreatment of explants with glutathione inhibit embryogenesis, but increase shoot formation. (Dutta Gupta and Datta, 2002) When glutathione was added to cultures of *Dianthus caryophyllus*, both embryogenesis and shoot formation proliferated. (Agrawal and Purohit, 2012) It has been used in media at concentrations of 0.1, 0.5, and 1mM. (Dutta Gupta and Datta, 2002; Agrawal and Purohit, 2012) As mentioned previously, cultures of *J. curcas*, the addition of 25mg/L of glutathione in combination with 10mg/L of ascorbic acid proved beneficial for proliferating shoots and increasing antioxidant enzymes, like superoxide dismutase, while control shoots exhibited higher H_2O_2 and phenolics. (Misra, et al., 2010)

DTT is a reagent used to keep SH groups in a reduced state and in turn reduces disulfides. It is often used in place of β-mercaptoethanol because it is less toxic and pungent; although still

quite potent. As an antioxidant it can reduce or even prevent browning via lowering the redox potential when used as a pretreatment of explants. (Torres, 1989) Studies have shown that DTT can reduce the accumulation of peroxidase in cultures, thereby inhibiting browning and along with it improve callus formation, shoot growth, and rooting. (Tang, et al., 2004) DTT also reacts with the phenols released after explant cutting and restore enzymatic activity of cultures. (Narayanaswamy, 1994) This has been observed in the reduction of browning in pretreated and cultured explants of *Strelitzia reginae* using 0.04% DTT. (Yinghui, 2008) As of present, no studies of its use in *J. curcas* cultures have been reported.

Other antioxidants include α-tocopherol (vitamin E), which has been reported to inhibited lipid peroxide formation. (Yinghui, 2008) The addition of vitamin E to cultures of *J. curcas* resulted in sturdier shoots with an increased number of leaves. (Misra, et al., 2010) One more antioxidant used is malonic acid. It has been shown to initiate callus growth when used as a pretreatment for conifer explants at a concentration of 1g/L. (Bonga, 1981) Its action is not combating any oxidation, but based primarily on activating plant tissue morphogenesis. Also, it is known that phenolic compounds are produced through the malonic acid pathway. (Jha and Ghosh, 2005) Therefore, the addition of malonic acid may act as a product in a negative feedback system that stops the production of phenolic compounds.

There are several other chemicals used to regulate browning, such as activated charcoal and polyvinylpyrrolidone (PVP), which are added to media and bind to phenolic compounds through adsorption. This directly reduces the leaching of phenolic compounds to other explants in culture, but has no systemic effect on cultures themselves. Typically, PVP is preferred over activated charcoal because it is less messy to work with and activated charcoal settles in media. When activated charcoal does not subside the effects of browning PVP may be used in

concentrations of 250-1000 mg/L. (Beyl, 2005) Lastly, the MES buffer used in protoplast solutions may also be used in media to prevent browning, due to its pH range of 5.5-6.7. Generally, plant tissue culture media is adjusted to 5.8 $\pm$ 0.1, therefore browning may be reduced solely by using MES as a buffer to keep the pH within this optimum range. The addition of 1g/L of MES was shown to reduce browning in cultures of *Sideritis trojana*. (Çördük and Aki, 2011)

Browning can also be reduced by adjusting environmental factors before, after, and during culture. These include the pretreatment of explants by submersion for several minutes up to 24 hours in an antioxidant solution. Similarly, explants may also be cut while being submersed in an antioxidant solution. By limiting the exposure of air to the explant while cutting the effects of browning may be subsided. Another environmental factor that may be adjusted to reduce browning is the exposure to light during culture. Many cultures are grown in complete darkness for the first stages of development. This is mainly due to photooxidation, which states that overexposure to light promotes phenolic oxidation and leads to browning. Culturing in the dark results in a decrease of photosynthetic activity and chlorophyll content, while carotenoid pigments remain. Also, culturing in darkness helps to maintain the pH of cultures media. Lastly, transferring cultures to fresh media every 3-7 days instead of the traditional 2 weeks will lessen browning by simply relieving the cultures from the surrounding phenolic compounds released into the media.

Further Research

The potential for advancements in controlling browning of *J. curcas* tissue cultures with antioxidants may be achieved by observing biomass, performing chlorophyll analysis, and examining peroxidase activity. Biomass may be recorded as fresh weight or dry weight. However, dry weight would give a better estimate of the increase in tissue growth by reducing

the water weight from the calculation. Nonetheless, fresh and dry net weights should be congruent with its respective increase or decrease within each condition tested. Growth rates can be determined by subtracting final weights from initial weights of fresh or dry calli over time. A growth index (GI) may also be calculated by applying the following formula for each condition tested: $GI = (W_F - W_I) / W_I$; where W_F and W_I represent the final and initial weights. (Dodds and Roberts, 1985; Godoy-Hernández and Vázquez-Flota, 2006) Any effects of antioxidants on biomass of browning in tissue culture may reveal future additions to optimize conditions for culture.

Chlorophyll analysis of calli may be done by preparing an extract of tissue in a solvent, such as acetone, and reading the absorbencies at 645nm and 663nm for chlorophyll *a* and chlorophyll *b* respectively. (Parwata et al., 2012; Yamada and Sato, 1983) Calculating the amount of chlorophyll may be determined using a number of equations with varying coefficients. Explicitly Arnon's equation is used, but more recently the equations by Lichtenthaler and Porra have more specific coefficients. A study of chlorophyll in *J. curc*as used Lichtenthaler's formula of chlorophyll *a* (µg/mL) = $12.25\ A_{663} - 2.79\ A_{646}$ and chlorophyll *b* (µg/mL) = $21.50_{646} - 5.10\ A_{663}$. (Lichtenthaler and Buschmann, 2001; Parwata et al., 2012; Pompelli, et al., 2010) Chlorophyll content of *J. curcas* varies throughout literature. One of these studies showed *J. curcas* leaf chlorophyll content was at 0.039 mg/g whereas under optimum watering conditions it was 0.042mg/g. (Parwata, et al., 2012) A study revealed higher chlorophyll readings under optimum conditions, such as 2.34 mg/g. (Jamil, et al., 2009) The carotenoid content may also be determined using Lichtenthaler's equation by recording absorbencies at 470nm and using the following formula: $c_{(x+c)}$ (µg/mL) = $(1000\ A_{470} - 1.82\ c_a - 85.02\ c_b)/198$. (Lichtenthaler and Buschmann, 2001) Carotenoid content is important to understanding browning because it is

responsible for scavenging oxidants and may serve as photoprotective factor in plant cells under stress. (Pompelli, et al., 2010)

Peroxidase activity is also significant in studying the effects of browning in that its level of content is directly related to signs of stress in plants. Hence a higher level of peroxidase may indicate a reaction to an abundance of oxidants attacking cell components. To measure peroxidase activity an extract another extract must be made, but within a buffer solution at a pH of 7.0 that is ideal for the peroxidase enzyme. To quantify the amount of peroxidase in the extract the absorbance can be read by adding H_2O_2 to catalyze a reaction along with the addition of the substrate guaiacol. When, guaicol reacts with H_2O_2 it becomes oxidized and produces tetraguaiacol which is brown in color that has an absorbance of 470nm. (Liu, et al., 2011; Verma, et al., 2014) The intricacy of testing peroxidase activity is first establishing a baseline of the extract volume. This is found by measuring the absorbance of increasing volumes of extract from 0.1mL to 1mL every 30 seconds for 5 minutes. The volume with the highest R^2 value, which is most linear over time, is chosen for further testing. Any peroxidase activity found may only be relative to the amount of peroxidase under the normal conditions of a control group. There have been up to 35 different types of peroxidase enzymes found in plants. (Cowen, 1990) Recently, a novel thermal stable peroxidase was discovered in *J. curcas*, however its activity is inhibited by DTT. (Cai, et al., 2012)

CHAPTER IV

CONCLUSION

Although *J. curcas* already bears many attributes as an oilseed crop for biodiesel production such as, drought tolerance, ability to grow on marginal land, and is non-edible. It still has the potential for advancements. Improving the species, whether it be through the fusion of protoplasts to form polyploids or by transformation via insertion of genes, requires first and foremost the establishment of a working culture protocol. The addition of antioxidants to protoplast culture solutions may reduce stress induced by browning and increase viability. Candidates for antioxidants may be determined by investigating their effects on browning in *J. curcas* tissue culture for further use in protoplast culture systems. Successful protoplast to plant regeneration in *J. curcas* could make it possible to manipulate this species through genetic engineering. With this stated, it still remains that "protoplast isolation and culture is still empirical, and as such appears to be more an artistic method than a scientific one." (Evans and Bravo, 1983)

REFERENCES

Agrawal M, Purohit S (2012) Changes in antioxidant enzymes activity during *in vitro* morphogenesis of carnation and the effect of antioxidants on plant regeneration. World Journal of Science and Technology 2(7): 87-92

Ahmad I, Hussain T, Ashraf I, Nafees M, Maryam, Rafay M, Iqbal M (2013) Lethal effects of secondary metabolites on plant tissue culture. American-Eurasian Journal of Agriculture & Environmental Sciences 13(4): 539-547

Azad MAK, Yokota S, Ishiguri F, Yoshizawa N (2006) Plant Regeneration from mesophyll protoplasts of a medicinal plant, *Phellodendron amurense* Rupr. *In vitro* Cellular & Development Biology, Plant 42(6): 502-507

Baldi A, Bisaria VS, Srivastava AK (2007) Biotechnological approaches for the production of some promising plant-based chemotherapeutics. In Kayser O, Quax W, eds, Medicinal Plant Biotechnology, Volume 1, Wiley-VCH, Weinheim, pp 117-147

Balestri E, Luccarini G, Cinelli F (2001) Isolation of Leaf Protoplasts from Pancratium maritimum L. and two other dune plants: Possible applications. Journal of Coastal Research 17(1): 188-194

Basha SD, Sujatha M (2009) Genetic analysis of *Jatropha* species and interspecific hybrids of *Jatropha curcas* using nuclear and organelle specific markers. Euphytica 168: 197-214

Bengochea T, Dodds JH (1986) Plant Protoplasts: A Biotechnological Tool for Plant Improvement. Chapman and Hall Ltd, London

Beyl CA (2005) Getting started with tissue culture: Media preparation, sterile technique, and laboratory equipment. In Trigiano RN, Gray DJ, eds, Plant Development and Biotechnology, CRC Press, Boca Raton, pp 19-37

Bonga, JM (1981) Organogenesis in vitro of Tissues from Mature Conifers. In Vitro 17(6): 551-518

Buendía-González L, Orozco-Villafuerte J, Cruz-Sosa F, Chávez-Ávila VM, Vernon-Carter EJ (2007) Clonal propagation of mesquite tree (Prosopis laevigata Humb. & Bonpl. Ex Willd. M. C.Johnston). I. Via cotyledonary nodes. *In Vitro* Cellular & Developmental Biology. Plant 43(3): 260-266

Butcher DN, Ingram DS (1976) Plant Tissue Culture. Edward Arnold, London

Cai F, OuYang C, Duan P, Gao S, Xu Y, Chen F (2012) Purification and characterization of a novel thermal stable peroxidase from *Jatropha curcas* leaves. Journal of Molecular Catlysis B: Enzymatic 77: 59-65

Chawla HS (2003) Plant Biotechnology: A Practical Approach. Science Publishers, Inc, Enfield

Çördük N, Aki C (2011) Inhibition of browning problem during micropropagation of *Sideritis trojana* bornm., an endemic medicinal herb of Turkey. Romanian Biotechnological Letters 16(6): 6760-6765

Cowen R (1990) Droopy plants drop hints of enzyme's role. Science News 137(6)

Cutler AJ, Saleem M, Wang H (1991) Cereal protoplast recalcitrance. *In Vitro Cellular & Developmental Biology. Plant* 27(3) 104-111

Davey MR, Anthony P, Power JB, Lowe KC (2006) Isolation, culture, and plant regeneration from leaf protoplasts of *Passiflora*. In Loyola-Varga VM, Vázquez-Flota FA, eds, Plant Cell Culture Protocols 2nd Ed., Humana Press Inc., Totowa, pp 201-210

Deore AC, Johnson TS (2008) High-frequency plant regeneration from leaf-disc cultures of *Jatropha curcas* L.: an important biodiesel plant. Plant Biotechnology Reports 2: 7-11

Dodds JH, Roberts LW (1985) Experiments in Plant Tissue Culture 2nd Edition. Cambridge University Press, Cambridge

Evans DA, Bravo JE (1983) Protoplast isolation and culture. In Evans DA, Sharp WR, Ammirato PV, Yamada Y, eds, Handbook of Plant Cell Culture, Volume 1, Techniques for Propagation and Breeding. Macmillan Publishing Co., New York, pp 124-176

Evans DA, Bravo JE (1983) Protoplast isolation and culture. In Giles KL, ed, Plant Protoplasts, Supplement 16, Academic Press, New York, pp 33-53

Frazão LA, Cenciani K, Chiavegato MB, Clemente CC, Feigl BJ, P. Cerri CE (2010) Land use in production of raw materials for biofuels. In R Lal, B.A. Stewart, eds, Soil Quality and Biofuel Production, CRC Press, Boca Rotan, pp 93-113

Fukunaga Y and King J (1977) Effects of different nitrogen sources in culture media on protoplast release from plant cell suspension culutres. Plant Science Letters 11: 241-250

George EF, Davies W (2008) Effects of the physical environment. In George EF, Hall MA, De Klerk G, eds, Plant Propagation by Tissue Culture, Ed 3 Vol 1. Springer, Dordrecht, pp 423-464

Godoy-Hernández G, Vázquez-Flota FA (2006) Growth measurements: Estimation of cell division and cell expansion. In Loyola-Varga VM, Vázquez-Flota FA, eds, Plant Cell Culture Protocols 2nd Ed., Humana Press Inc., Totowa, pp 51-58

González-Vallejo EB, Morales F, Cistué L, Abadía A, Abadía J (2000) Plant Physiology 122(2): 337-344

Gu H, Jiang Y, Zhou L, Gao J (2011) Reactive extraction and in situ self-catalyzed methanolysis of germinated oilseed for biodiesel production. Energy Environmental Science 4: 1337-1344

He Y, Guo X, Lu R, Niu B, Pasapula V, Hou P, Cai F, Xu Y, Chen F (2009) Changes in morphology an biochemical indices in browning callus derived from *Jatropha curcas* hypocotyls. Plant Cell, Tissue and Organ Culture 98: 11-17

Heller J (1996) Physic Nut. *Jatropha curcas* L. Promoting the conservation and use of underutilized and neglected crops. Institute of Plant Genetics and Crop Plant Research, Getersleben / International Plant Genetic Resources Institute, Rome

Jamil S, Abhilash PC, Singh N, Sharma PN (2009) *Jatropha curcas*: A potential crop for phytoremediation of coal fly ash. Journal of Hazardous Materials 172: 269-275

Jha TB, Mukherjee P, Datta MM (2007) Somatic embryogenesis in *Jatropha curas* Linn., an important biofuel plant. Plant Biotechnology Reports 1: 135-140

Joshi M, Mishra A, Jha B (2011) Efficient genetic transformation of *Jatropha curcas* L. by microprojectile bombardment using embryo axes. Industrial Crops and Products 33: 67-77

Kane ME (2005) Shoot culture procedures. In Trigiano RN, Gray DJ, eds, Plant Development and Biotechnology, CRC Press, Boca Raton, pp 145-157

Kapur R, Saleem M, Harvey BL, Cutler AJ (1993) Oxidative metabolism and protoplast culture. *In Vitro* Cellular & Developmental Biology. Plant 29(4) 200-206

Kennedy BF, De Filippis LF (2004) Tissue degradation and enzymatic activity observed during protoplast isolation in two ornamental grevillea species. *In Vitro* Cellular & Developmental Biology, Plant 40(1): 119-125

Kohli A, Raorane M, Popluechai, Kannan U, Syers JK, O'Donnel AG (2009) Biofuels: *Jatropha curcas* as a novel, non-edible oilseed plant for biodiesel. In Ferry N, Gatehouse AMR, eds, Environmental Impact of Genetically Modified Crops, CAB International, Oxfordshire, pp 296-324

Kumar R, Elangovan V (2010) Assessment of the volumetric attributes of Eri silkworm (*Philosamia ricini*) reared on different host plants. International Journal of Science and Nature 1(2): 156-160

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): 1-10

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

Lichtenthaler HK, Buschmann C (2001) Current Protocols in Food Analytical Chemistry. F4.31—F4.38 Supplement 1

Liu C, Liu Y, Guo K, Fan D, Li Guoquing, Sheng Y, Yu L, Yang R (2011) Effect of drought on pigments, osmotic adjustment and antioxidant enzymes in six woody plant species in karst habitats of southwestern China. Environmental and Experimental Botany, 71: 174-183

Maes WH, Achten WMJ, Reubens B, Raes D, Samson R, Muys B (2009) Plant-water relationships and growth strategies of *Jatropha curcas* L. seedlings under different levels of drought stress. Journal of Arid Environments 73: 877-884

Malabdi RB, Van Staden J (2005) Role of antioxidants and amino acids on somatic embryogenesis of *Pinus patula*. *In Vitro* Cellular & Developmental Biology, Plant 41(2): 181-186

Misra P, Toppo DD, Gupta N, Chakrabarty D, Tuli R (2010) Effect of antioxidants and associate changes in antioxidant enzymes in controlling browning and necrosis of proliferating shoots of elite *Jatropha curcas* L. Biomass and Bioenergy 34(12): 1861-1869

Murch SJ, Saxena PK (2005) Journey of a Single Cell to a Plant. Science Publishers, Inc., Enfield, NH

Narayanaswamy S (1994) Plant Cell and Tissue Culture. Tata McGraw-Hill, New Dehli

Nassar SA, El-Ahmady SH, Nassar AH, Al-Azizi MM (2013) Studying the possible biotransformation of the cytotoxic diterpenoid paclitaxel using *Jatropha curcas* cell suspension culture. European Journal of Medicinal Plants 3(2): 241-253

Neumann K, Kumar A, Imani J (2009) Plant Cell and Tissue Culture- A Tool in Biotechnology: Basics and Application. Springer, Berlin

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

Pahl G (2005) Biodiesel: Growing a New Energy Economy. Chelsea Green Publishing Company, White River Junction

Pan ZG, Liu CZ, Murch SJ, Saxena PK (2005) 2004 SIVB Congress symposium proceedings "Thinking outside the cell": Optimized chemodiversity in protoplast-derived lines of St. John's Wort (*Hypericum perforatum* L.) *In Vitro* Cellular & Developmental Biology, Plant 41(3): 226-231

Papadakis AK, Siminis CI, Roubelakis-Angelakis KA (2001) Reduced activity of antioxidant machinery is correlated with suppression of totipotency in plant protoplasts. Plant Physiology 126(1): 434-444

Parmar HK, Johari M, Rathore BS (2014) Anthelmintic activity of latex of *Jatropha curcas* (ratanjot). International Journal of Pharma Sciences and Research 5(3): 100-101

Parwata IGMA, Indradewa D, Yudono P, Ketonegoro BD, Kusmarwiyah R (2012) Physiological responses of *Jatropha* to drought stress in coastal sandy land conditions. Makara Journal of Science 16(2): 115-121

Pitkin RB (1990) Introductory Biology Laboratory Manual. Shippensburg University, Shippensburg, PA.

Pompelli MF, Barata-Luís R, Vitorino HS, Gonçalves ER, Rolim EV, Santos MG, Almeida-Cortez JS, Ferreira VM, Lemos EE, Endres L (2010) Photosynthesis, photoprotection and antioxidant activity of purging nut under drought deficit and recovery. Biomass and Bioenergy 34: 1207-1215

Prabakaran AJ, Sujatha M (1999) *Jatropha tanjorensis* Ellis & Saroja, a natural interspecific hybrid occurring in Tamil Nadu, India. Genetic Resources and Crop Evolution 46: 213-218

Prakash AH, Rao KS, Kumar MU (1997) Plant regeneration from protoplasts of *Capsicum annuum* L. cv. California Wonder. Journal of Biosciences 22(3): 339-344

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

Rao KS, Prakash AH (1995) A simple method for the isolation of plant protoplasts. Journal of Biosciences 20(5): 645-655

Ross IA (1999) Medicinal Plants of the World: Chemical Constituents, Traditional and Modern Medicinal Uses. Humana Press, New Jersey

Scheffran J (2010) Criteria for a sustainable Bioenergy infrastructure and lifecycle. Mascia PN, Scheffran J, Widholm JM, eds, Plant Biotechnology for Sustainable Production of Energy and Co-products, Springer, Heidelberg, pp 409-443

Shiba T, Mii M (2005) Plant regeneration from mesophyll- and cell suspension-derived protoplasts of *Dianthus acicularis* and characterization of regenerated plants. *In Vitro* Cellular & Developmental Biology, Plant 41(6): 794-800

Shrivastava S, Banerjee M (2008) *In vitro* Clonal propagation of physic nut (*Jatropha curcas* L.): Influence additives. International Journal of Integrative Biology 3(1): 73-79

Sujatha M, Makkar HPS, Becker K (2005) Shoot bud proliferation from axillary nodes and leaf sections of non-toxic *Jatropha curcas* L. Plant Biotechnology Reports 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

Thorpe T, Stasolla C, Yeung EC, de Klerk G-J, Roberts A, George EF (2008) The components of plant tissue culture media II: Organic additions, osmotic and pH effects, and support systems. In George EF, Hall MA, De Klerk G, eds, Plant Propagation by Tissue Culture, Ed 3 Vol 1. Springer, Dordrecht, pp 115-173

Torres KC (1989) Tissue Culture Techniques for Horticulture Crops. Van Nostrand Reinhold, New York

Tremblay L and Tremblay FM (1994) Biotechnology in Agriculture and Forestry 29. In Bajaj YPS, ed, Springer-Verlag, Berlin, pp 115-128

Tudses N, Premjet S, Premjet D (2014) Optimal conditions for high-yield protoplast isolations of *Jatropha curcas* L. and *Ricinus communis* L. American-Eurasian Journal of Agriculture & Environmental Sciences 14(3): 221-230

Varshney A, Sudhakar Johnson T (2009) Efficient plant regeneration from immature embryo cultures of *Jatropha curcas*, a biodiesel plant. Plant Biotechnology Reports (4): 139-148

Veilleux RE, Compton ME, Saunders JA (2005) Use of protoplasts for plant improvement. In Trigiano RN, Gray DJ, eds, Plant Development and Biotechnology, CRC Press, Boca Raton, pp 213-224

Verma KK, Singh M, Gupta RK, Verma CL (2014) Photosynthetic gas exchange, chlorophyll fluorescence, antioxidant enzymes, and growth responses of *Jatropha curcas* during flooding. Turkish Journal of Botany, 38: 130-140

Wilson RF and Hildebrand DF (2010) Engineering status, challenges and advantages of oil crops. In Mascia PN, Scheffran J, Widholm JM, eds, Plant Biotechnology for Sustainable Production of Energy and Co-products, Springer, Heidelberg, pp 209-248

Yamado Y and Sato F (1983) Selection for Photoautotrophic Cells. In Evans DA, Sharp WR, Ammirato PV, Yamada Y, eds, Handbook of Plant Cell Culture, Volume 1, Techniques for Propagation and Breeding. Macmillan Publishing Co., New York, pp 489-500

Yinghui D (2008) Biological functions of antioxidants in plant transformation. *In Vitro Cellular & Developmental Biology. Plant* 44(3): 149-161

BIOGRAPHICAL SKETCH

Christopher S. Lukaszewski grew up in a military family that relocated frequently. He was born in Ulm, Germany; relocated to Lackland AFB, Texas, then Fort Gordon, Georgia, and onto Fort Hood, Texas. He attended Hutto High School in rural central Texas, earned college credits from Temple Junior College, and later at Austin Community College. He attended Concordia University Austin and then transferred to Concordia Portland to complete his bachelor's degree in biology at which he worked as a lab assistant in archaeological digs, organic chemistry, and genetics labs. After graduation, he attended Portland State University and volunteered at the Tualatin National Wildlife Refuge where he was awarded an AmeriCorps scholarship as a youth leader and received a naturalist certification. Then, he was accepted to The University of Texas Rio Grande Valley master's degree program in biology where he became employed as a teaching assistant. Throughout his time there he was granted one research assistantship to work on his thesis project, another to assist in writing a biology laboratory textbook, and a presidential scholarship. During his last semesters, he worked as a biological technician at the USDA APHIS PPQ where research was done on Molecular Genetic Analysis of the Citrus Leprosis Virus Vector, Brevipalpus (Acari: Tenuipalpidae). Now, he works at Texas A&M AgriLife as a biological technician on several projects, such as Using Sugarcane as a Biofactory for Therapeutic Proteins, Enhancing Drought Tolerance in Two Major Food crops, Sugarcane and Rice, and Improved Sugarcane Varieties Through Biotechnology. His contact information is 1001 N. Sal St. Apt. B, Edinburg, TX 78541.