Summary and Conclusion

Identifying an alternative fuel source has become imperative. *Jatropha curcas* has all the properties to enable it to be considered as a promising alternative fuel source. Systematic scientific studies will help to understand the plant better. This will also help to device ways to increase its innate potential. Seed oil content of the plant ranges between 22-44%. This large variation in the oil content, unavailability of quality planting material and absence of seed banks jeopardize its commercial success. These issues could be addressed in multiple ways. One of the methods of choice of doing so is transgenic studies. In this study, a holistic approach has been attempted at increasing the oil yield from the plant. For this, tissue culture and somatic embryogeneis protocols have been developed, over-expression of DGAT has been attempted and RAPD profiling of oil content has been reported in this thesis. Micropropagation is looked as an answer for raising such plants which have been touted as economically beneficial but have not been cultivated or whose germplasm is not maintained. During the time of undertaking this study, *Jatropha curcas* was not a cultivated variety, its germplasm was not known, agronomical practices were not known and there was a lot of discrepancy with regards to its oil content. At such juncture plant tissue culture holds a lot of promise. Moreover, a standardized tissue culture protocol is a pre-condition for undertaking transgenic studies. Hence, micropropagation of *J. curcas* was studied. Direct and indirect organogenesis was studied. Direct plant regeneration without an intervening callus phase is a more reliable method for multiplication or clonal propagation. Direct organogenesis was attempted through nodal explants. Apical and axillary buds, tender, thin, green twigs cut below 2^{nd} and 3^{rd} nodes were taken as explants. The culture medium comprised of MS medium supplemented with 2.2 μM BAP and 4.9 μM IBA. Nodal explants produced single shoots within 10-12 days. Explants were sub cultured on MS medium supplemented with 2.22 μM BAP and 4.92 μM IBA. Multiple shoot formation was observed within a period of 30-40 days. Multiplication of shoots was obtained in the same combination. The shoots were then kept on MS basal medium for rooting. Roots were observed after nearly three weeks in MS basal medium. For indirect organogenesis a range of combination of BAP (4.5 μM - 27.0 μM) and IBA (3.0 μM -7.5 μM) were used. Curling of enlarged leaf discs was

first observed followed by callus appearance at the cut margins. Callus morphology and relative response of callusing was studied. Buds in clusters (3-5) started emerging from various locations of the callus, mostly underneath the explants. First bud initiation was observed in MS medium supplemented with 27.0 μM BAP + 3.0 μM IBA. It was also the most effective combination for shoot bud initiation and proliferation. In the present study, tweaking cytokinin levels did not have an effect on organogenesis while low IBA levels did influence organogenesis. Low levels of IBA play a key role in callus formation, bud initiation and multiple shoot formation. Rooting was observed after three weeks of transfer in the rooting medium (MS Basal medium). It could be concluded that auxins play a greater role than cytokinins in organogenesis of *J.curcas.*

Somatic embryogenesis forms other integral part of micropropagation studies. Direct, indirect somatic embryogenesis through solid media studies and liquid suspension culture studies was undertaken in this investigation. Somatic embryogenesis acts as a powerful tool for genetic improvement of any plant species because of its single cell origin. The suspension liquid culture system allows the study of different physiological and biochemical characteristics such as growth parameters, plant growth regulators effect, nutrient uptake and maturation capacity, somatic embryo morphology etc. In the current study the roles of a few factors that have an influence on the performance of explants in culture conditions have been discussed. Age of seeds is known to be an important parameter influencing the outcome of cells in culture. Here, immature and mature seeds were used to obtain first cotyledonary leaves which then served as explants. It was observed that mature seeds could give rise to cotyledonary leaves upon initiation in callus inducing media, whereas immature seeds failed to do the same. Seed viability was the next parameter found to have a bearing on the outcome of the experiments. It was observed that seeds stored at room temperature did not germinate and their cotyledons were degenerated. Unviable seeds were treated with GA10 ppm to break seed dormancy however; it did not help. Hence for all experiments seeds after collection were stored at -20°C until use. Seasonal cues like environmental conditions also have an effect on the performance of explants in culture conditions. In this study, embryogenic calli formation was found to be higher in the months of January to April. It could be explained by the fact that season dependent metabolism is retained

in tissue culture conditions and consequently, it is hard to break down the dormant state. The role of auxins in morphogenesis is evident from the fact that the cells which respond to auxin revert to a dedifferentiated state and begin to divide. For liquid suspension culture studies, the cotyledonary leaves were initiated on MS supplemented with varied concentration of 2, 4-D (0.4, 0.8, 1.2 mg/l) and NAA (5mg/l, 10 mg/l, 15 mg/l) for a period of 30 days followed by their transfer to no auxin media. Early embryogenic structures like globular, heart shaped and torpedo shaped cells were seen. Once in suspension culture, it was observed that the cell number was initially low for first 4-5 days, the cell number increased within 10-12 days and then a gradual decrease was observed after 20 days. For solid media studies short time auxin pulse treatment was used wherein the cotyledonary leaves were initiated on MS +2, 4-D (0.4, 0.8, 1.2 mg/l + 2% coconut water and 0.4, 0.8 and 1.2 mg/l +10% coconut water and 2.4, 3.6 and 4.0 mg/l) and MS+ NAA for a period of seven days or fourteen days followed by their transfer to no auxin medium. Various embryogenic structures were observed. There are atleast two stages in somatic embryogenesis, stages requiring and inhibited by auxins. Hence, auxin levels were checked on day 4, day 7 and day 14. On day 4 pre-embryogenic masses were not observed whereas on day 7 pool of embryogenic and non-embyrogenic structures were observed. On day 14 most of the calli had turned embryogenic. Hence, day 4 calli was considered as non-embryogenic calli (NE) and day 7 and day 14 had both embryogenic (E) and non-embryogenic characteristics. Total auxin levels were estimated and it was found that a significant increase is seen in total auxin in embryogenic(E) calli(7 days and 14 days) as compared to non-embryogenic calli (NE)(4 days) in various media combinations studied. It could be concluded from the present study that 2, 4-D alone at higher concentration (4.0 mg/l) is a preferred medium to induce embryogenesis in *J. curcas* as compared to lower 2, 4-D levels with coconut water. This could be due to the fact that coconut water is an undefined medium and hence may interfere with reproducibility of procedure. This is the first report of liquid suspension culture studies of *Jatropha curcas* and direct as well as indirect somatic embryogenesis.

Jatropha seeds contain 22-44% oil in its seeds. Main constituent of oil is TAG (Triacylglycerol). DGAT (Diacyl glycerol acyltransferase) is the enzyme that catalyzes the committed step of TAG

synthesis from DAG (Kennedy pathway). A correlation between DGAT transcript level and oil accumulation has been reported in common oilseeds as well as in plant species that accumulate unusual fatty acids. Binary vector pE172 contains DGAT2 gene under a seed specific promoter and binary vector pE309 contains DGAT2 gene under a constitutive promoter. Transformation of gene of interest was carried out through triparental mating. It was confirmed by restriction digestion. In species where low transformation efficiencies are expected, the study of the effect of several factors by comparing the percentage of recovered transformed plants may prove unsuccessful because limited numbers of transformants are produced. Therefore, it becomes important to evaluate the influence of diverse factors on the efficiency of T-DNA transfer. *Agrobacterium* infection time was studied. Fifteen and twenty minutes of immersion time of explants in *Agrobacterium* culture was studied and it was found that twenty minutes is more effective time period for immersion as compared to fifteen minutes. Evaluation of selective and bactericidal antibiotics was done and it was found that 20mg/L kanamycin is effective as a selective agent. Preculturing explants prior to inoculation and co-cultivation with *Agrobacterium* has been shown to improve genetic transformation frequencies in many plants. Hence, for both leaf explants and somatic embryos seven days of pre-culture was preferred. More so in case of somatic embryos as seven days pre-culture would enable to generate a few somatic embryos which would then be susceptible for *Agrobacterium* infection during co-cutivation. In the present study, co-cultivation for 4 days yielded maximum transformation efficiency.

Genetic diversity of six different *J.curcas* genotypes was studied using RAPD. Pre-requisite for RAPD is to obtain a good quality DNA. Hence, a protocol for successful DNA isolation from *J.curcas* was standardized and a modified protocol using CTAB was devised. Out of 37 primers used to study the genetic diversity of 6 *Jatropha curcas* accessions, 7 primers could generate reproducible amplification products. These primers yielded 95 amplified bands/fragments. Eleven unique alleles were detected with a total of five primers in five genotypes. The putatively similar bands originating for RAPDs in different individuals may not necessarily be homologous, although they may share the same size in base pairs. Accessions from MSU and TNAU clustered together as per Jaccard's similarity coefficient.

In conclusion direct and indirect organogenesis from different explants has been achieved. For the first time liquid suspension culture studies for *J.curcas* has been reported in this study. Solid media studies were also undertaken for somatic embryo formation and maturation. Successful *Agrobacterium* mediated transformation of leaf discs and somatic embryos of *J.curcas* have been achieved. Existing genetic variation in six different *J.curcas* genotypes has been studied through RAPD.For the same a new modified protocol for DNA isolation has been developed. Genotype from The M.S.University of Baroda and Mettupalayam fell in the same cluster.

The studies reported here can be used to cultivate *J. curcas* with greater economic returns.

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ORAL AND POSTER PRESENTATIONS

1. **Shukla P**, Makwana V, Bhatt D and Robin P, Preliminary studies on Tissue culture of *Jatropha curcas,* presented at the National Symposium on Plant Biotechnology for Conservation, Characterization and Crop Improvement, February 8-10,2008, Udaipur

2. **Shukla P**, Makwana V and Robin P, "Somatic embryogenesis in *Jatropha curcas*: an attempt" presented at the 1st International conference on 'New Frontiers in Biofuels', organized by Delhi Technological University on January 18-19, 2010, New Delhi, India.

3. **Purna Shukla**, Mamta Bangera, Vijay Makwana, Pushpa Robin, Standardization of Agrobacterium mediated transformation of *Jatropha curcas*, oral presentation at Anveshan 2010 organised by DST

4. Abstract titled," Agrobacterium mediated transformation of *Jatropha curcas*" accepted at the **Gordon Research Seminar**, New Hampshire, USA.

5. **Purna Shukla**, Neha Gandhi, Amruta Sonawane, Pushpa Robin, Correlating Auxin levels with embryogenic potential during somatic embryogenesis in *Jatropha curcas* clonal propagation, PTCA, St. Xavier's College, Ahmedabad, 19-21 January, 2012

6. Article titled" Efficient method for obtaining high frequency regenerants of biofuel crop *Jatropha curcas*" under communication.

AWARDS

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1. Makwana V, **Shukla P** and Robin P (2010). GA application induces alteration in sex ratio and cell death in *Jatropha curcas,* Plant Growth Regulation. 61:121–125.

2. Makwana V, **Shukla P** and Robin P (2010). Comparing potential of GA and 2, 4-D in increasing fruit yield from *Jatropha curcas*, Journal of Biofuels. 1:157-162

Biotechnological approach for improvement of oil content in *Jatropha curcas*

A THESIS SUBMITTED TO

THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY

BY

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STATEMENT UNDER O. Ph.D. 8/(iii) OF
THE M. S. UNIVERSITY OF BARODA, VADODARA

This is to certify that the work presented in this thesis is original and was obtained from the studies undertaken by me under the supervision of **Dr. Pushpa Robin**

Vadodara — Purna Shukla
Date: — Candidate

This is to certify that the work presented in the form of a thesis in fulfillment of the requirement for the award of the Ph. D. degree of The M. S. University of Baroda by **Ms. Purna Shukla** is her original work. The entire research work and the thesis have been built up under my supervision.

Vadodara
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Dedicated to my parents.....

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Purna Shukla

Dwindling fossil fuel reserves coupled with increased carbon dioxide emissions driving climate change have shifted world focus to reduce fossil fuel dependence. Mitigation of this problem could be achieved by increased promotion of renewable energy sources like promoting bioenergy, including biofuels. Recent interest and curiosity about the potential of biomass to lead the energy sector has shown a ray of hope for millions. However, there are certain genuine concerns and uncertainties which could be answered through strong scientific research, sustainable solution and good policy in hand. The energy demand of the transport and telecommunication sector which utilizes more than 30% of current energy needs to be fulfilled without much ado. Biofuels have this potential as only a few changes would be required in the distribution infrastructure. The added advantage of opting for biofuels as an energy source for an agricultural nation like India is its promising farm income support enabling rural development. Depending on biofuels alone to address climate change and energy security is a little overwhelming; nevertheless increased demand for biofuels creates a huge market for agricultural products.

Jatropha curcas has gained popularity as a biofuel crop as the oil present in seeds could be easily converted to biodiesel. Other advantages include its capability to grow on dry, wasteland with marginal nutrition and irrigation. Unlike other sources like sugarcane, rapeseed etc. its non-edible and hence does not compete with food crop for land and water and thereby retaining the food security. The plant finds a lot of traditional applications as medicinal use, oil for lighting, soap production and many more. It produces a toxin and hence is not grazed by cattle thereby promoting its use as a hedge plant. It can withstand soil erosion. The oil can be directly used in engines without any modification. There are a few bottlenecks which have hindered its growth as a biodiesel plant. These include a large variation in the oil content, skewed ratio of male: female flower, making its yield limited. Most of the *Jatropha* currently grown is toxic which renders the seedcake unsuitable as cattle fodder and may also pose as human hazard. The knowledge of agronomy practices about *Jatropha* is still lacking and hence poses a major drawback for mass cultivation. In nature it propagates through seeds and quality planting material is not available. The production of disease free plants by tissue culture has yet not been commercialized. It could be stated that crop improvement is at an early stage and now efforts to increase oil yield through

genetic variation studies are on its way. Breeding programmes like grafting or inter-specific hybridization for higher oil yield may take nearly two to three years to develop.

Various field trials of *Jatropha* cultivation in sub Saharan Africa and South Asia have revealed that a holistic approach which encompasses *Jatropha* production, oil extraction and utilization in rural communities appear as the most viable approach (Brittaine and Lutaladio, 2010). *Jatropha* biofuel production may seem to be a boon for those semi-arid and remote areas which have little opportunity for alternative farming strategies.

The high expectation to see *Jatropha* as a commercially viable option requires scientific research in improving its genetic potential for oil yield, large availability of quality planting material and know-how of its agronomic practices. In this thesis an attempt is made to bring *Jatropha curcas* to the forefront by addressing the problem holistically.

1.0 Energy scenario in India:

India has been rated as the fourth largest energy consumer of the world following United States, China and Russia. The need of a voraciously growing economy cannot be satisfied by one energy source but many are required to satisfy the need of millions. This is of concern for those involved in energy sector, science and technology. More so when the global economy hits at an all time low and India's energy demand on a spiraling spree. This is exemplified by increased vehicle ownership and higher energy demand by the transport sector, telecommunication etc. According to the Oil and Gas Journal (OGJ, 2011), India has approximately 5.7 billion barrels of proven oil reserves as of January 2011, the second largest amount in the Asia-Pacific region after China. India produced roughly one million barrels per day (bbl/d) of total liquids in 2010, of which 0.75 bbl/d was crude oil. The country consumed 3.2 million barrels per day (bbl/d) in 2010. Given these facts how do we meet our energy demands?

The dual problem of ever increasing oil consumption and low production has left India increasingly dependent on imports to meet its petroleum demand. In 2010, India was the world's fifth largest net importer of oil, importing more than 2.2 million bbl/d or about 70 percent of consumption. In such a scenario it becomes the need of the hour to step up our efforts to look for alternative sources of energy. Alternative energy source is an energy source that can be used instead of fossil fuels. It is usually a renewable source of energy that could be used should fossil fuels run out. There was a time when nuclear power was seen as the answer. Huge amounts of power could be produced from a small amount of Uranium. However, when it was known to produce radioactive waste which is not only dangerous to health but also has a half life of hundreds of years it was realised as no sustainable option.

1.1 Renewable sources of energy:

Renewable energy resources are natural resources that can be replenished by natural processes at a rate comparable or faster than its rate of consumption by humans. Solar radiation, wind and hydroelectricity are permanent resources and are considered as renewable resources of energy as they do not have the hazard of non-availability. Renewable resources may also mean commodities such as wood, paper and leather, if harvesting is performed in a sustainable manner.

1.1.1 Advantages of using natural sources of energy:

- They are inexhaustible – they will always be available – they are renewable
- They are clean and will not damage the Earth
- There are several types – so one or more of them is present in each country
- Most natural sources can be used on a small scale and serve local needs therefore cutting costs of transmitting the energy

1.1.2 Solar energy

India has an average annual temperature between 25°C – 27.5 °C. This means that India has huge solar potential. The sunniest parts are situated in the South/East coast, from Calcutta to Madras. Solar energy derived from sun is also known as the oldest source of energy to be used on earth. In tropical country like ours it finds its use even today in drying tons of materials mostly in the rural belts. It is estimated to generate 35 MW of power from 1 sq km area. There is technological advancement taking place with each passing day thereby increasing the use of solar energy in cooking, lighting, water heating open air drying etc. High end applications such as solar vehicles, desalination plants etc. are picking up by leaps and bounds. Photovoltaic cells and concentrated solar power are two methods of choice of converting solar energy into power.
However, the biggest limitation is the initial startup cost. For solar energy to become one of the front runners, what is required is research, use of inexpensive technology and low capital input.

1.1.3 Wind Energy

India seconds Germany as the worlds emerging market for wind energy. The wind power potential of India is about 45,000 MW. States like Gujarat, Andhra Pradesh, Karnataka, Madhya Pradesh and Rajasthan are having more than 5000 MW potential each. Advantages of wind energy is it being environment friendly, clean and safe, has the lowest gestation period when compared to conventional energy, low operating costs since no fuel consumption is involved and setting up costs are comparable to conventional forms.

The major limitation is unavailability of strong, dependable winds most of the time as it is a prerequisite to generate power. Due to this energy from wind machines is also considered intermittent as it is not continuously generated. Electricity produced by wind power sometimes fluctuates in voltage causing difficulty in linking its power. Avian mortality is another matter of concern.

1.1.4 Hydro power

The hydroelectric power refers to the energy produced from flowing water. In India, hydro power is the most dominant renewable energy source. It has the potential to generate 15,000 MW of energy. The north eastern states of India like Arunachal Pradesh, Assam, Mizoram and Manipur receive highest annual rainfall and the western belt between Mumbai and Mahe are the ones to receive highest annual rainfall hence they serve as ideal locations for such an establishment. The major disadvantage of hydro plant is discontinuous electricity generation.

1.1.5 Biomass

Biomass remains the oldest means of energy used by humans along with solar energy. In olden times fire was generated using wood or leaves, which is basically a biomass. Biomass includes solid biomass and biogas. Sugarcane bagasse used in agriculture, pulp and paper residues in forestry and manure in livestock residues are the most common forms of biomass used. However, the limitation of such processes is low efficiency due to heat loss to the surroundings.

1.1.6 Bio-fuels

Biofuels by definition involves fuel generation from sources of biological origin for example ethanol generated from sugarcane bagasse, diesel generated from algae or *Jatropha*, gas generated by burning material of biological origin like municipal waste, domestic waste or manure etc. India has more than 50 million Ha of wasteland, which could be utilized for cultivating plants. Biofuels are also called as next generation sustainable fuels. The main advantage of using biofuels is low generation of noxious chemicals like CO_2, SO_x, NO_x etc. Of all

the biofuels present today biodiesel is fast gaining attention of industrialists, technologists and academicians alike.

1.2 Biodiesel

In 1885, Dr. Rudolf Diesel built the first diesel engine and ran it on vegetable oil. He first displayed his engine at Paris in 1900 and amazed everyone when he ran the patented engine on easily available hydrocarbon fuel - which included gasoline and peanut oil. In 1912, he made a statement which remains the most quoted one in today's time as *"the use of vegetable oils for engine fuels may seem insignificant today. But such oils may in the course of time become as important as petroleum and the coal tar products of present time."*

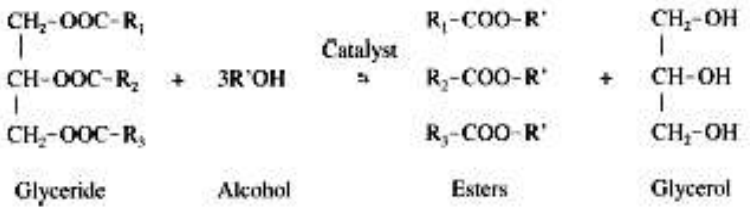
Scientists discovered that a simple chemical process could reduce the viscosity of vegetable oils and it was shown to work well in modern engine. ***This fuel was called Bio-Diesel.*** Since then the technical developments to improve its properties as engine fuel have been completed. Plant oil is highly valued as Bio fuel "Diesel" and has transformed into Bio Diesel in well-developed nations.

1.2.1 Biodiesel Definition

Biodiesel is defined as alkyl esters of long chain fatty acids. It can be derived from natural, renewable biological sources such as vegetable oils like Sunflower, Canola or *Jatropha*. Biodiesel operates in compression ignition engines like petroleum diesel thereby requiring no essential engine modifications. Biodiesel can be made from new or used vegetable oil and animal fat. Unlike fossil diesel, pure biodiesel is biodegradable, nontoxic and essentially free of sulphur and aromatic compounds.

1.2.2 Chemistry of biodiesel production

Biodiesel is produced by transesterification of triglycerides in to smaller, straight chain molecules of methyl esters, using an alkali, acid or enzyme as catalyst. There are three stepwise reactions with intermediate formation of diglycerides and monoglycerides resulting in the production of three moles of methyl esters and one mole of glycerol from triglycerides. The overall reaction is:



Alcohols such as methanol, ethanol, propanol, butanol and amyl alcohol are used in the transesterification process. Methanol and ethanol are used most frequently. Methanol because of its low cost, physical and chemical advantages is the most preferred. Stoichiometric molar ratio of alcohol to triglycerides required for transesterification reaction is 3:1.

1.2.3 Advantages of biodiesel

- Produced from sustainable / renewable biological sources
- Ecofriendly and oxygenated fuel
- Sulphur free, less CO, HC, particulate matter and aromatic compounds emission
- Income to rural community
- Fuel properties similar to the conventional fuel
- Used in existing unmodified diesel engines
- Reduce expenditure on oil imports
- Non toxic, biodegradable and safe to handle
- Decrease Global Warming

Other advantage of biofuels is generation of market for agricultural products and stimulation of rural development. This would mean more opportunities for farmers and more rural employment thereby helping poverty alleviation. This would help farmer to generate capital and be energy independent. At the national level, biofuel generation would give rise to new industries, new technologies, new jobs and new markets. India has also accepted a future with biodiesel and has declared an effective biofuel policy in which biodiesel, primarily from *Jatropha*, would meet 20% of the diesel demand beginning with 2020. The focus in India is primarily on biodiesel as diesel is presently the most important vehicle fuel and its demand is growing at rapid rate.

1.2.4 Disadvantages of biodiesel

Despite the many positive characteristics of Biodiesel, there are also many disadvantages.

Energy Output: Biodiesel has a lower energy output than traditional fuels and therefore require greater quantities to be consumed in order to produce the same energy level.

Production Carbon Emissions: Several studies have been conducted to analyze the carbon footprint of biodiesel, and while they may be cleaner to burn, there are strong indications that the process to produce the fuel – including the machinery necessary to cultivate the crops and the plants to produce the fuel – has hefty carbon emissions.

High Cost: To refine biodiesel to more efficient energy outputs and to build the necessary manufacturing plants to increase biofuel quantities will require a high initial investment.

Food Prices: As demand for food crops such as corn grows for biodiesel production, it could also raise prices for necessary staple food crops.

Water Use: Massive quantities of water are required for proper irrigation of biofuel crops as well as to manufacture the fuel, which could strain local and regional water resources. Many scientists are now proposing to look at the water footprint of a plant to decide its viability.

Availability: Biofuel are not yet widely available for consumer purchase and most vehicles are not equipped to run on biofuel products. Limited availability reduces the desirability of biofuel as alternative energy sources.

1.3 Putative plants for bio-diesel

- ***Jatropha curcas*** **(ratanjyot)**
- *Pongamia pinnata* (karanj)
- *Calophyllum inophyllum* (nagchampa)
- *Hevea brasiliensis* (rubber)
- *Calotropis gigantia* (ark)
- *Euphorbia tirucalli* (sher)
- *Boswellia ovalifololata* (shallaki)
- *Azadirachta indica* (neem)

All the above mentioned plants have been cultivated or found in the wild with various medicinal and other economical prospects. Due to their appreciable seed oil content, they are considered as potential biodiesel sources. The properties of oil from these plants have been studied. Of all the plants, *Jatropha* is considered the most potent source. The properties of *Jatropha* oil is given in table 1.3.

1.4 Reason for selecting *Jatropha curcas* for the present study:

- Its seed oil content is high (22-44 %) in comparison to other putative plants
- It can easily grow in arid region (20 cm rainfall) and this potentiates its role as a solution of crop for the saline wastelands. It shows its adaptability to varied agro-climatic conditions and soil types
- It is not foraged by animals and hence needs no special care to keep them safe
- It has many medicinal properties and different parts of the plant have been utilized from generations as traditional medicines
- Short gestation period: 2 years
- Long productive life: 50 years
- Close physico-chemical properties to petro-diesel

1.5 About *Jatropha*:

1.5.1 Taxonomy, Botany and Ecology:

Kingdom: **Plantae**
Division: **Magnoliophyta**
Class: **Magnoliopsida**
Order: **Malpighiales**
Family: **Euphorbiaceae**
Subfamily: **Crotonoideae**
Tribe: **Jatropheae**
Genus: ***Jatropha***

Figure 1.1: Image of *Jatropha curcas*

Jatropha curcas is a biofuel plant belonging to Euphorbiaceae family and contains approximately 170 known species. Linnaeus (1753) was the first to name the physic nut *Jatropha* L. in "Species Plantarum" and this is still valid today. The genus name *Jatropha* derives from the Greek word *jatr´os* (doctor) and *troph´e* (food), which implies medicinal uses. By definition, it's a small tree or large shrub with a height of three to five meters which under favorable conditions could reach up to eight or ten meters.

The physic nut has alternately arranged shallow lobed leaves with the young ones showing pigmentation while the mature leaves retain the green color. Seedling upon germination gives rise to a central taproot, four lateral roots and a few secondary roots. As true for Euphorbiales, the branches of the physic nut contain latex. Though it has been claimed that branches serve as fuelwood, it hardly is true as they are hollow and its soft wood has little fuelling properties. *Jatropha* is a monoecious shrub, bearing male and female flowers on the same plant. The inflorescence is formed terminally on the branch and possesses an unequal ratio of male to female plants. Raju and Ezradanum, 2002 reported a male:female ratio of 29:1. Physic nut depends on insects like bees, flies, and thrips for its pollination. Breeding system plays a critical role in deciding the route of plant evolution and hence breeding (Grant, 1981). The results of breeding system indicated 32.9% fruit setting under selfing and 89.7% under natural pollination in *Jatropha*. The high fruit setting under open pollination revealed that the plant is capable of producing fruits through selfing and cross-pollination. Such a breeding system represents facultative cross-pollination (Dhillon et al, 2006). However, the fruit sets of artificial self-pollination, artificial cross-pollination and natural cross-pollination were 87.93%, 86.66% and 76.42%, respectively, which indicated that *Jatropha curcas* was self compatible and showed a tendency to cross-pollinate (Qing et al, 2007). The ability to self pollinate through geitonogamy is considered to be adaptive for *Jatropha curcas* for colonization (Raju and Ezradanum, 2002). 50% of female flowers set fruit with 53% fecundity rate, 32% apomixis rate and 2:3 seed-ovule ratio (Bhattacharya et al, 2005; Abdelgadir et al, 2008) this suggests that fruit production can be increased by manipulating biological processes of pollination and growth. Fruits are formed in bunches of 10 or more. They are fleshy, ovoid and greenish at the beginning, turning yellow and finally brown as they dehydrate with ageing. Each fruit contains three black seeds, occasionally having two or four seeds. Fruiting takes place after 90 days of flowering. The oil content in

Jatropha seed is reported to be in the ranges from 22 to 44 % by weight of the seed and ranges from 45 to 60% weight of the kernel (Chhetri et al, 2008). The plant attains complete maturity at the end of four to five years with maximum seed yield. The physic nut is diploid with 2n=22 chromosomes (Heller, 1996).

It is now widely known that *Jatropha* was introduced in Asian sub-continent through Portuguese sea farers. This is a non-edible oil-bearing plant widespread in arid, semi-arid and tropical regions of the world. *Jatropha* is a drought resistant perennial tree that grows in marginal lands and can live over 50 years. Temperature is an important aspect of climate. *Jatropha* can be grown in wide range of soils in tropical and subtropical parts of the globe with a temperature average of more than 20°C. *Jatropha curcas* can be grown potentially over wastelands, which require re-vegetations. *Jatropha curcas* is a wild growing hardy plant well adapted to wide range of pH or moisture levels. It can be grown well on degraded soils having low fertility and moisture and also on stony, gravelly or shallow and even on calcareous soils. For economic returns, a soil with moderate fertility is preferred. In low rainfall areas and in prolonged rainless periods, the plant sheds its leaves as a counter to drought. Its water requirement is extremely low and it can stand long periods of drought by shedding most of its leaves to reduce transpiration loss (Kumar and Sharma, 2008). The emergence of seed requires hot and humid climate. It can be cultivated successfully in the regions having scanty to heavy rainfall. Misra and Misra (2010), citing Francis et al, 2005 stated that marginal areas of semiarid regions which are frequently subjected to dry and hot conditions, are not amenable for growth of most other crops however, *Jatropha* grows well under this climate. This species may have potential as an excellent model for the physiological and molecular mechanisms involved with plant resistance to combined abiotic stresses (Misra and Misra, 2010). *Jatropha* usually grows below 1400 meters of elevation from sea level and requires a minimum rainfall of 250mm, with an optimum rainfall between 900-1200mm (Chhetri et al, 2008).

1.5.2 Toxicity:

Though most of *Jatropha* plants found in the wild in Asian sub-continent are toxic in nature, there is one Mexican variety which is non-toxic in nature. In Mexico, White Winged Dove,

chickens or pigs consume the seeds and boiled or roasted seeds are used to prepare traditional dishes (Gubitz et al, 1999). However, seeds of *J.curcas* are in general toxic to humans and animals. The toxic nature of *Jatropha* plants is due to the toxin curcin. Curcin is similar to ricin, the toxic protein of castor bean (*Ricinus communis*) (Heller, 1996). All parts of the plant contain this toxin and therefore animals cannot graze it, however, the most toxic part is the seed. Apart from curcin it also contains phorbol esters, diterpene inhibitors and trypsin inhibitors. Chance ingestion of seeds by humans has varied effects from gastro-intestinal irritation to lethality. Numerous feeding experiments with different animal species showed abdominal pain, diarrhea, respiratory problems and imbalance. Histopathological findings include gastrointestinal inflammation, necrosis of the liver, heart and kidneys as well as hemorrhages in the liver (Gubitz et al, 1999). One or two seeds upon ingestion lead to nausea and diarrhea whereas consumptions of more seeds may even lead to death following excessive dehydration. However, these poisonous and anti-nutritional properties of the seeds are exploited in traditional medicine for de-worming and as a purgative (Brittaine and Lutaladio, 2010). All the by-products of these toxic varieties like biodiesel; seed cake etc. are toxic in nature. The use of seed cake as animal feed is therefore not possible as it is still found to contain thermostable toxic diterpenes bound to it. However, it might find use after laboratory-scale detoxification.

1.6 Agronomy:

1.6.1 Plantation:

Complete germination of seed is achieved within 9 days. Adding manure during the germination has a negative effect on germination, but it is favorable if applied after germination is achieved. It can be propagated by cuttings, which yields faster results than multiplication by seeds. The flowers only develop terminally (at the end of a stem), so a good ramification (plants presenting many branches) produces the greatest amount of fruits. The plants are self-compatible. Another productivity factor is the ratio between female and male flowers within an inflorescence; more female flowers mean more fruits. *Jatropha curcas* thrives on a mere 250 mm (10 in) of rain a year, and only during its first two years, it need to be watered in the closing days of the dry

season. Ploughing and planting are not needed regularly, as this shrub has a life expectancy of approximately forty years. While *Jatropha curcas* starts yielding fruits from an age of 9–12 months, the best yields are obtained only after 2 – 3 years time. If planted in hedges, the productivity of *Jatropha* is 0.8 kg to 1.0 kg of seed per meter of live fence. The seed production is around 3.5 tons / hectare (Seed production ranges from about 0.4 tons per hectare in first year to over 5 tons per hectare after 3 years).

1.6.2 Crop establishment and Propagation Methods:

The selection of planting material should be from cuttings or seed that have proven, over several seasons, to have high yield and seed oil content under the same irrigation and fertilization conditions that are proposed for the new plantation. Seed from high-yielding *Jatropha* plants are not generally available, due to the fact that the out-crossing seed selected from productive plants may or may not result in high-yielding and high-quality plants (Brittaine and Lutaladio, 2010). With good moisture conditions, germination needs 10 days. The seed shell splits, the radicula emerges and four little peripheral roots are formed. Soon after the development of the first leaves, the cotyledons wither up and fall off. Further growth is sympodial. In permanently humid equatorial regions, flowering occurs throughout the year. Fruit development needs 90 days from flowering until seeds mature. Further development corresponds to rainy seasons: vegetative growth during the rainy season and little increment during the dry season. Old plants can reach a height of up to 5 m. With good rainfall conditions, nursery plants bear fruit after the first rainy season, with directly seeded plants bearing for the first time after the second rainy season. With vegetative propagation, the first seed yield is higher (Heller, 1996). Heller (1996) proposed two methods of propagation namely generative and vegetative.

Generative propagation (seeds)

- direct seeding – seeding depth, date and quality of the seed
- transplanting – type and length of precultivation, planting date

Vegetative propagation (cuttings)

- direct planting – character of cuttings (length, diameter, age), cutting time, storage, fungicide treatment, planting time and depth
- transplanting – as with direct planting of cuttings and precultivation of seeds.

Propagation from seed: Pre-cultivation in nurseries, sown in either nursery beds or containers enables better germination and survival of seedlings through control over moisture, shade, soil, weeds, pests and diseases. Seeds should be sown three months before the start of the rains in polyethylene bags or tubes. The bags should be long enough to avoid unduly restricting taproot growth (Brittaine and Lutaladio, 2010). The bags or cells should be filled with free-draining growing media containing organic matter (such as 1:1:1 sand-soil-manure or 1:1:2 sand soil-compost) and well watered prior to sowing (Achten, 2008). Pre-treatment to soften or break the seed coat will enhance germination. Seedlings after germination may be planted out after two to three months, after reaching a height of 30–40 cm and before taproot development becomes overly restricted. Nursery shade should be gradually removed for hardening off the plants before they are transplanted to the field (Brittaine and Lutaladio, 2010).

Vegetative Propagation using cuttings: Heller (1996) states that the advantage of using cuttings is their genetic uniformity, rapid establishment and early yield. The disadvantage is the scarcity of material, and the cost of harvesting, preparation, transport and planting of the woody stems, compared to seeds. A further disadvantage is that cuttings do not produce a taproot, meaning there is less capacity for the plant to reach soil water and nutrient reserves with correspondingly lower potential yields, although the effect of this, for different environments, has not yet been determined. The absence of a taproot makes for less stability on exposed windy sites, and cuttings compete more for water and nutrients with intercrops. Seedling-raised plants would be a better choice in this situation and for agroforestry systems. Poorer longevity may be expected for plantations established using cuttings. Brittaine and Lutaladio, 2010 further summarized the advantages and disadvantages of different methods of propagation as shown in table 1.1

Table 1.1: Alternative Propagation Methods

PARENT MATERIAL	ADVANTAGES	DISADVANTAGES
Seed-sown directly in the field	Cheapest method. Good taproot development.	Lower survival rate of seedlings. Least successful method of propagation. Poor uniformity of growth. Variable productivity of the progeny. More weeding required in the field.
Seed- nursery raised in poly bags	Control of seedling environment. Fewer losses. More uniform plants.	Higher costs than direct seeding. Variable productivity of the progeny. Seedling tap root development may be impaired by the poly bag.
Seed- nursery raised in seedbed	As above. No restriction of taproot. Lower transport costs.	Higher costs than direct seeding. Variable productivity. Higher losses at planting out of bare root seedlings.
Vegetative cuttings – planted directly in the field	Clones give more uniform productivity and potentially higher yield per ha. Yields sooner than seed raised plants	Sufficient cutting of good plants may be difficult and costly to source. Lack of a taproot means poor soil anchorage, less capacity to extract water and nutrients, less suited to intercropping. Shorter productive life of the plantation. Larger cuttings needed to ensure survival.
Vegetative cuttings – nursery raised in poly bags	As above. Fewer losses and more uniform plants. Mini cuttings may be used where parent material is scarce.	As above. Higher costs than planting cuttings directly.
Vegetative cuttings – nursery raised in seed bed	As above. Lower transport costs from nursery to field.	As above. Higher losses when planted out.
Tissue culture	Clonal. Uniform productivity. Develops taproot. Rapid multiplication of new plants.	High cost. Newly developed protocols not yet commercially available.

1.6.3 Spacing of plants:

5 to 6 kg of seeds is needed for planting a land of 1 hectare. The distance between two rows and that between two plants is maintained ideally as 2 meters. With this spacing about 2500 plants could be accommodated per hectare. On rainfed wastelands, high-density plantations at 2 x 1meter or 1.5 x 1.5 meter accommodating 5000 or 4444 plants per hectares were shown to be productive (Gubitz et al, 1999).

1.6.4 Best Agronomic practices to increase seed yield

1. Generation of elite planting materials from clonal seed blocks
2. Ensuring high yielding population with optimum plant density
3. Building the plant architecture by pruning
4. Use of plant growth regulants
5. Practicing integrated Nutrient Management (Use of fertilizers, VAM, Biofertilizers includes Azospyrillum, phosphobacteria, Zn / K mobilizer)
6. Use of Biocontrol agents Viz. Trichoderma / Pseudomonas
7. Manipulation of flowering by irrigation practices
8. Keeping Bee hives
9. Intercropping with seasonal crops to get income during gestation period

1.7 Uses of *Jatropha* tree and its products:

Jatropha plant has been used for its different properties in different communities of the world. It has been used as an ornamental plant in Africa and America, grown in gardens for their ornamental foliage and flowers. It is also commonly grown as a live hedge around agricultural fields as animals do not browse it.

- Erosion control and eco-restoration: *Jatropha* has proven effective in reducing the erosion of soil by rainwater. The taproot anchors the plant in the ground while the profusion of lateral and adventitious roots near the surface binds the soil and keeps it from being washed out by heavy rains. *Jatropha* also improves rainwater infiltration when planted in lines to form countour bunds (Brittaine and Lutaladio, 2010). *Jatropha* hedges reduce wind erosion by lessening wind velocity and binding the soil with their surface roots (Henning, 2004).

- Livestock Barrier: In the fields, one can find *Jatropha* plant as a fence to protect the crop under cultivation. Due to the toxic nature of the plants its not grazed by animals and cattle thereby protecting the crop from damage caused due to grazing of animals. Hedges planted very close together (5cm) form a barrier that is impenetrable even by chickens.
- Medicinal and Insecticidal properties: In India, Africa and in Latin America various parts of *J.curcas* have been used in traditional medicine. In Africa seeds are used as purgative while leaves are used as Haemostatic. In Mali leaves are used as a treatment for malaria. Leaf decoction when applied externally is used as treatment for rheumatism and inflammation. Seeds find their use as contraceptive and abortifacient. Root decoction is drunk against pneumonia, syphilis and as a purgative. The use of *J.curcas* oil was done for the control of insect pests and it seemed to be a promising alternative to hazardous chemicals as reported by Solsoloy, 1993. The author also studied the effect of *J.curcas* oil extracts on cotton bollworm *Helicowerpa armigera* and on cotton flowerweevil *Amorphoidea lata* .Crude oil of *J.curcas* showed more potential than its methanolic extract in control of sorghum pests *Sesamia calamistis* and *Busseola fusca* (Mengual, 1997).
- Potential for industrial use: *Jatropha* oil has very high saponification value and is being extensively used for making soap in India and other countries. At present *Jatropha curcas* oil is being imported to meet the demand of cosmetic industry. In China, a varnish is prepared by boiling the oil with Iron oxide. In village, it is used as an illuminant as it burns bricants and candles as in case of castor oil. It is used for wool spinning in England. The protein content *Jatropha* oil cake may be used as raw material for plastics and synthetics fibres. It would also be advantageous to make use of *Jatropha* oil as hydraulic oil (Gubitz et al, 1999).

1.8 Potential value of *Jatropha curcas* seeds

The analysis of *Jatropha curcas* seeds show that it contains; moisture 6.62; protein 18.2; fat 38.0; carbohydrates 17.30; fibre 15.50; and ash 4.5% (Gubitz et al, 1999). The oil content is 22 to 44% in the seeds and 50 to 60% in the kernel. The oil contains 21% saturated fatty acids and 79% unsaturated fatty acids. It has also been found that there are some chemical elements in the seeds which possess poisonous and purgative properties and render the oil non edible for human consumption. The seeds of *Jatropha curcas* form within seedpods. Each seedpod typically

contains three seeds (figure1.2). The typical mass and composition of the seeds is detailed in table 1.2. In addition to being a valuable source of oil, the seeds are also rich in protein. The protein composition of *Jatropha curcas* seed meal has been analyzed, and it has been shown to compare favorably with soybean meal (Makkar et al, 1998a, b), containing a good balance of essential amino acids, with the exception of lysine. The seeds of most tested varieties of *Jatropha curcas* are inedible, and remain so after heat-inactivation treatments used in seed-meal processing (Heller, 1996). Consequently, the protein rich seed meal of *Jatropha curcas* is not used as animal feed. The prices of seed oil and meal fluctuate depending on supply (harvest) and demand. Although oil is more valuable than meal, the seed meal is potentially a valuable commodity. The ability to use *Jatropha curcas* meal as animal feed not only improves the economics of *Jatropha curcas* production, but also means the crop would produce both fuel and feed.

Table 1.2: Range of average seed mass, oil content, and protein content reported for *Jatropha curcas* seeds (Makkar et al, 1998a, b; Martinez-Herrera et al, 2006; Rao et al, 2008).

	Reported ranges
Average seed mass	450–860 mg
Testa (shell) (%)	30–40%
Kernel (%)	60–70%
Avera Average oil content	
Whole seed	39–37%
Kernel	44–62%
Protein content	
Kernel	22–35%
Seed meal after oil extraction	48–64%

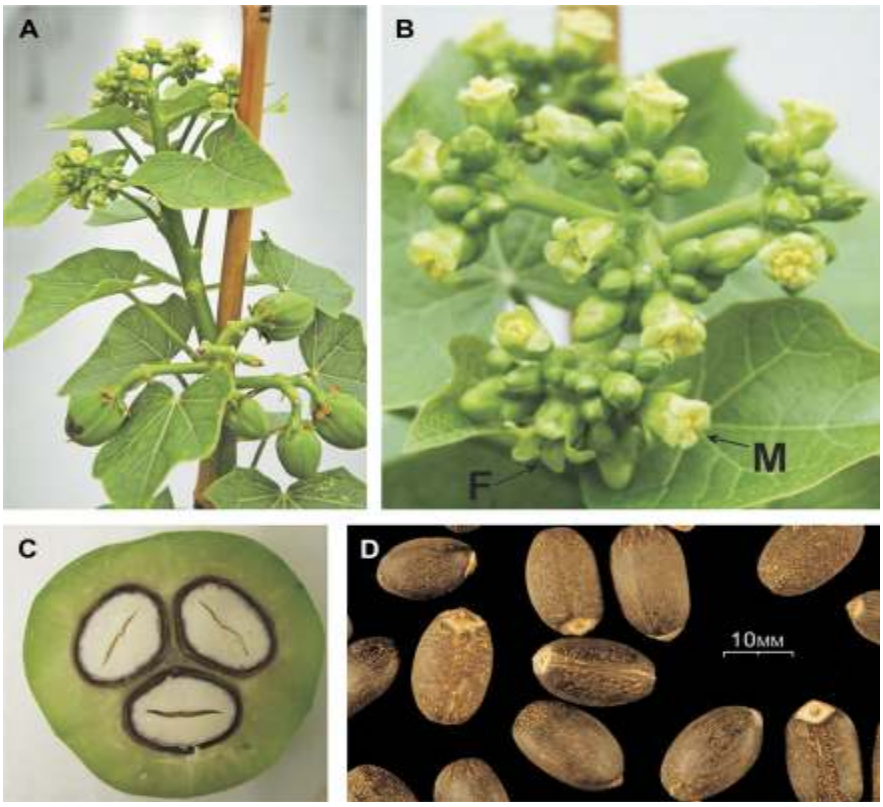


Figure 1.2: Images of *Jatropha curcas* (A) Young *Jatropha curcas* plant with both flowers and developing seedpods. (B) *Jatropha curcas* inflorescence containing both male staminate flowers (M) and female pistillate flowers (F). (C) Cross-section of a *Jatropha curcas* seedpod containing three developing seeds (D) Mature seeds of *Jatropha curcas*

1.8.1 *Jatropha curcas* Oil as an energy source

Jatropha curcas oil gives esters of 16-18 chain length compared to Diesel that has hydrocarbon with 8-10 carbon atoms per molecule. This makes *Jatropha* oil much more viscous than diesel and a fuel with lower ignition quality (cetane number). For these reasons, using the oil directly in engines had not been fully tested over long periods. In Europe, plant oils are usually transesterified (with alcohol and hydroxide) to produce bio-diesels with properties similar to mineral diesel. This reduces their viscosity and increases their cetane number (table 1.3). However, this requires considerable investment and currently it is not cost effective. A principle reason is that the price of crude has been dropping in relative terms over the last decade. As of present, *Jatropha* oil is not as cost effective as diesel, except in exceptional circumstances. A

systematic study of the plant as fuel source and options to improve its inherent property is necessary.

Table 1.3: Physical and Chemical properties of diesel and *Jatropha curcas* oil blend (Pramanik, 2003)

Properties	Diesel	*Jatropha curcas* oil
Density (gm/cc), 30°C	0.836–0.850	0.93292
Kinematic viscosity (cSt), 30°C	4–8	52.76
Cetane No.	40–55	38
Flash point °C	45–60	210
Calorific value, MJ/kg	42–46	38.2
Saponification value	–	198
Iodine No.	–	94

1.8.2 Fuel properties

The important chemical and physical properties of *Jatropha curcas* oil have been determined by standard methods and compared with diesel (table 1.3). The heating value of the vegetable oil is comparable to the diesel oil and the cetane number is slightly lower than the diesel fuel. However, the kinematic viscosity and the flash point of *Jatropha curcas* oil are several times higher than the diesel oil.

1.8.3 *Jatropha* oil blend with diesel

Viscosity of vegetable oil needs to be brought to the specified range. This is brought about by diluting or blending it with other fuels like alcohol or petro-diesel. *Jatropha* oil has been blend with diesel oil in varying proportions with intention of reducing its viscosity and to bring it close to petro-diesel. The high viscosity of *Jatropha curcas* oil has been decreased drastically by partial

substitution with diesel oil (table 1.4). The viscosity of the vegetable oil was decreased on increasing the diesel content in the blend. Though a substantial decrease in viscosity and density was observed with 70:30 or 60:40 *Jatropha*/diesel (J/D) blends, the viscosity and density remain a lot higher than that of diesel. A reduction of viscosity to 55.56% and 62.13% was obtained with 70:30 and 60:40 J/D blends, respectively. Therefore, 70–80% of diesel may be added to *Jatropha curcas* oil to bring the viscosity close to diesel fuel and thus blends containing 20–30% of *Jatropha curcas* oil can be used as engine fuel without preheating (Pramanik, 2003).

Table 1.4: Properties of *Jatropha curcas* oil–diesel blends (Pramanik, 2003)

% of *J. curcas* oil (v/v)	% of diesel fuel (v/v)	Density (g/cc), 30°C	Viscosity (cSt), 30°C	Viscosity reduction (%)	Observation
70	30	0.9	23.447	55.56	Stable mixture
60	40	0.89	19.222	62.13	Stable mixture
50	50	0.853	17.481	66.86	Stable mixture
40	60	0.88	13.953	73.55	Stable mixture
30	70	0.871	9.848	81	Stable mixture
20	80	0.862	6.931	86.86	Stable mixture

1.8.4 Lipid and Fatty acid composition of seed oil and triacylglycerols

Triacylglycerol (TG), the dominant lipid present in *Jatropha curcas* seed (table 1.5), is converted to its esters for use as biodiesel. The TGs have three fatty acids and the compostion of these in *Jatropha curcas* seed oil is well characterized. Fatty acid composition is presented in table 1.6.

Both saturated and unsaturated fatty acids are present in seed oil. The predominant fatty acid present in *Jatropha* seed oil are Oleic acid, Linoleic acid, Stearic acid and Palmitic acid. Among them, Oleic and Linoleic acid are the major ones.

Tabel 1.5: Percentage of Oil classes and lipid composition of *Jatropha curcas* seed oil

Composition	Percentage (%)
Unsaponifiable	3.8
Hydrocarbons esters	4.8
Triacylglycerol	**88.2**
Free fatty acid	3.4
Diacylglycerol	2.5
Sterols	2.2
Monoacylglycerols	1.7
Polar lipids	2

Table 1.6 Fatty Acid composition of seed oil of *Jatropha curcas* (Larson &Graham, 2001)

Fatty acid		Percentage (%)
Myristic acid	14:00	0 - 0.1
Palmitic acid	16:00	14.1-15.3
Stearic acid	18:00	3.7- 9.8
Arachidic acid	20:00	0- 0.3
Behenic acid	22:00	0- 0.2
Palmitoleic acid	16:01	0- 1.3
Oleic acid	18:01	**34.3- 45.8**
Linoleic acid	18:02	**29.0- 44.2**

Though *Jatropha* oil has a beneficial Fatty acid composition, high oil content and can easily be converted and used to replace fossil fuel, it has not been exploited largely because of certain drawbacks, which are:

- Lack of good quality and quantity of planting material, no authentic source for seeds, and no plantation bank has yet been developed.
- Low seed yield due to a Female: Male flower ratio of 1:25-1:30 in the wild.
- Great variability in oil yield from 22% - 44%.

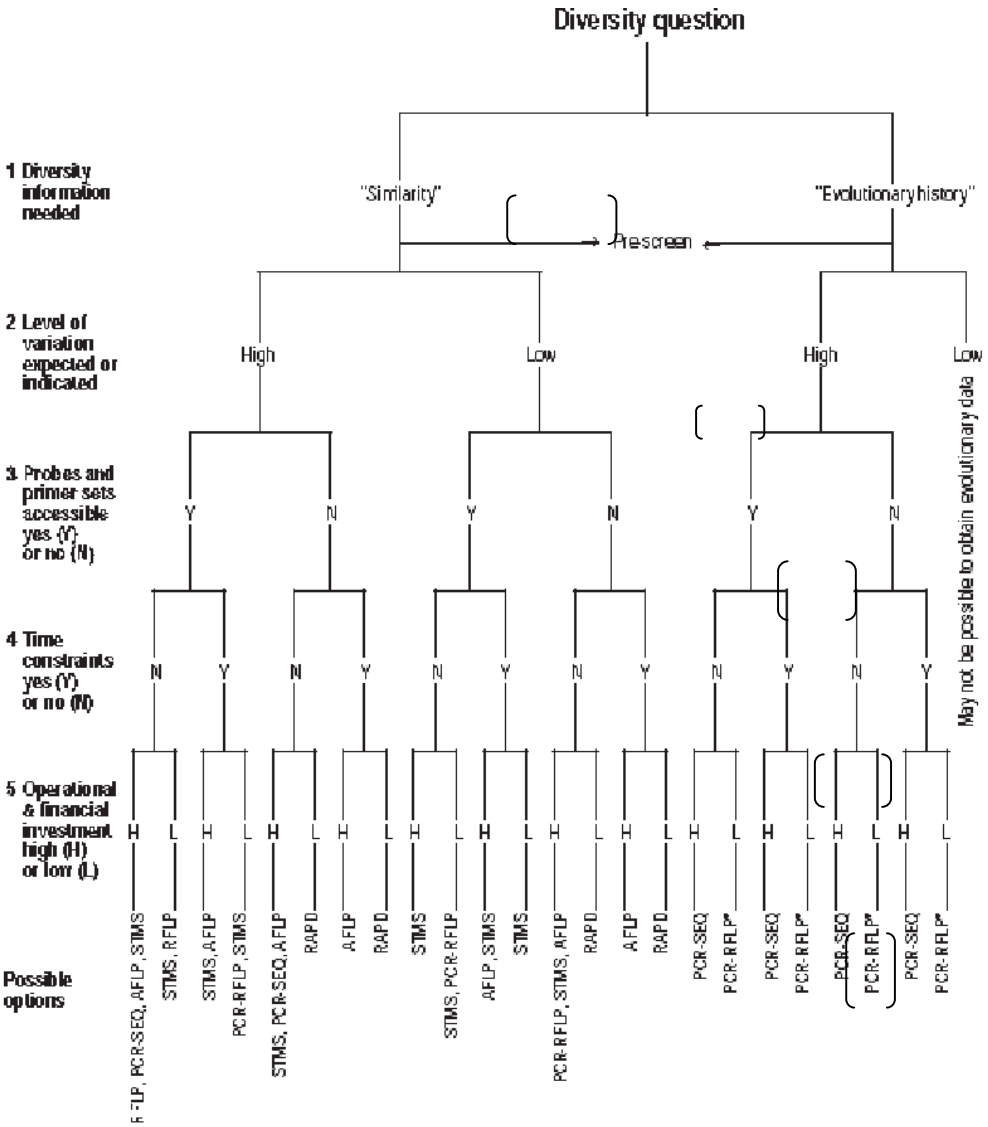
1.9 Existing Genetic variation:

Jatropha curcas is not a native of Asia. It was believed to be brought to Asia by Portuguese sea farers from its place of origin- Central America. Hence, it is not a cultivated variety. It grows as a 'wild' one. This results in a large variation in its growth, seed content, seed weight, oil content and many other physiological parameters. Being of cross-pollinating nature, variation seems to be naturally higher. In the present investigation, similar observations were made in the state of Gujarat regarding its use as hedge plant, limited or no cultivation prior to its popularity, very limited knowledge regarding its oil content, agronomical practices and its commercial exploitation. As it started gaining popularity many farmers, agricultural industries and academia

alike initiated studies on making it a commercial success. With a few years of basic research it became evident that there exits high variation in different accessions present in the state. The Government of India initiated studies on this biofuel crop in collaboration with many national laboratories. As stated by Basha and Sujatha, 2007, the programmes sponsored by various agencies in different countries have a common mandate of survey of *Jatropha* plantations, selection of candidate plus phenotypes, establishment of seed production areas, evaluation, establishment of high-tech nurseries and vegetative multiplication gardens and progeny trials of high yielding plantations. The success of these programmes lie in the identification of genetically divergent material and development of genetically superior stocks. An understanding of the extent of genetic diversity is critical for the success of a breeding programme. As mentioned, *Jatropha* species being cross pollinated results in large variation thereby improving the scope for breeding programmes. However, the major constraint in achieving higher quality of oil yield for this crop are lack of information about its genetic variability, oil composition, and absence of suitable ideotypes for different cropping systems. Research on this species has lagged behind than that of other crop of this family like *Ricinus*. Therefore, improvement of this crop is needed through utilization of available genetic diversity (Gupta et al, 2008). In this thesis an attempt has been made to study the genetic diversity existing in *J.curcas* plantation. Since variability is a prerequisite for selection programme, it is necessary to detect and document the amount of variation existing within and between populations (Ganesh Ram et al, 2008). The conservation and use of plant genetic resources are essential to the continued maintenance and improvement of agricultural and forestry production and, thus, to sustainable development and poverty alleviation. Molecular genetics has an important role to play in many aspects of conservation such as characterizing plant genetic diversity for purposes of improved acquisition, maintenance and use (Karp et al, 1997). Molecular markers can be classified into three categories and stated as per their level of analysis. The first is morphological markers based on phenotypic analysis, followed by biochemical markers which analyze the gene product and finally comes the genetic/molecular marker which analyses at the DNA level. With the development of the polymerase chain reaction (PCR), in particular, numerous molecular technologies have been, and still are being, developed, which can be used for the detection, characterization and evaluation of genetic diversity. These techniques vary in the way that they resolve genetic differences, in the

type of data that they generate, in the taxonomic levels at which they can be most appropriately applied, and in their technical and financial requirements (Karp et al, 1997). The concept of genetic markers dates back to the nineteenth century when Gregor Mendel first used phenotype based markers in his work on pea plants. Since, then there has been an advent in the discovery of different kinds of markers. Budak et al, 2004 cited Stromberg et al, 1994 stating about cases where the selection is based only upon the phenotypic values, breeders commonly confront the problem of genotype environment interaction (GEI), which may mask favorable genotypes making selection more difficult. Biochemical markers have a disadvantage of being under the influence of the DNA sequence. So, ultimately molecular marker, which by definition is a DNA sequence that is readily detected and whose inheritance can be easily monitored, is the preferred marker. Molecular markers are based on naturally occurring DNA polymorphism, which forms basis for designing strategies to exploit for applied purposes. A marker must be polymorphic i.e. it must exist in different forms so that chromosome carrying the mutant genes can be distinguished from the chromosomes with the normal gene by the marker it carries. Genetic polymorphism is defined as the simultaneous occurrence of a trait in the same population of two discontinuous variants or genotypes. An ideal DNA marker should be (i) highly polymorphic in nature as it is polymorphism that is investigated in genetic diversity studies (ii) co-dominant in nature as it allows determination of homozygous and heterozygous states of diploid organisms (iii) frequently occurring in the genome (iv) neutral in behavior, easy, cheap (v) highly reproducible (Kumar et al, 2009)

Table 1.7: Molecular tools in plant genetic resources conservation:a guide to the technologies



Decision making chart for the selection of molecular screening techniques taken from Karp et al, 1997

1.10 Molecular Marker techniques:

Basic marker techniques can be broadly classified into two categories. The first is non-PCR based technique or hybridization based technique and second as PCR based technique.

1.10.1 Non-PCR based technique:

1) RFLP (Restriction Fragment Length Polymorphism)

It was the first molecular marker technique reported by Botstein et al, 1980. In this technique, the chromosomal DNA is digested with restriction enzymes where the distance between two restriction sites vary among different individuals. This generates a banding pattern which can be identified by probes. RFLP's are co-dominant markers. They are useful markers for population studies and diversity classification, provided that sufficient polymorphisms can be detected in the species under study (Karp et al, 1997). Because of their presence throughout the plant genome, high heritability and locus specificity, the RFLP markers are considered superior. The technique is not very widely used because it is time consuming, involves expensive and radioactive/toxic reagents and requires large quantity of high quality genomic DNA (1-10 µg). The requirement of prior sequence information for probe generation increases the complexity of the methodology. These limitations led to the conceptualization of a new set of less technically complex methods known as PCR-based techniques.

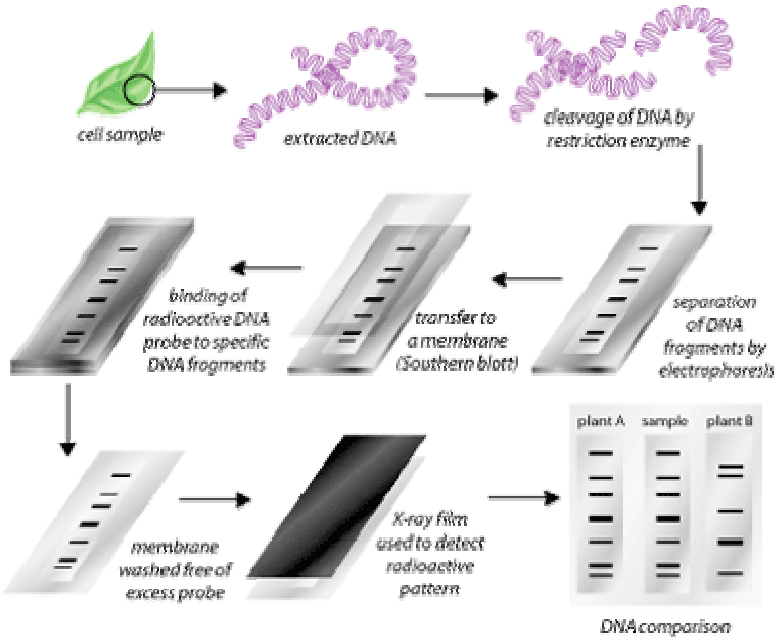


Figure 1.3: Schematic representation of RFLP taken from www.scq.ubc.ca

1.10.2 PCR-based techniques:

The invention of PCR has revolutionized scientific research. It is easy and quick in operation. Next to it is the use of random primers which enabled development of genetic markers without prior knowledge of the genome under investigation. These days PCR is widely used in roughly two types of research namely (i) arbitrary primed PCR or multi locus profiling technique (ii) sequence targeted PCR techniques.

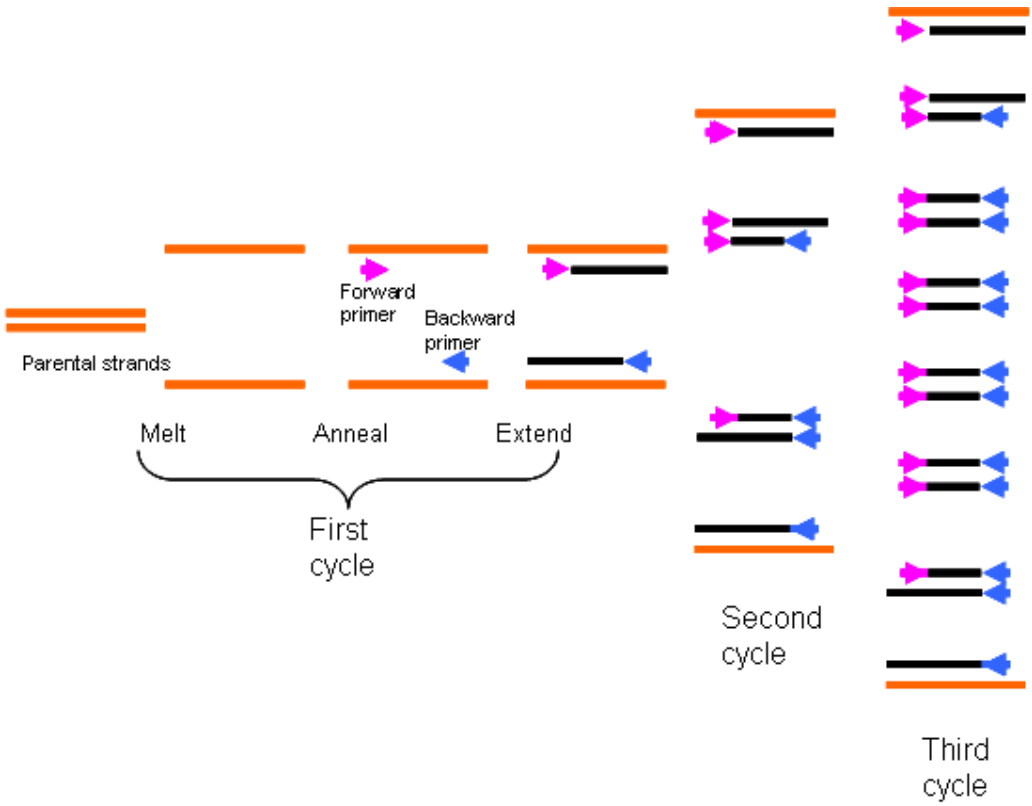


Figure 1.4: A schematic representation of PCR taken from http://biochemistryforlife.wikispaces.com/

1.10.2.1 Arbitrary Primed PCR:

a) RAPD (Random amplification of polymorphic DNA)

b) AFLP (Amplified Fragment Length Polymorphism)

a) RAPD: This is a PCR based technique first described by Williams et al, 1990. RAPD is a technique based on the amplification of genomic DNA with single primers of arbitrary nucleotide sequence. These primers detect polymorphisms in the absence of specific nucleotide sequence information, and the polymorphisms function as genetic markers, and can be used to construct genetic maps. In this reaction, a single species of primer anneals to the genomic DNA at two different sites on complementary strands of DNA template. If these priming sites are within an amplifiable range of each other, a discrete DNA product is formed through thermo cyclic amplification. On an average, each primer directs amplification of several discrete loci in the

genome, making the assay useful for efficient screening of nucleotide sequence polymorphism between individuals (William et al, 1990). RAPDs are DNA fragments amplified by PCR using short synthetic primers (generally 10 bp) of random sequence. These oligonucleotides serve as both forward and reverse primer, and are usually able to amplify fragments from 1–10 sites simultaneously. The polymorphisms arise due to variation in primer annealing. Each product is derived from a region of the genome that contains two short segments in inverted orientation, on opposite strands that are complementary to the primer (Kumar et al, 2009). The major advantage of RAPD is that it is quick and easy and can work in the absence of any genomic sequence information. Since it is a PCR based technique very low DNA quantity (5-50 ng) is sufficient to generate a RAPD profile. Since the technique employs random primers which are commercially available it doesn't require sequencing primers. The major drawback of the method is that the profiling is dependent on the reaction conditions so may vary within two different laboratories and as several discrete loci in the genome are amplified by each primer, profiles are not able to distinguish heterozygous from homozygous individuals (Bardakci, 2001). They are dominant markers and hence have limitations in their use as markers for mapping.

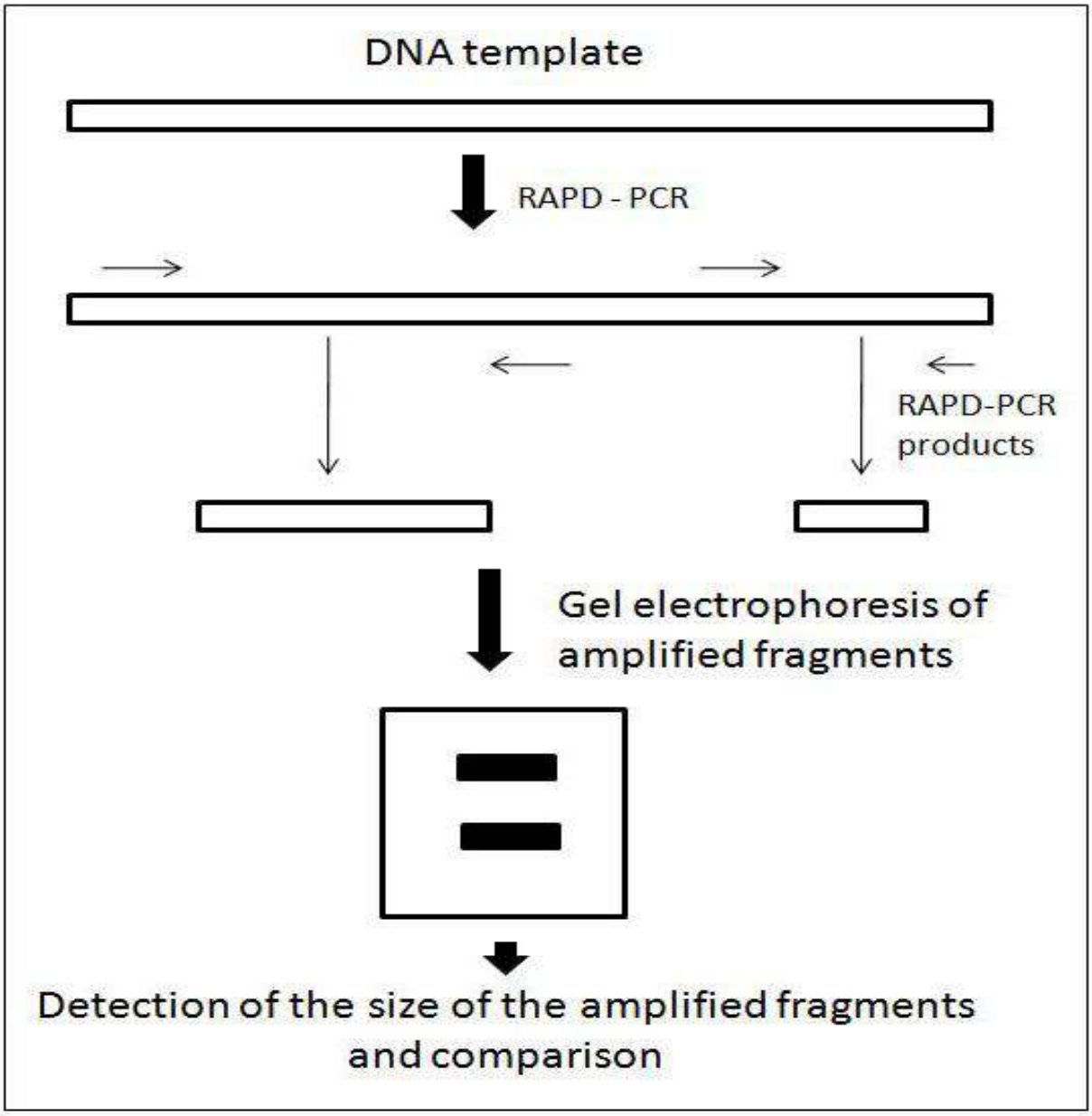


Figure 1.5: Principle of RAPD-PCR technique taken from Arif et al, 2010

b) AFLP: It is an intermediary technique between RFLP and PCR. In this technique genomic DNA is subjected to restriction digestion as in RFLP and the restriction fragments so generated are amplified by PCR. AFLP involves the restriction of genomic DNA, followed by ligation of adaptors complementary to the restriction sites and selective PCR amplification of a subset of the

adapted restriction fragments. These fragments are viewed on denaturing polyacrylamide gels either through autoradiographic or fluorescence methodologies (Vos et al, 1995). AFLP therefore involves both RFLP and PCR. The PCR primers consist of a core sequence (part of the adapter), and a restriction enzyme specific sequence and 1–5 selective nucleotides (the higher the number of selective nucleotides, the lower the number of bands obtained per profile). The AFLP banding profiles are the result of variations in the restriction sites or in the intervening region. The AFLP technique simultaneously generates fragments from many genomic sites (usually 50–100 fragments per reaction) that are separated by polyacrylamide gel electrophoresis and that are generally scored as dominant markers (Kumar et al, 2009). The strength of AFLP technique is that it generates fingerprints of any DNA regardless of its source, and without any prior knowledge of DNA sequence. Most AFLP fragments correspond to unique positions on the genome and hence can be exploited as landmarks in genetic and physical mapping (Agarwal et al, 2008). Disadvantages include the need for purified, high molecular weight DNA, the dominance of alleles, and the possible non-homology of co-migrating fragments belonging to different loci. In addition, due to the high number and different intensity of bands per primer combination, there is the need to adopt certain strict but subjectively determined criteria for acceptance of bands in the analysis. Special attention should be paid to the fact that AFLP bands are not always independent. For example, in case of an insertion between two restriction sites the amplified DNA fragment results in increased band size. This will be interpreted as the loss of a small band and at the same time as the gain of a larger band (Kumar et al, 2009).

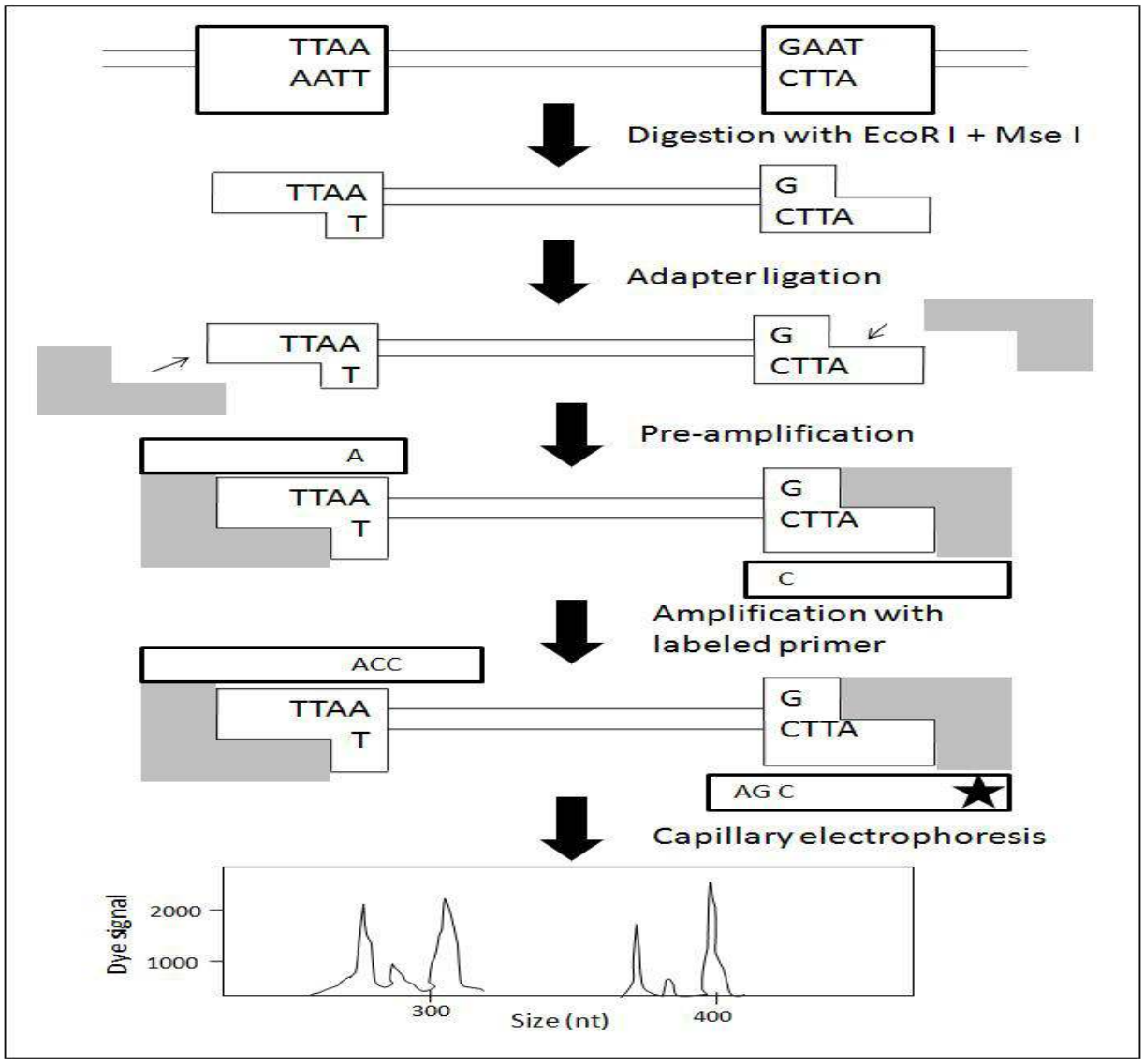


Figure 1.6: A schematic flow chart showing the principle of the AFLP method taken from Arif et al, 2010

1.10.2.2 Sequence targeted PCR based markers

With the advent of high-throughput sequencing technology, abundant information on DNA sequences for the genomes of many plant species has been generated ESTs of many crop species have been generated and thousands of sequences have been annotated as putative functional genes using powerful bioinformatic tools. In order to correlate DNA sequence information with

particular phenotypes, sequence- specific molecular marker techniques have been designed (Agarwal et al, 2008).

Microsatellite based marker technique

Microsatellite or short tandem repeats or simple sequence repeats are monotonous repetitions of very short (one to five) nucleotide motifs, which occur as interspersed repetitive elements in all eukaryotic genomes. Variation in the number of tandemly repeated units is mainly due to strand slippage during DNA replication where the repeats allow matching via excision or addition of repeats. As slippage in replication is more likely than point mutations, microsatellite loci tend to be hypervariable. Microsatellite assays show extensive inter-individual length polymorphisms during PCR analysis of unique loci using discriminatory primer sets. The PCR amplification protocols used for microsatellites employ loci-specific either unlabelled primer pairs or primer pairs with one radiolabelled or fluorolabelled primer. Analysis of unlabelled PCR products is carried out using polyacrylamide or agarose gels. The employment of fluorescent labelled microsatellite primers and laser detection (e.g. automated sequencer) in genotyping procedures has significantly improved the throughput and automatisation. However, due to the high price of the fluorescent label, which must be carried by one of the primers in the primer pair, the assay becomes costly. Microsatellites are highly popular genetic markers because of their co-dominant inheritance, high abundance, enormous extent of allelic diversity, and the ease of assessing SSR size variation by PCR with pairs of flanking primers. The reproducibility of microsatellites is such that, they can be used efficiently by different research laboratories to produce consistent data.

Single Nucleotide Polymorphism

Single nucleotide variations in genome sequence of individuals of a population are known as SNPs. They constitute the most abundant molecular markers in the genome and are widely distributed throughout genomes although their occurrence and distribution varies among species. The SNPs are usually more prevalent in the non-coding regions of the genome. Within the coding regions, an SNP is either non-synonymous and results in an amino acid sequence change or it is synonymous and does not alter the amino acid sequence. Synonymous changes can modify mRNA splicing, resulting in phenotypic differences. Improvements in sequencing technology and availability of an increasing number of EST sequences have made direct analysis of genetic

variation at the DNA sequence level possible. Majority of SNP genotyping assays are based on one or two of the following molecular mechanisms: allele specific hybridization, primer extension, oligonucleotide ligation and invasive cleavage. High throughput genotyping methods, including DNA chips, allele-specific PCR and primer extension approaches make single nucleotide polymorphisms (SNPs) especially attractive as genetic markers. They are suitable for automation and are used for a range of purposes, including rapid identification of crop cultivars and construction of ultra high-density genetic maps.

From the introduction given above it is clear that though *Jatropha curcas* is a crop with immense potential as a biofuel plant, variation in genetic material and lack of high quality planting material act as an impediment in its success.

1.11 To bring *Jatropha* to the forefront

Inspite of its obvious advantages, one of the major reasons why biodiesel has not been able to directly compete with petro-diesel in the current global market is its limited availability due to it not being produced on a large scale. Widespread production can be promoted by

Increased cultivation: What makes *Jatropha* especially attractive to India is that it is drought-resistant and can grow in saline, marginal and even otherwise infertile soil, requiring little water and maintenance. It is highly adaptable and easy to propagate; a cutting taken from a plant and simply pushed into the ground will take root. Hence bringing in more wastelands under *Jatropha* cultivation will help in increasing yield and hence solve problems of fuel and optimum utilization of waste or barren lands. This in turn will also help in generating employment for the rural folk.

Increased number of seeds: *Jatropha* plant has a skewed male to female flower ratio of 28:1 due to which the seed production per plant is less (Raju and Ezradanum, 2002) Increase in seed number can be achieved by manipulating the ratio of various plant growth regulators to increase the ratio of female to male flowers. This will result in an increase in the number of seeds eventually increasing the total oil content.

Increased oil content per seed: Seed oil content in *Jatropha* ranges between 22-44%. Seed oil content can be increased by genetic engineering, thus leading to higher oil content per plant and per hectare of cultivated area. By using these methods, it is possible to increase oil production per plant and finally lead to augmentation in the amount of biodiesel produced. For example, if seed yield per plant is 9 kg and average yield of oil per kg of seeds is 350 grams, it will lead to an oil yield of 3.15 kg per plant. Increasing seed yield to 12 kg and oil yield per kg of seed to 450 grams, will lead to an oil yield of 5.4 kg per plant ; an increase of 71.42 % in oil yield.

1.11.1 Increasing oil content per seed

Seed triacylglycerol (TAG) biosynthesis is located in the endoplasmic reticulum with glycerol-3-phosphate and fatty acyl-coenzyme A (CoAs) being the primary substrates. There are three acyltransferases and a phosphohydrolase involved in the plant storage lipid bioassembly, namely glycerol-3-phosphate acyltransferase (GPAT, EC 2.3.1.15), *lyso*-phosphatidic acid acyltransferase (LPAT, EC 2.3.1.51), phosphatidate phosphohydrolase (PAPase, EC 3.1.3.4), and diacylglycerol acyltransferase (DGAT, EC 2.3.1.20). The three acyl transferases catalyse the stepwise acylation of the glycerol backbone with the final step being the acylation of *sn*-1, 2-diacylglycerols (DAGs) by DGAT to form TAGs, a biochemical process known as Kennedy pathway. (Kennedy, 1961; Barron and Stumpf, 1962; Stymne and Stobart, 1987).

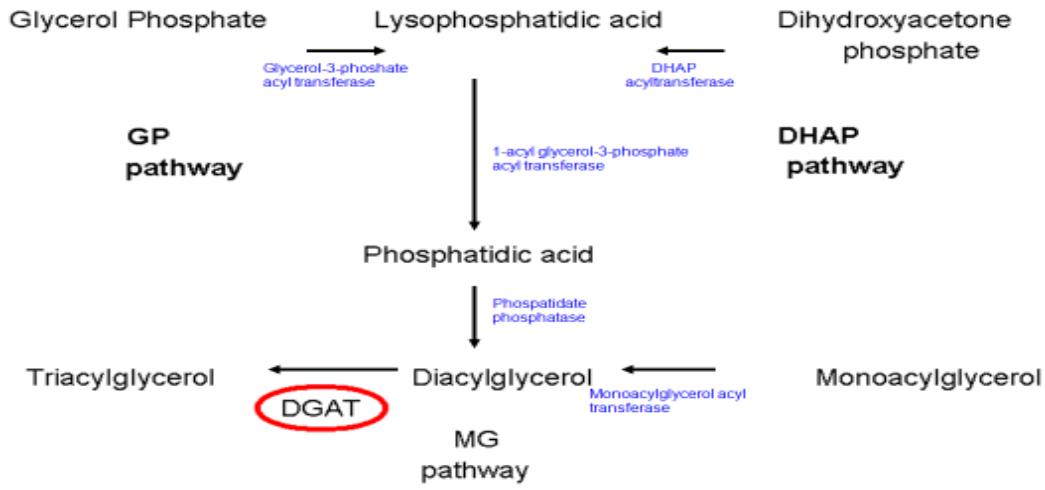


Figure 1.7: Kennedy Pathway

1.11.2 Targeting DGAT

The acyl CoA dependent acylation of *sn*-1,2-DAG as catalysed by DGAT is the only enzyme in the traditional Kennedy pathway that is exclusively committed to TAG biosynthesis. The biochemical properties of microsomal DGAT have been examined in a number of plants systems (Frentzen, 1993), including developing seeds (Cao and Huang, 1987; Bernerth and Frentzen, 1990; Vogel and Browse, 1996) and embryo cultures (Taylor et al, 1991,1992; Weselake et al, 1991; Little et al, 1994) of *Brassica napus.* In general, studies with developing seeds indicate that DGAT activity increases rapidly during the active phase of oil accumulation and then decreases markedly as seed lipid content reaches a plateau. (Tzen et al, 1993; Weselake et al*,* 1993).

A number of studies with both mammalian (Mayorek et al, 1989; Tijburg et al, 1989) and plant (Ichihara et al, 1988; Perry and Harwood, 1993a, 1993b; Settlage et al, 1995; Perry et al, 1999) systems have suggested that DGAT may catalyze a rate limiting reaction in TAG bio-assembly. Hence increasing the amount of this enzyme may increase TAG biosynthesis thus leading to increase in oil content per seed. In this work it is proposed to increase the expression of DGAT in *Jatropha* to increase its oil content. Genetic modification of *Jatropha* with regards to increasing oil content has not been reported so far. However, similar work has been done in *Arabidopsis* where seed specific over-expression of an *Arabidopsis* cDNA encoding DGAT resulted in an enhancement of seed oil content and seed weight. (Jako et al, 2001). He et al, 2004 have reported the cloning and characterization of a cDNA encoding DGAT from castor bean. Increase in levels of DGAT enzyme can be brought about by increased expression of the DGAT gene. This can be done by cloning cDNA of DGAT gene into a binary vector and its transformation into *Jatropha* followed by its expression under a seed specific strong promoter. There are different methods for plant transformation, which are

1.11.3 Electroporation

In this technique, protoplasts, intact cells or tissue (callus, immature embryo, and inflorescence) are incubated in buffer solution containing DNA and subjected to high-voltage electrical pulses.

DNA migrates through pores in the membrane and integrates into the plant genome (Sorokin et al, 2000)

Advantages- This technique is a simple, fast and inexpensive way for transient and stable transformation of different tissues.

Disadvantages- It has a low efficiency and requires careful optimization; also the range of tissues that can be transformed is narrow as compared to particle bombardment.

1.11.4 Microprojectile Bombardment

In this technique Tungsten or gold particles are coated with DNA and accelerated towards target plant tissues using a special apparatus called a particle gun. These accelerated particles punch holes in the plant cell wall and usually penetrate only1-2 cell layers. The DNA-coated particles go either near or in the nucleus, where the DNA comes off the particles and integrates into plant chromosomal DNA (Klein et al, 1987).

Advantages- It is one of the most versatile and effective technique. It is also environmentally friendly.

Disadvantages-low efficiency of transformation, low survival of bombarded cells caused by mechanical damages, DNA is not protected during bombardment, high number of transgene copies, random intracellular target.

1.11.5 Microinjection

Using a micromanipulator and an inverted microscope, DNA is injected into cells or protoplasts by a syringe. (Holm, 2000).

Advantages-Possibility for optimization of amount of DNA delivered to a single plant cell, precision and predictability of the DNA delivery place.

Disadvantages - A single cell receives DNA during a single injection event. It is an expensive technique and requires exceptional manual capabilities. The above methods have been used widely in plants but have the following disadvantages:

1. A relatively low efficiency of transformation
2. Integration of many transgene copies into the plant genome

3. Rearrangements of the transgene copies and chromosomes
4. Transgene silencing

1.11.6 *Agrobacterium tumefaciens* mediated transformation:

This has become the method of choice for plant molecular biologists. In this method, co-cultivation of plants or plant explants with *Agrobacterium* is done and the gene of interest is transferred by T-DNA mediated transfer from bacterial cell to the plant genome. The phytopathogenic bacterium *Agrobacterium tumefaciens* genetically transforms plants by transferring a portion of its resident Ti plasmid, the T-DNA (Transferred DNA) to the plant cells (Gelvin, 2000). It naturally infects wounded plant sites and causes the formation of crown gall tumors (De la Riva et al, 1998). Virulent strains of *A. tumefaciens* harbor large plasmids of size 140-235 kb (Zaenen et al, 1974).

1.12 Natural transformation by *Agrobacterium tumefaciens*

A successful genetic transformation of an organism requires three components; target cells to be transformed, a vector to carry a foreign gene into the target cell's nucleus, and the transformed cell's ability to stably transmit the new genetic information from generation to generation. Having a whole plant regeneration system in hand meant that the only element needed for complete genetic transformation of a plant is a vector to carry a foreign gene to a target cell. That was provided by a modification of a naturally occurring genetic engineering system found in *Agrobacterium tumefaciens*. The molecular mechanism of *A. tumefaciens* infection was shown to use its T-plasmid as a vector to genetically transform the host cell (Dilworth, 2009). *A. tumefaciens* has the exceptional ability to transfer a particular DNA segment (T-DNA) of the tumor-inducing (Ti) plasmid into the nucleus of infected cells where it is then stably integrated into the host genome and transcribed, causing the crown gall disease. T-DNA contains two types of genes: the oncogenic genes, encoding for enzymes involved in the synthesis of auxins and cytokinins and responsible for tumor formation; and the genes encoding for the synthesis of opines. These compounds, produced by condensation between amino acids and sugars, are synthesized and excreted by the crown gall cells and consumed by *A. tumefaciens* as carbon and

nitrogen sources. Outside the T-DNA, are located the genes for the opine catabolism, the genes involved in the process of T-DNA transfer from the bacterium to the plant cell and the genes involved in bacterium-bacterium plasmid conjugative transfer. Virulent strains of *A. tumefaciens* and *A. rhizogenes*, when interacting with susceptible dicotyledonous plant cells, induce diseases known as crow gall and hairy roots, respectively. These strains contain a large mega plasmid (more than 200 kb) which plays a key role in tumor induction and for this reason it was named Ti plasmid, or Ri in the case of *A. rhizogenes*. Ti plasmids are classified according to the opines, which are produced and excreted by the tumors they induce. During infection the T-DNA, a mobile segment of Ti or Ri plasmid, is transferred to the plant cell nucleus and integrated into the plant chromosome. The T-DNA fragment is flanked by 25-bp direct repeats, which act as a *cis* element signal for the transfer apparatus. The process of T-DNA transfer is mediated by the cooperative action of proteins encoded by genes determined in the Ti plasmid virulence region (*vir* genes) and in the bacterial chromosome (De la Riva et al, 1998).

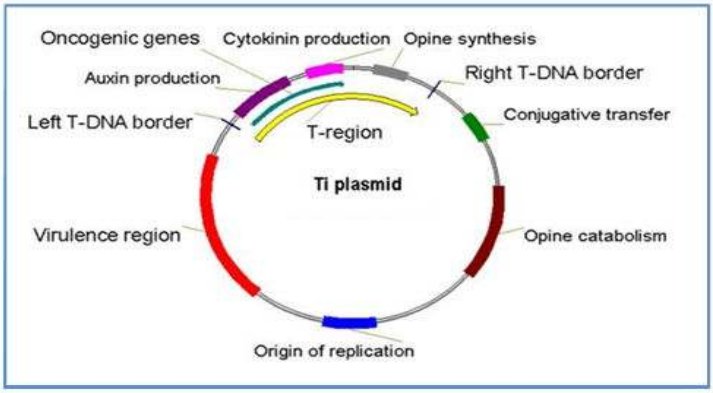


Figure 1.8: Pictorial representation of Ti plasmid

1.12.1 Ti Plasmid

The Ti plasmid consists of the following regions:

1) Right T-DNA border sequence and Left T-DNA border sequence. These are imperfect direct repeats flanking the T-DNA region. They are not transferred to the plant genome but are essential components in the transfer process.

2) Origin of replication which lies outside the T-DNA and allows the Ti plasmid to be stably maintained in *A. tumefaciens*.

3) Gene for opine catabolism which lies outside the T-DNA region. Opines are unique and unusual condensation products of an amino acid and keto acid. They are synthesized within the crown gall and then secreted. They can be used as a carbon and nitrogen source by an *A. tumefaciens* carrying a Ti plasmid bearing the gene for catabolism of that particular opine. This is therefore a unique mechanism that has evolved where each strain of *A. tumefaciens* genetically transforms only those plant cells which produce a compound that it alone is able to utilize (Glick, 2003).

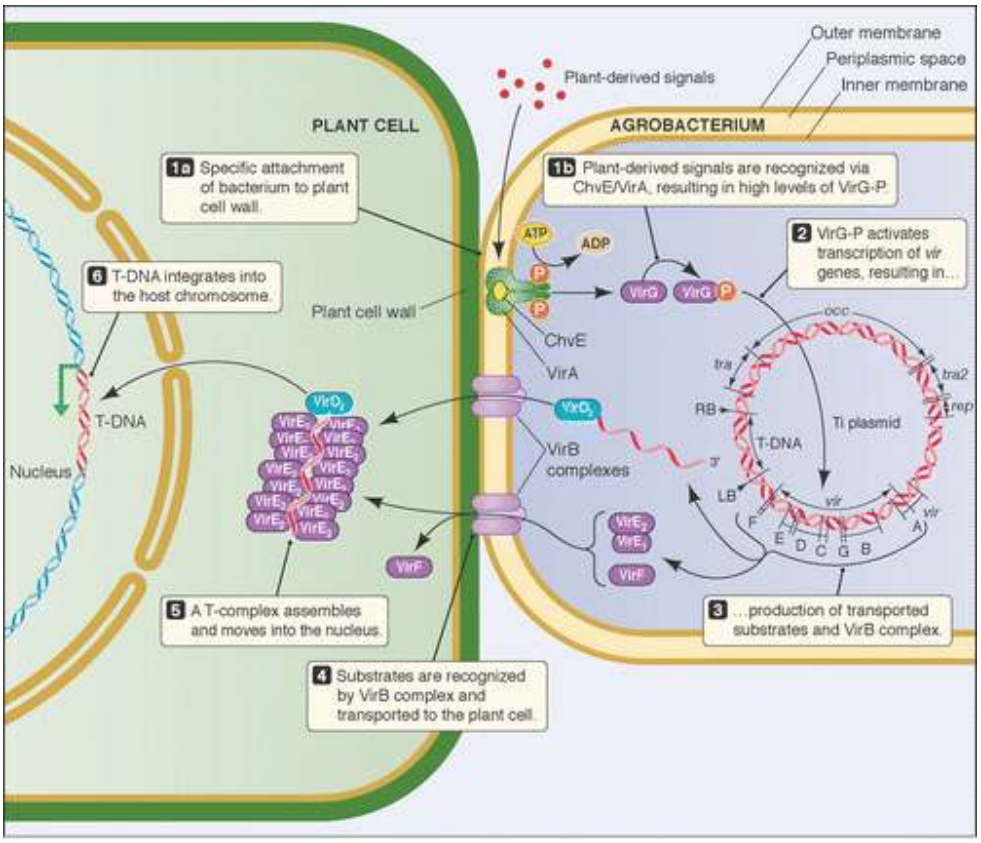
4) T-DNA region which consists of oncogenic genes namely those for auxin and cytokinin production. These phytohormones are responsible for the tumorous growth which results due to a disturbance in the levels of the phytohormones. It also contains gene for opine synthesis. All genes of the T-DNA have promoter sites and polyadenylation sites that are eukaryotic in nature (Ooms et al, 1981).

5) The vir (virulence) genes that lie outside the T-DNA region encodes virulence (Vir) proteins process the T-DNA region from the Ti-plasmid, producing a 'T-strand'. After the bacterium attaches to a plant cell, the Tstrand and several types of Vir proteins are transferred to the plant through a transport channel. Inside the plant cell, the Vir proteins interact with the T-strand, forming a T-complex. This complex targets the nucleus, allowing the T-DNA to integrate into the plant genome and express the encoded genes as shown below.

1.12.2 The T-DNA transfer process

The transfer of T-DNA from *A.tumefaciens* to plant cells is a complex process entailing several steps like bacterial colonization, induction of bacterial virulence system, generation of T-DNA transfer complex, T-DNA transfer and integration of T-DNA into plant genome.

Bacterial colonization is the essential early step which takes place when the bacteria attaches to the surface of the plant cell. Polysaccharides of the bacterial cell wall are found to play a significant role in bacterial colonization. Along with that chromosomal 20 kb *att* locus genes are also required for successful bacterial colonization (De la Riva et al, 1998).



AR McCullen CA, Binns AN. 2006. Annu. Rev. Cell Dev. Biol. 22:101–27

Figure 1.9: Schematic representation of plant bacterial interaction

For induction of bacterial virulence system T-DNA transfer is required. The T-DNA transfer is mediated by products encoded by the 30-40 kb *vir* region of the Ti plasmid. This region is composed by at least six essential operons (*vir A, B, C, D, E, G*) and two non-essential operons (*virF, virH*). The number of genes per operon differs, *virA, virG* and *virF* have only one gene; *virE, virC, virH* have two genes while *virD* and *virB* have four and eleven genes respectively. The only constitutive expressed operons are *virA* and *virG*, coding for a two-component (VirA-VirG) system activating the transcription of the other *vir* genes (De la Riva et al, 1998). VirA and VirG proteins function as members of a two component sensory-signal transduction genetic regulatory system. VirA is a periplasmic antenna that senses the presence of particular plant phenolic compounds that are induced on wounding. In coordination with the monosaccharide

transporter ChvE and in the presence of the appropriate phenolic and sugar molecules, VirA autophosphorylates and subsequently transphosphorylates the VirG protein. VirG in the nonphosphorylated form is inactive; however, on phosphorylation, the protein helps activate or increase the level of transcription of the *vir* genes, most probably by interaction with *vir*-box sequences that form a component of *vir* gene promoters.

Together with the VirD4 protein, the 11 VirB proteins make up a type IV secretion system necessary for transfer of the T-DNA and several other Vir proteins, including VirE2 and VirF. VirD4 may serve as a "linker" to promote the interaction of the processed T-DNA/VirD2 complex with the VirB-encoded secretion apparatus. Most VirB proteins either form the membrane channel or serve as ATPases to provide energy for channel assembly or export processes.

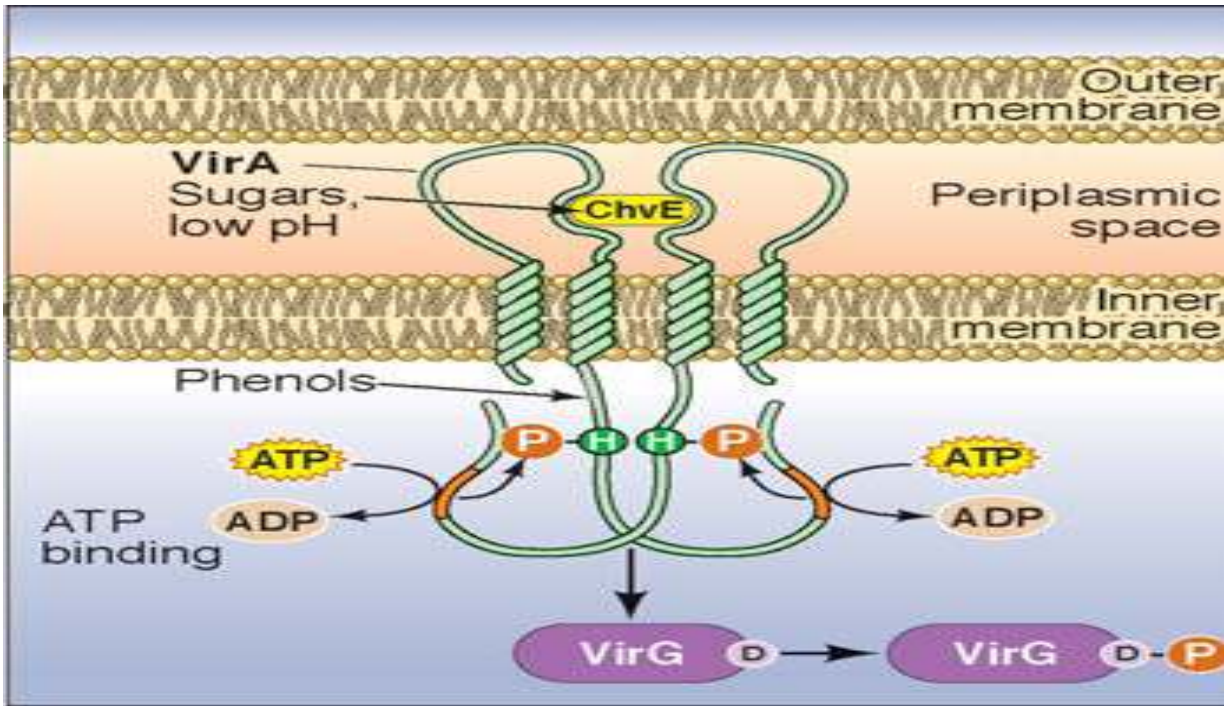


Figure 1.10: The ChvE/VirA/VirG signal transducing system by Mc Cullen and Binns, 2006

Several proteins, including VirB2, VirB5, and possibly VirB7, make up the T-pilus. VirB2, which is processed and cyclized, is the major pilin protein. The function of the pilus in T-DNA transfer remains unclear; it may serve as the conduit for T-DNA and Vir protein transfer, or it

may merely function as a “hook” to seize the recipient plant cell and bring the bacterium and plant into close proximity to effect molecular transfer (Gelvin, 2003).

For generation of T-DNA transfer complex activation of *vir* genes produces single-stranded (ss) molecules representing the copy of the bottom T-DNA strand. Any DNA placed between T-DNA borders will be transferred to the plant cell, as single strand DNA, and integrated into the plant genome. These are the only cis acting elements of the T-DNA transfer system (De la Riva et al, 1998). The VirD2 functions as an endonuclease excising the T-DNA strand from the Ti plasmid. These two proteins VirD2 and VirE2 have been proposed to constitute, with the T-strand, a “T-complex” that is the transferred form of the T-DNA. Thus, it is possible that one function of VirE2 is to form a pore in the plant cytoplasmic membrane to facilitate the passage of the T-strand. Because of its attachment to the 5’ end of the T-strand, VirD2 may serve as a pilot protein to guide the T-strand to and through the type IV export apparatus. Once in the plant cell, VirD2 may function in additional steps of the transformation process. VirD2 contains nuclear localization signal (NLS) sequences that may help direct it and the attached T-DNA to the plant nucleus. The NLS of VirD2 can direct fused reporter proteins and in vitro-assembled T-complexes to the nuclei of plant, animal, and yeast cells. Furthermore, VirD2 can associate with a number of *Arabidopsis* importin-α proteins in an NLS-dependent manner, both in yeast and in vitro systems. VirE2 is a non-sequence-specific single-stranded DNA binding protein. In *Agrobacterium* cells, VirE2 probably interacts with the VirE1 molecular chaperone and may therefore not be available to bind T-strands. However, when bound to single-stranded DNA, VirE2 can alter the DNA from a random-coil conformation to a shape that resembles a coiled telephone cord This elongated shape may help direct the T-strand through the nuclear pore. VirE2 also contains NLS sequences that can direct fused reporter proteins to plant nuclei As with VirD2, VirE2 interacts in yeast with *Arabidopsis* importin- α proteins in an NLS-dependent manner. Finally, VirE2 may protect T-strands from nucleolytic degradation that can occur both in the plant cytoplasm and perhaps in the nucleus (Gelvin, 2003).

1.13 Use of *Agrobacterium* in biotechnology

Genetic experiments indicated that a particular class of plasmids, the Ti (and later Ri) plasmids, were responsible for tumorigenesis (Larebeke et al, 1974) and that a portion of these plasmids, the T-DNA, was transferred to plant cells and incorporated into the plant genome (Chilton et al, 1977). It was thus obvious to propose that Ti plasmids be used as a vector to introduce foreign genes into plant cells.

However, Ti plasmids are very large and T-DNA regions do not generally contain unique restriction endonuclease sites not found elsewhere on the Ti plasmid. Therefore, one cannot simply clone a gene of interest into the T-region. Scientists have therefore developed a number of strategies to introduce foreign genes into the T-DNA. These strategies involved two different approaches:

1) Cloning the gene, by indirect means, into the Ti plasmid such that the new gene is *cis* to the virulence genes on the same plasmid

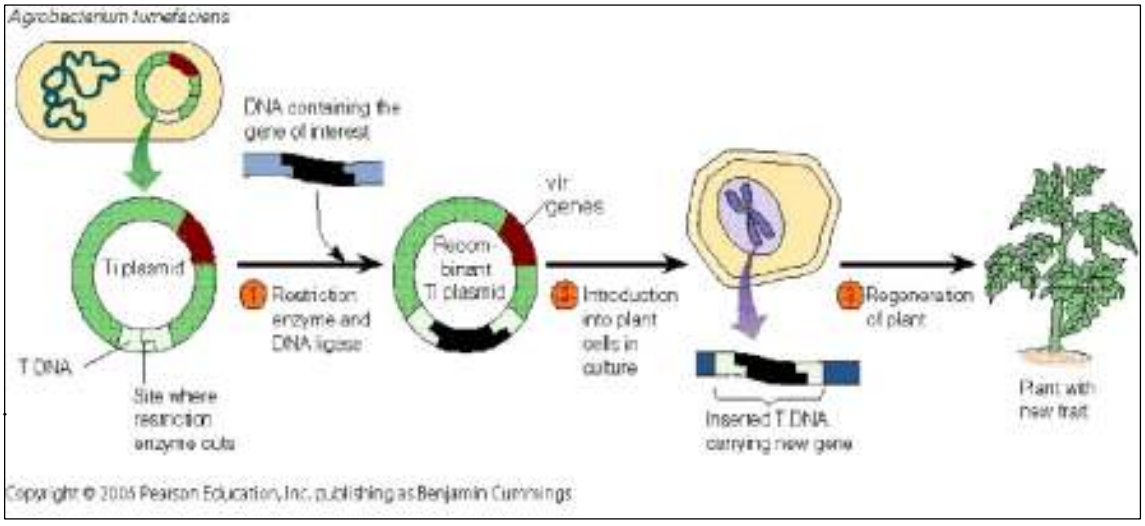


Figure 1.11: Cloning of gene of interest into Ti plasmid and its use in plant transformation

2) Cloning the gene into a T-region that is on a separate replicon from the *vir* genes. T-DNA binary vectors are a class of vectors which contain two different origins of replication such that they can replicate in two different hosts. For eg. pCAMBIA 1305.2 which can replicate in *E. coli*

and *Agrobacterium tumefaciens* is replicated to a high copy number in *E.coli* following which it is transformed into *Agrobacterium tumefaciens* strain containing disarmed Ti plasmid (Ti plasmid with tumourigenic genes excised out) which contains the *vir* genes that encode the Vir proteins.

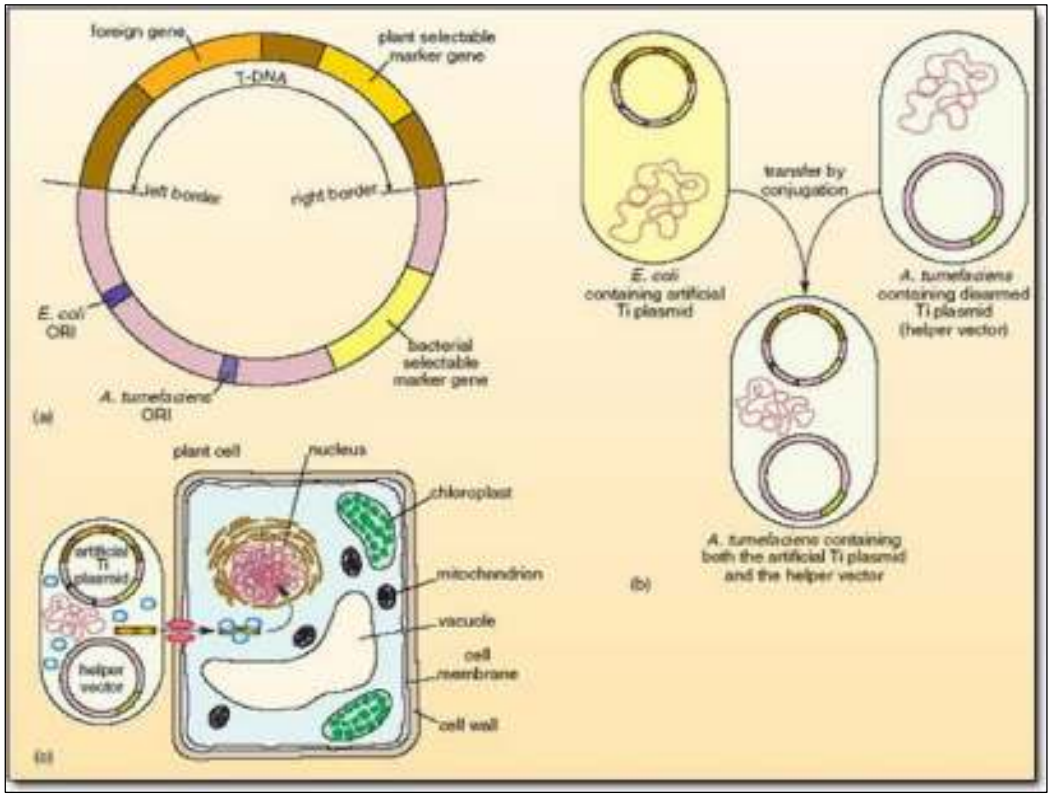


Figure 1.12: Plant transformation using binary vectors

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Tissue Culture Studies For Development of Regeneration Protocol for *Jatropha*

2.0 Introduction

Jatropha curcas is a drought resistant shrub belonging to the genus Euphorbiaceae. It has been touted as a biodiesel plant. The first commercial application of its seed oil was reported from Lisbon where the oil was used for soap production and for lighting lamps. The press cake was used as fertilizer for potatoes (Gubitz et al, 1999).

Today, *Jatropha curcas* is mainly cultivated as a biofuel crop on marginal land for poverty alleviation. *Jatropha* has also been exploited for its medicinal properties. The seeds of this plant are used as a purgative, sap for wound healing, decoction of leaves for malarial treatment and many more.

In India it is generally planted as a fence around the fields to protect it against grazing cattle and it is also found to reduce soil erosion. In Comore islands, in Papua New Guinea and in Uganda, *Jatropha curcas* plants are used as a support for Vanilla plants and as a source of shade for Coffee plants in Cuba (Henning, 2004).

However, its commercial success as a biofuel plant is still elusive. Major bottlenecks for the same are significant variations in seed yield and oil content, low seed viability and germination rate. For vegetative propagation to be successful, it would require availability of quality planting material throughout the year. However, this is limited by unavailability of quality planting material and propagation being affected by climatic changes.

Therefore, improvement in programmes of popularizing *J. curcas* by modern methods of agro-biotechnology is of interest worldwide. This has increased the importance of developing tissue culture protocols to facilitate large scale production of true-to-type plants. These protocols could also be used for the improvement of the species by genetic engineering techniques. *In vitro*

regeneration techniques offer a powerful tool for germplasm conservation, mass multiplication of true-to-type plants and genetic transformation (Kumar and Reddy, 2010).

Genetic transformation approach allows introduction of novel genes thereby helping in introducing agronomically important traits. The availability of an efficient regeneration system is a prerequisite for utilizing this approach. For successful genetic modification, efficient production of transgenic plants, effective and fast regeneration system is imperative. Though several reports on regeneration from various explants in *J. curcas* exist, not many reports on its genetic transformation are present. This is due to the lack of efficient protocols to regenerate whole plants through *in vitro* regeneration from the transformed tissue.

2.1 Literature Studies

Few regeneration protocols have been reported for *J. curcas* using different explants like leaves (Sujatha and Mukta, 1996; Sujatha et al, 2005; Deore and Johnson 2008; Reddy et al, 2008; Khurana-Kaul et al, 2010; Divakara et al, 2010); shoot tips (Rajore and Batra, 2005); nodes and axillary nodes (Sujatha et al, 2005); petiole (Sujatha and Mukta, 1996, Kumar and Reddy, 2010, Kumar et al, 2011); hypocotyls (Sujatha and Mukta, 1996); cotyledons (Kumar et al, 2010) etc. Both direct and indirect plant regeneration systems have been reported. All the above stated reports have mentioned the use of Murashige and Skoog's (MS) medium (1962). Along with the salts of MS media, phytohormones are also added. Plant hormones do not function in isolation within plant, instead, function in relation to each other. Hormone balance is apparently more important than the absolute concentration of any one hormone. Both cell division and cell expansion occurs in actively dividing tissue, and of the two what predominates is decided by the ratio of cytokinin and auxin. The ratio of hormones in the media also decides the pattern of overall growth and morphological changes. In light of this, the hormone differentials in experimental media should also have an effect on the growth and development of excised explants (Mineo, 1990). The role of specific hormones for specific stages of tissue culture is well defined. Several studies have reported the use of plant growth regulators used at various stages like shoot bud induction, elongation, rooting etc. for *J.curcas.* Apart from the kind of hormone its concentration is also critical. For *J.curcas* use of varied ranges of cytokinins like TDZ

(Thiadiazuron), BAP (Benzyl amino purine), Kn (Kinetin) and auxins like IBA (Indole-3-butyric acid), NAA (Naphthalene acetic acid), IAA (Indole-3-acetic acid) have been used. Apart from phytohormones certain media additives have also been found to play a crucial role in achieving regeneration. Sujatha and Mukta (1996) reported regeneration from various explants like hypocotyls, petiole and leaf explants with a range of cytokinins like zeatin, kinetin and N6 Benzyladenine either singly or in combination with indole-3-butyric acid (IBA). This study also compared the performance of different leaves with respect to their position in tissue culture where they reported that the third leaf was more responsive as compared to the fourth leaf. Independent of the explants type, direct adventitious shoot bud induction was recorded highest on MS medium with 2.22 µM BA and 4.9 µM IBA. Half strength MS, MS basal salts without auxin, MS in combination with IAA, NAA or IBA was used to induce rooting and 80% rooting efficiency was reported. Rajore and Batra (2005) reported shoot tip culture on MS with 8.87 µM BAP and 2.85 µM IAA. They also reported the incorporation of adenine sulphate, glutamine and activated charcoal as media additives. They reported rooting on half-MS with 2.46-24.6 µM IBA. Half strength MS in combination with 3.0 mg/l IBA favored root formation in the same study. Sujatha et al, 2005 reported shoot bud induction from axillary nodes of *Jatropha curcas* on MS with 2.3-46.5 µM Kn, 2.2-44.4 µM BA and 2.3-45.4 µM TDZ individually. Leaf segments were cultured on MS +8.9 µM BA +2.5 µM IBA. Incorporation of TDZ was found to favor shoot bud induction as compared to other cytokinins. The elongated shoots were rooted on medium comprising of half strength MS basal salts and supplemented with 5.4 µM NAA. MS medium supplemented with 22.19 µM BAP, 2.32 µM Kn and 0.57 µM IAA facilitated shoot bud induction from nodal explants of *J.curcas* (Kalimuthu et al, 2007). Effectiveness of TDZ in inducing direct organogenesis from leaf discs of *J.curcas* was first reported by Deore and Johnson (2008). Though earlier reports suggested direct organogenesis with BAP and IBA, Khurana-Kaul et al, 2010 reported intermediate callus with the use of the same combination, however, they also reported the effectiveness of TDZ along with IBA. In this study they varied $CuSO_4$ levels and found it to be more effective at ten times higher concentration than that of MS salts. This resulted in significant improvement in shoot bud induction. The role of TDZ in significantly influencing shoot bud induction in *J.curcas* was also substantiated by Kumar and

Reddy, 2010; Kumar et al, 2011. They also mentioned the orientation of the explant as an important factor for shoot bud formation.

Shoots can be derived either through differentiation of non-meristematic tissues known as adventitious shoot formation or through pre-existing meristematic tissues known as axillary shoot formation. Both the approaches require synergistic interaction of physical and chemical factors. A successful plant regeneration protocol requires appropriate choice of explant, age of the explants, definite media formulations, specific growth regulators, genotype, source of carbohydrate, gelling agent and other physical factors including light regime, temperature, humidity, etc. Genotype is one of the main factors that influence the organogenic response of cultures in different plant species. Among the different factors affecting *J.curcas* regeneration, the genotypic dependence is ranked quite high (Mukherjee et al, 2011).

From the above discussion it is evident that a need to derive a genotype dependent effective regeneration protocol is imperative and that would pave the way for further genetic transformation studies.

2.2 Materials and Methods

2.2.1 Plant Material and general culture conditions

Jatropha curcas plants grown on The M.S. University Campus were used as source of explants. Tissue culture media were prepared as per specifications in Murashige and Skoog (MS) (1962) having 30g/L sucrose. The pH of the media was adjusted to 5.6 ± 0.1 using 1N NaOH or 1N HCl and supplemented with 7g/L agar. Media was autoclaved at 15 psi at 121ºC for 20 minutes. Growth conditions were maintained at 26±2°C with 16 h photoperiod with flux density of 30 µmol m^{-2} s^{-1} by cool white fluorescent tubes (Philips, India).

2.2.2 *Jatropha curcas* explants

Young leaves at 3rd and 4th node from the apex were collected from 1.5-2 year old plants grown on campus. The explants were thoroughly washed with tap water for nearly half an hour followed by washing with soap solution. The explants were surface sterilized with 0.1%

mercuric chloride for 2 to 3 minutes followed by five rinses with sterile distilled water. The detailed protocol for explant surface sterilization of *J.curcas* is presented in section 2.3.1. Leaves were then excised into small pieces of 1x1cm and inoculated on Murashige & Skoog (MS) basal medium. After a week these leaf discs were transferred to media containing different combination of BAP and IBA for regeneration. They were observed periodically and their properties were recorded. A week after inoculation on MS basal medium, the explants were cultured on (4.5 μM-27.0 μM) BAP and (3.50 μM-7.5 μM) IBA.

For experiments on culture initiation from axillary buds, tender, thin, green twigs cut below 2nd and 3rd nodes were taken as explants. Each explant would contain one apical bud and 2 to 3 axillary buds. Nodes after collection were pretreated and sterilized for 6 minutes using 0.1% mercuric chloride trimmed to 1.0-1.5 cm and cultured. The culture medium comprised of MS medium supplemented with 2.2 μM BAP and 4.9 μM IBA. These cultures were then sub-cultured on MS medium supplemented with 8.9 μM BAP and 2.9 μM IAA with adenine sulphate (100mg/l) and glutamine (100 mg/l) as an addendum. The cultures were incubated at 26 ± 2 ºC under a 16 h photoperiod using cool, white fluorescent light (30 μmol m^{-2} s^{-1}).

2.2.3 Data Analysis

Experiments were set in completely randomized design. All the experiments were repeated thrice and had ten replicates with single explants. Observations on number of explants forming callus, bud initiation and number of explants forming multiple shoots per explants were recorded. All data obtained were subjected to analysis of variance and significant differences in values were calculated according to Duncan's Multiple Range Test (DMRT).

2.3 Results and Discussion

2.3.1 Standardization of surface sterilization treatment

Surfaces of plant parts carry a wide range of micro-organisms. Micro-organisms as common companion to plant roots have been well documented but there have been fewer reports describing their existence in the aerial parts of plants. For this reason the plant shoot tissues, especially the meristems, have widely been considered sterile. Nevertheless the vascular tissues

are regularly colonized by bacteria and some fungi are known to inhabit the leaves of plants (Pirttila et al, 2000). For these reasons microbial contamination is one of the most serious problems of tissue culture. This has been reviewed exhaustively by Leifert and Cassells, 2001. There are two major ways of achieving axenic cultures. The first and convenient procedure is to produce the explants under aseptic conditions for example aseptically germinated seedlings. For the second type i.e. production of explants under non-aseptic conditions, conditions vary. The first step should be removal of dirt and debris from the plant tissue followed by washing with a detergent solution. Detergent is removed by several rinses in distilled water prior to sterilization. Contamination with microorganisms is considered to be the single most important reason for losses during *in vitro* culture of plants. During sterilization, the living plant material should not lose its biological activity and only contaminants should be eliminated; therefore explants are surface sterilized only by treatment with disinfectant solution at suitable concentrations for a specified period (Oyebanji et al, 2009). The disinfectants widely used are sodium hypochlorite, ethanol, calcium chloride, mercuric chloride etc. Sodium hypochlorite is usually purchased as laundry bleach. Its exact mode of action is unknown however, it (NaOCl) is known to form hypochlorite (HOCl) which has bactericidal activity. It is readily available and can be diluted to proper concentrations. A balance between concentration and time must be determined empirically for each type of explant to prevent phytotoxicity. The standard protocol for these procedures can be found at our University website (https://www.msu.edu/course/css/451/).

Though ethanol is a powerful sterilizing agent, it is extremely phytotoxic. Therefore, its use in surface sterilization of plant material is restricted to a few seconds or minutes. Its use also depends upon the nature of plant material for example very young and tender tissue like young leaves, young petioles etc. are given a very short treatment. Generally 70% ethanol is used prior to treatment with other compounds.

To enhance effectiveness in sterilization procedure, a surfactant like Tween 20® is frequently added to the sterilizing solution (and in some laboratories mild vacuum is applied during the procedure); in general, the sterilizing solutions containing the explants are continuously stirred during the sterilization period (https://www. msu.edu/course/css/451/).

Mercuric chloride is extremely toxic to plants and humans and must be disposed off with care. Since mercury is phytotoxic, it is critical that many rinses be used to remove all traces of the mineral from the plant material. In a nutshell, to enhance the effectiveness of sterilization procedure surfactant (e.g. Tween 20) may be frequently added to the sodium hypochlorite, a mild vacuum may be used during the procedure and the explants are often shaken or continuously stirred (https://www. msu.edu/course/css/451/). A successful tissue culture protocol starts with effective explant sterilization (Dodds and Roberts, 1985).

The aim of the present investigation was to find out a suitable surface sterilization protocol that would ensure maximum explants survival with minimum contamination and also be cost effective and time efficient. Surface sterilization was followed as per standard procedure with 0.1 % mercuric chloride with different time duration for nodes and leaves, however they turned brown and no growth was observed in the cultures. Reducing the concentration and/or duration of exposure resulted in heavy fungal contamination. Initially variation in pre-treatment and mercuric chloride time duration was done to study its effect on achieving axenic cultures. Leaf explants dried and finally died owing to longer duration of exposure to mercuric chloride. Plant species with rich secondary metabolites such as *Jatropha curcas*; have proved to be difficult for mass propagation through tissue culture (Roy, 1995).

As shown in table 2.1, APSA (all purpose surface adjuvant) was used as a surfactant. In the pre-treatment virosyl (10%) was used to enhance the process of surface sterilization. However, no improvement was seen in survival of explants. It has been reported that the use of field grown plants as a direct source of explant material for the production of 'clean' *in vitro* plantlets presents a major challenge (Webster et al, 2003).

Results reported in table 2.1 shows that a combination of APSA, labwash, Bavistin, streptocyclin and virosyl with 0.1% mercuric chloride for 6 - 10 min did not yield good results. So in the next set of experiments NaOCl was added as pretreatment II and Bavistin, streptocyclin and ciprofloxacin were used in pretreatment III (table 2.2). The time of pretreatment to mercuric chloride was decreased to 1 min for leaves and 6 min for nodes.

Table 2.1: Explant survival and contamination with varied pretreatment conditions

Pretreatment I	Pretreatment II	Pretreatment III	Sterilant Concentration and duration of treatment	Number of Explants inoculated	Number of explants contaminated
Labwash +APSA	---	IPA (70%) (1 minute)	0.1% $HgCl_2$ (10minutes)	30 (N) 30 (L)	30 (N) 30 (L)
Running tap water (30 minutes) ↓ Labwash +APSA	---	IPA (70%) (1 minute)	0.1% $HgCl_2$ (8 minutes)	30 (N) 30 (L)	30 (N) 30 (L)
Labwash +APSA ↓ Bavistin+ Streptocyclin (O/N)	APSA+ Vacuum Infiltration	---	0.1% $HgCl_2$ 8 minutes-N 6 minutes-L	30 (N) 30 (L)	30 (N) 30 (L)
Labwash +APSA ↓ Virosyl (10%) (30 minutes) ↓ Bavistin + Streptocyclin (O/N)	APSA+ Vacuum Infiltration	---	0.1% $HgCl_2$ 8 minutes-N 6 minutes-L	30 (N) 30 (L)	23 (N) 30 (L)

Key: Nodes (N), Leaves (L), Overnight (O/N), All Purpose Surface Adjuvant (APSA), Isopropanol (IPA)

Variation in time duration of mercuric chloride as well as sodium hypochlorite in pre-treatment were tried to obtain sterile node and leaf explants. NaOCl was incorporated in the pre- treatment step to reduce fungal contamination. Time of exposure to $HgCl_2$ was also varied.

Table 2.2: Explant survival and contamination with NaOCl in pretreatment and varying $HgCl_2$ duration

Pretreatment I	**Pretreatment II**	**Pretreatment III**	**Sterilant concentration and duration of treatment**	**Number of Explants inoculated**	**Number of explants contaminated**
Running tap water ↓ Labwash + APSA	3% Sodium Hypochlorite (7 minutes)	IPA (70%) (1 minute)	0.1% $HgCl_2$ 8 minutes-N 6 minutes-L	30 (N) 30 (L)	15 (N) 30 (L)
Running tap water ↓ Labwash + APSA	3% Sodium Hypochlorite (6 minutes)	IPA (70%) (1 minute) ↓ Bavistin + Streptocyclin (2 hours)	0.1% $HgCl_2$ 6 minutes-N 5 minutes-L	30 (N) 30 (L)	19 (N) 10 (L)

Pretreatment I	Pretreatment II	Pretreatment III	Sterilant concentration and duration of treatment	Number of Explants inoculated	Number of explants contaminated
Running tap water ↓ Labwash + APSA	3% sodium Hypochlorite 6 minutes-N 2 minutes-L	IPA (70%) (I minute) ↓ Bavistin+ streptocyclin (2 hours)	0.1% $HgCl_2$ 7 minutes-N 1 minute-L	50 (N) 30 (L)	25 (N) 30 (L)
Running tap water ↓ Labwash + APSA	3% sodium Hypochlorite 6 minutes-N 2 minutes-L	Bavistin+ streptocyclin (O/N) ↓ IPA (70%) (I minute)	0.1% $HgCl_2$ 6 minutes-N 1 minute-L	45 (N) 30 (L)	45 (N) 30 (L)

Pretreatment I	Pretreatment II	Pretreatment III	Sterilant concentration and duration of treatment	Number of Explants inoculated	Number of explants contaminated
Running tap water ↓ Labwash + APSA	3% sodium Hypochlorite 6 minutes-N 1 minute-L	Bavistin+ ciprofloxacin (2 hours) ↓ IPA (70%) (I minute)	0.1% $HgCl_2$ 6 minutes-N 1 minute-L	40 (N) 70 (L)	31 (N) 70 (L)
Running tap water ↓ Labwash + APSA	3% sodium Hypochlorite 6 minutes-N 1 minute-L	Bavistin+ ciprofloxacin (1 hours) ↓ IPA (70%) (I minute)	0.1% HgCl2 6 minutes-N 30seconds-L	40 (N) 60 (L)	30 (N) 36 (L)

However, both NaOCl and mercuric chloride were proving detrimental to the survival of explants. Hence, NaOCl was removed from the pre-treatment step and Bavistin, a systemic fungicide was included as a pre-treatment and its treatment time was standardized to 2 hours. The use of antibiotics in certain growing cultures was found to be inhibitory. Also $HgCl_2$ treatment duration was modified and survival of axenic cultures increased. Webster et al, 2003 cited Barrett & Cassells, 1994 stating that uninformed use of antimicrobial chemicals (such as antibiotics) may cause phytotoxicity, retard explants growth and encourage the buildup of resistance. Furthermore, even though some antibiotics may give comparatively high activity

when tested on defined bacteriological media these results are not usually replicable on the complex tissue culture media and so the expected results are usually elusive.

Table 2.3: Explants survival and contamination with varying time of exposure to fungicide and $HgCl_2$

Pretreatment I	**Pretreatment II**	**Pretreatment III**	**Sterilant concentration and duration of treatment**	**Number of explants inoculated**	**Number of explants contaminated**
Running tap water ↓ Labwash + APSA	Bavistin+ Streptocyclin (O/N)	IPA (70%) (1 minute)	0.1% $HgCl_2$ 7 minutes-N 1 minute-L	30 (N) 30 (L)	30 (N) 29 (L)
Running tap water ↓ Labwash + APSA	Bavistin (1.5 hours)	IPA (70%) 1minute- (N) 30 seconds-(L)	0.1% $HgCl_2$ 3 minutes-N 1 minute-L	125 (N) 43 (L)	96 (N) 2 (L)

Pretreatment I	Pretreatment II	Pretreatment III	Sterilant concentration and duration of treatment	Number of explants inoculated	Number of explants contaminated
Running tap water ↓ Labwash + APSA	Bavistin (2 hours)	IPA(70%) 1minute-(N) 30 seconds-(L)	0.1% $HgCl_2$ 3minutes-N 1 minute-L	75 (N)	9 (N)
Running tap water ↓ Labwash + APSA	Bavistin (2 hours)	IPA(70%) 1 minute-(N) 30 seconds-(L)	0.1% $HgCl_2$ 3minutes-N 1 minute-L	82 (N)	12 (N)

Explant contamination is a function of several plant and environmental related factors such as plant species, age, explant source and prevailing weather condition. Despite the best timing and selection efforts it is almost impossible to eliminate contamination from *in vitro* grown plants (Webster et al, 2003). From the above results it was concluded that after rinsing the explants in running tap water, they should be washed with detergent followed by Bavistin treatment for 2 hours. Once these pretreatments were over, explants were treated with 70% IPA for one minute

to 30 seconds for nodes and leaves respectively. Surface sterilization was done using 0.1% $HgCl_2$for 4 minutes for nodes and 1 minute for leaves.

2.3.2 Indirect organogenesis through leaf discs

The regeneration of plants through an intermediate callus phase is called "indirect regeneration." The explants (meristematic tissue) dedifferentiate into callus, an unorganized growth of dedifferentiated cells. Group of cells in callus reorganize to form meristemoid, similar to meristem tissue. This then redifferentiates to form shoot buds, which finally regenerates to plantlets. In this study, adventitious shoot regeneration from leaf explants was attempted.

Figure 2.1: Callus initiation at the cut margin

It is a known fact that a balance between auxin and cytokinin normally induces effective organogenesis. Though the nature of interaction between the two plant growth regulators is not completely understood, cell division seems to be regulated by their interactions affecting different phases of cell cycle. While auxins are known to exert an effect on DNA replication, cytokinin exerts some control over the events leading to mitosis (Pasternak et al, 2000). Therefore normal cell divisions would require synchrony between S phase and cell division suggesting that auxin and cytokinin levels in culture be carefully matched. Reports have shown the importance of BAP and IBA in inducing organogenesis from leaf discs (Sujatha and Mukta, 1996; Rajore and Batra, 2005; Sujatha et al, 2005). In the current investigation a range of combination of BAP (4.5 µM - 27.0 µM) and IBA (3.0 µM -7.5 µM) were used for indirect organogenesis. Curling of enlarged leaf discs was first observed followed by callus appearance at the cut margins (figure 2.1).

No significant difference was observed in callusing and regeneration frequency from the explants derived from 3rd and 4th expanding leaf. This is in contrast to the result reported by Sujatha and Mukta, 1996 wherein 50% regeneration was seen in leaf discs obtained from 3rd expanding leaf and 30% from the 4th expanding leaf.

Figure 2.2: Emerging shoot buds from callus

Callus morphology and relative response of callusing was studied. It was observed that in MS media supplemented with both BAP and IBA at highest levels i.e. 27.0 µM and 7.5 µM respectively, callus formation was very less and much slower as compared to other combinations. BAP levels as low as 9.0 µM with 5.0 µM IBA also failed to induce sufficient callus (table 2.4).

Table 2.4: Various media combinations studied for callus formation in leaf explants of *J.curcas*

BAP/IBA (µM)	Total No of explants	Number of explants showing callus	Callus Morphology within a week/ after week	Presence of Callus	Time of Callus appearance	Relative response to Callus formation
4.5/3.0(B)	30	27	White/Green + White	Over entire margin	Within two weeks time	++
13.0/3.0(C)	30	30	White/Green + White	Over entire margin + bulb of Callus at edge	Immediately after a week	+++
27.0/3.0(D)	20	18	White/Green + White	Over entire margin + bulb of Callus at edge + small calli over midrib	Initial fast Development of Callus followed by slow development	++

BAP/IBA (µM)	Total No of explants	Number of explants showing callus	Callus Morphology within a week/ after week	Presence of Callus	Time of Callus appearance	Relative response to Callus formation
13.0/5.0(F)	05	18	White/Green + White	Over the surface	Initially slow then fast	+++
27.0/5.0(G)	05	29	Green + White	Over entire leaf margin	Appeared after two weeks	+++
4.5/7.5(H)	04	16	Green + White	On one entire side	Appeared after two weeks	+++
27.0/7.5(J)	06	24	Green + White	On one side	Appeared after two weeks or so	+

Explants on MS medium supplemented with lower levels of IBA (3.0µM and 5.0 µM) and higher levels of BAP gave maximum callus formation. Similar results were obtained in MS fortified with high levels of IBA in combination with low BAP. Though MS media containing highest levels of either BAP or IBA showed massive callus formation no organogenesis was observed. Even on sub-culturing on respective media it continued to form callus. In the medium

supplemented with 13.0 µM BAP + 7.5 µM IBA and 27.0 µM BAP + 7.5 µM IBA, callus turned brown and dried after first passage. By reducing IBA to 3.0 µM in combination with all three concentrations of BAP, organogenesis was observed after 45 days in culture. In the present study, tweaking cytokinin levels did not have an effect on organogenesis while low IBA levels did influence organogenesis. Low levels of IBA play a key role in callus formation, bud initiation and multiple shoot formation. This is also substantiated by Kalimuthu et al, 2007 wherein it has been reported that high concentrations of auxins are generally inhibitory to morphogenesis, and the use of an appropriate auxin-cytokinin ratio is essential to obtain proper shoots and root primordia. This could be suggestive of the importance of lower levels of IBA in inducing organogenesis. Buds in clusters (3-5) started emerging from various locations of the callus, mostly underneath the explants as shown above in figure 2.2. First bud initiation was observed in MS medium supplemented with 27.0 µM BAP + 3.0 µM IBA. It was also the most effective combination for shoot bud initiation and proliferation (table-2.5, figure 2.3).

Table 2.5: Effect of BAP and IBA on formation of buds

BAP/IBA (µM)	Number of buds per explants
	After 6 weeks
27.0/3.0	10 ± 2.35 [a]
13.0/3.0	8.0 ± 1.41 [a]
4.5/3.0	5.0 ± 0.94 [b]
27.0/5.0	2.0 ± 0.94 [c]
4.5/7.5	0.0 ± 0.0 [c,d]

Means in each column followed by same letters are not significantly different according to DMRT at α=0.05 at 12 degrees of freedom.

Figure 2.3: Shoot bud initiation and proliferation

In the current study, MS+27.0 μM BAP + 3.0 μM IBA resulted in 10 buds per regenerating calli. Out of these 9.33 buds showed proliferation on the same combination. Khurana-Kaul et al, 2010 reported 8.6 buds in MS+13.33 μM BAP + 2.46 μM IBA. Using similar combination in the present study also gave rise to 8.0 buds per regenerating calli. In the study by Khurana-Kaul et al, 2010 they have also shown the influence of TDZ in achieving higher number of shoot buds. While in the present study results have been achieved even in the absence of TDZ. Sujatha and Mukta, 1996 reported 2.22 μM BAP and 2.46 μM IBA to give 10.7 shoots per regenerating calli. A few other media combinations in the present study showed the same response a little later. As shown in table-2.6, there is a significant difference between all the media combinations studied except for the two containing isomolar concentration of IBA (3.0 μM) with varying BAP levels (13.0 μM and 27.0 μM). This indicates that the increased level of cytokinin fails to show its impact on initiation of organogenesis. However, after organogeneses have been successfully induced and shoot bud proliferation is observed increased IBA levels have little influence (table-2.6).

Table 2.6: Effect of BAP and IBA on proliferation of buds

BAP/IBA (μM)	Number of buds per explants
	After 10 weeks
27.0/3.0	9.33 ± 0.27^{a}
27.0/5.0	8.66 ± 0.54^{a}
13.0/3.0	6.00 ± 1.63^{b}
4.5/3.0	5.33 ± 1.90^{c}
4.5/7.5	0.0 ± 0.0^{d}

Means in each column followed by same letters are not significantly different according to DMRT at α=0.05 at 12 degrees of freedom

Of the four media combinations used in the study for formation of new buds, MS medium supplemented with 13.0 µM BAP +3.0 µM IBA did not show further response. However, other media combinations continued to show their effect on the number of shoot regenerants. table-2.7 depicts the mean length of regenerants with shoot length of more than 1.0 cm. In some explants, regenerants of more than 1.5-2.0 cm were obtained (figure 2.4).

Table 2.7: Effect of BAP and IBA on shoot length of bud regenerants

BAP/IBA (µM)	Mean shoot length	% frequency
4.5/3.0	>1.0cm	62.5
27.0/3.0	>1.0cm	55.5
27.0/5.0	>1.0cm	0

Shoot length of regenerants measuring more than 1.0 cm only have been considered.

Figure 2.4: Regenerants achieved from callus

Number of shoots per explant is presented in table-2.6. The MS medium supplemented with 4.5 µM BAP + 3.0 µM IBA and 27.0 µM BAP + 3.0 µM IBA are not significantly different according to DMRT (α=0.05). Kumar et al, 2010 achieved elongation on MS+ 4.5 µM BAP + 7.5 µM IBA with shoot length of 2.3-2.5cm.

The current investigation reports an effective combination for successful regeneration of shoots from leaf discs derived calli. It could be concluded that auxins play a greater role than cytokinins in organogenesis of *J.curcas.*

While wide changes in cytokinin levels did not yield significant changes in shoot bud induction, a small increment in auxin levels gave rise to appreciable changes in the same. Hence, it could be concluded that irrespective of BAP levels, low levels of IBA are essential in order to obtain multiple shoot regenerants per explant. Deore and Johnson (2008) have shown the synergistic role of thiadiazuron (TDZ) and BA in inducing shoots. They have also mentioned about reduced effect of BA in absence of TDZ. In the present study MS supplemented with 4.5 µM BAP + 3.0 µM IBA and 27.0 µM BAP + 3.0 µM IBA showed 100% frequency for callus formation, shoot bud induction and proliferation. Shoot elongation was studied in MS+8.8 µM BAP+ 2.5 µM IAA, MS+8.8 µM BAP+ 2.5 µM IAA+1.0 µM Kn, MS+22.0 µM BAP+ 2.5 µM IAA+1.0 µM Kn and MS+8.8 µM BAP+ 2.5 µM IAA+5.0 µM Kn. Out of these combinations studied, sprouting of new shoots was observed in MS+8.8 µM BAP+2.5 µM IAA+1.0 µM Kn and MS+22.0 µM BAP+ 2.5 µM IAA+1.0 µM Kn. MS+8.8 µM BAP+ 2.5 µM IAA+1.0 µM Kn also favored quick and effective growth of existing shoots along with MS+8.8 µM BAP+ 2.5 µM IAA+5.0 µM Kn. It is a widely known fact that root formation in *in vitro* grown plants is triggered or induced by external application of auxins. In the work reported on *J. curcas* MS in conjunction with NAA (Sujatha eta al, 2005) and IBA (Deore and Johnson, 2008) respectively has been used for root formation. In the present study, rooting was observed after three weeks of transfer in the rooting medium (figure 2.5).

Various media combinations were studied to induce rooting in regenerated shoots that had achieved length of more than 1.5-2.0 cm. The media combinations used to study rooting were MSB +11.0 µM NAA, MSB +21.0 µM NAA, MSB +50.0 µM NAA, Half strength MS+9.8 µM IBA+2.22 µM BAP, MS basal medium and direct transfer of the explants to vermiculite. Rooting could be achieved on MS basal medium. This is in contrast to the studies reported by Sujatha and Mukta, 1996 wherein rooting was observed on MS basal medium within 8-10 days with 88% rooting frequency. However, in the present investigation, more time was taken for root formation.

Figure 2.5: Root formation

2.3.3 Direct organogenesis in *J.curcas*

Direct shoot regeneration has been reported from a number of explants like shoot tip, axillary node, petiole, leaf disc, immature embryo, cotyledonary leaf, epicotyls, hypocotyls etc. of toxic and non-toxic varieties of *J.curcas* (Rajore and Batra, 2005; Sujatha et al, 2005; Kalimuthu et al, 2007; Deore and Johnson, 2008; Kumar and Reddy, 2010; Shrivastava and Banerjee, 2008; Kumar et al, 2010a, b). Of all these reports, only a few have reported direct organogenesis from shoot tips, nodes or axillary buds as explants (Rajore and Batra, 2005; Sujatha et al, 2005; Kalimuthu et al, 2007; Shrivastava and Banerjee, 2008). Direct plant regeneration without an intervening callus phase is a more reliable method for multiplication or clonal propagation. The plants produced by direct organogenensis may exhibit greater genetic stability than those produced via callus mediated organogenesis (Mukherjee et al, 2011). Murashige and Skoog's medium is the reported media for all the above mentioned studies on *J.curcas*. Plant growth regulators used for direct regeneration include TDZ, BA, Kn, IBA, IAA. These growth regulators were used at different levels either singly or in combination.

In the present study for experiments on culture initiation from axillary buds, tender, thin, green twigs cut below 2nd and 3rd nodes were taken as explants. Each explant containing one apical bud and 2 to 3 axillary buds were pretreated and surface sterilized with mercuric chloride. The culture medium comprised of MS medium supplemented with 2.2 µM BAP and 4.9 µM IBA. Nodal explants produced single shoots within 10-12 days (figure 2.6). Similar results have been reported by Kalimuthu et al, 2007 where MS basal medium was used for culture initiation. Further multiplication of shoots was achieved in MS fortified with BAP, Kn and IAA. Sujatha et al, 2005 reported the use of BA, Kn and TDZ. Except for one concentration of kinetin, none of the combination with the same showed any visible signs of explants differentiation except for bud emergence with very low axillary proliferation. It is also reported here that TDZ was more effective as compared to BA and Kinetin. It is a common fact that cytokinins and auxins were found essential for shoot multiplication. The higher concentration of auxins is generally inhibitory to morphogenesis and substitution of these reagents with an appropriate auxin-cytokinin ratio is essential to obtain proper shoot and root primordia (Kalimuthu et al, 2007). In the current investigation, explants were sub cultured on MS medium supplemented with 2.22 µM BAP and 4.92 µM IBA. Though apical bud is mostly the preferred explant for obtaining direct regeneration owing to its meristematic nature, it was found that apical bud was more prone to contamination. Multiple shoot formation was observed within a period of 30-40 days (figure 2.7). Multiplication of shoots was obtained in the same combination.

Figure 2.6: Emergence of new shoot bud

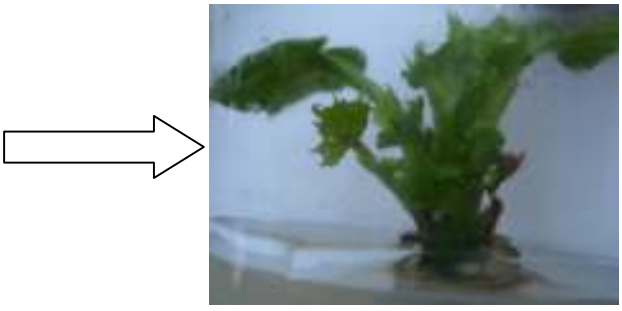

Figure 2.7: Multiplication of nodal culture

The media combinations used to study rooting were MSB +11.0 μM NAA, MSB +21.0 μM NAA, MSB +50.0 μM NAA, Half strength MS+9.8 μMIBA+2.22 μM BAP ,MS basal medium and direct transfer of the explants to vermiculite. The shoots were then kept on MS basal medium for rooting. Roots were observed after nearly three weeks in MS basal medium.

From the above studies it can be concluded that MS salts fortified with 2.2 μM BAP and 4.9 μM IBA successfully gave rise to new shoots and multiplication of shoots was also observed in the same.

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Study of Somatic Embryogenesis in *Jatropha curcas*: Liquid Suspension Culture and Solid Media Studies

3.0 Introduction

Jatropha curcas is a perennial shrub of the family Euphorbiaceae. It has gained importance due to the oil present in its seeds. The seed oil upon a simple esterification reaction can be converted to biodiesel. Thus, *Jatropha curcas* finds a place as a promising biofuel crop. However the average biodiesel yield per hectare is too low to make it economically viable. One of the reasons for this low yield is unavailability of quality planting material. Micropropagation is used to obtain multiple shoot culture of quality healthy plants. Multiple shoot culture technique involves a large number of steps making the procedure labor intensive and time consuming. Somatic embryos, on the other hand, have the capability to give rise to complete plantlets. Somatic embryogenesis (SE) can be initiated by two mechanisms: directly on explanted tissues, where plants are genetically identical (clonation), and indirectly (from unorganized tissues - callus). Embryo development follows a few discrete steps where pro-embryos develop via stages like globular stage, heart stage and a torpedo stage.

Various factors affect somatic embryogenesis. Role of carbon sources in regulation is discussed by Al-Khateeb, 2008. Level of endogenous hormones is considered to be one of the crucial factors determining embryogenic potential of explants (Feher et al, 2003). Auxin is one of the most important hormones in regulating SE in vitro (Cooke et al, 1993). Once embryogenesis has been induced, endogenous auxin synthesis begins via an alternate pathway. Several studies have revealed the importance of proper polar auxin transport as a prerequisite for normal morphogenesis beyond the globular stage. The removal or decrease in auxin acts as a trigger for somatic embryo development as it results in the inactivation of a number of genes enabling the embryogenesis program to proceed. This has been substantiated by the observations of Halperin and Wetherell, 1964; Borkird et al, 1986. Embryogenic cultures (E) have higher contents of

endogenous auxins than their Non- Embryogenic (NE) counterparts (Jimenez and Bangerth, 2001, Rajasekaran et al, 1987). However, there are a few reports in which no differences could be found between Embryogenic and NE cultures (Michalczuk et al, 1992).

3.1 Literature Studies

Regardless of whether the adult plant is an ephemeral weed or a long-lived sequoia tree, its developmental origin is essentially the same: a seed containing a simple mature embryo that displays an apical–basal axis of polarity and a radial pattern of concentric tissue layers perpendicular to the apical–basal axis. The axis of polarity has, at its top end, the primary shoot meristem, which is flanked by one or two cotyledons, and the primary root meristem at its bottom end. The basic body organization is influenced by potentially six different genomes namely, diploid zygote, triploid endosperm, diploid maternal tissue, haploid egg cell, diploid central cell and haploid sperm cells. During embryo development, the body axes and the basic body plan of the plant are laid down. This starts with establishment of the apical–basal axis, followed by establishment of the radial axis, and finally establishment of bilateral symmetry. Importantly, the root and shoot stem cell pools, which are essential for the quasi indefinite post-embryonic growth, are also specified during this early phase of pattern formation and morphogenesis. During the subsequent maturation phase, storage reserves accumulate and ultimately the embryo prepares for developmental arrest (De Smet et al, 2010). This ability to produce morphologically and developmentally normal embryos and, indeed, whole plants from undifferentiated somatic cells in culture, through the process of somatic embryogenesis, resides uniquely within the plant kingdom. Since the initial description of somatic embryo production from carrot callus cells more than 35 years ago (Steward et al, 1958), this unique developmental potential has been recognized both as an important pathway for the regeneration of plants from cell culture systems and as a potential model for studying early regulatory and morphogenetic events in plant embryogenesis. Somatic embryos are induced from cultured callus cells by a relatively simple manipulation of the culturing conditions, as summarized in figure3.1. In carrot, this generally involves (1) the establishment of a callus cell line, (2) the selection of an embryogenic subpopulation of the cultured cells through sieving or gradient fractionation, (3) the removal of auxin from the culture medium, and (4) the dilution of the cells to a relatively low

density. The overall embryogenic potential of a culture is highest when the culture is relatively young (i.e., within the first year of its life) and resides primarily within a subpopulation of the culture that has been termed proembryogenic masses" or PEMs by Halperin, 1966 (Zimmerman, 1993).

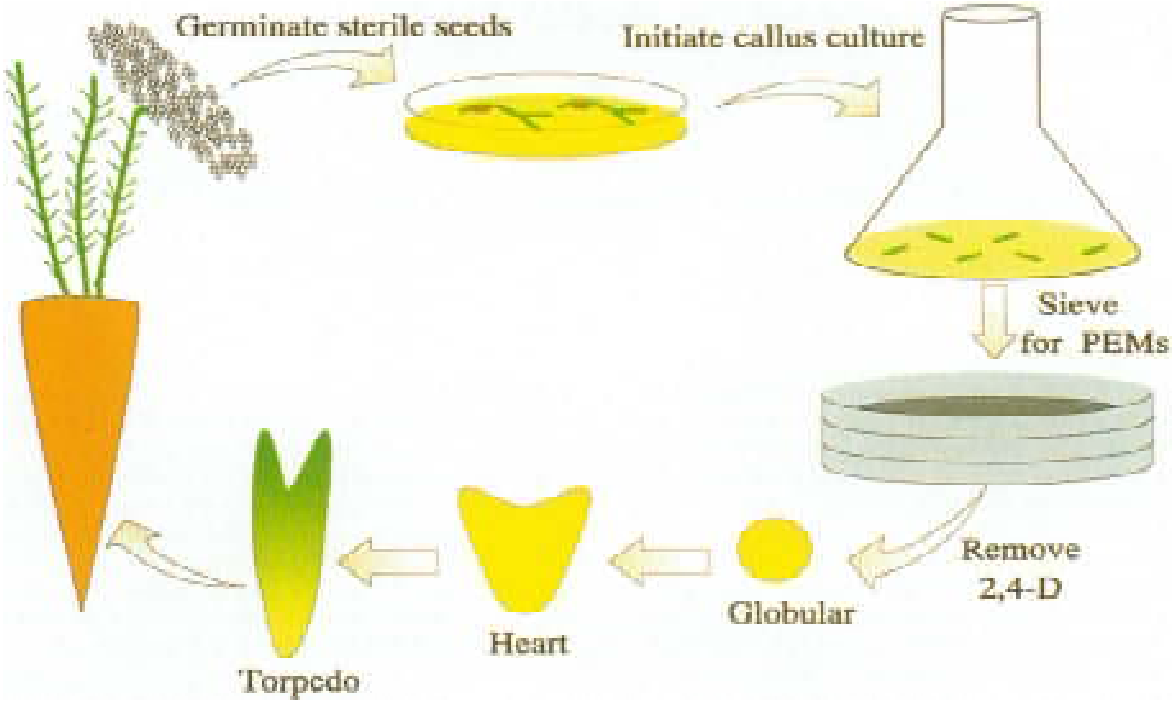

Figure 3.1: Summary of culturing of carrot somatic embryos (Zimmerman, 1993)

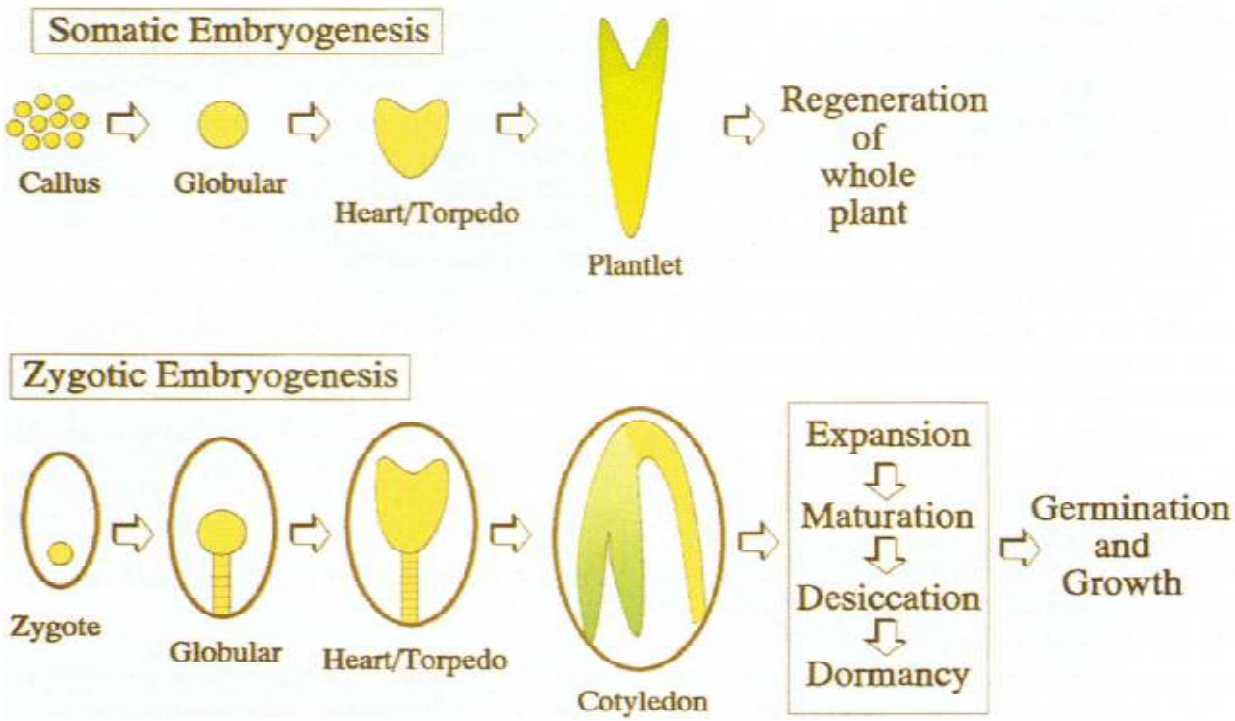

Figure 3.2: A comparison of somatic and zygotic embryogenesis (Zimmerman,1993)

Somatic embryogenesis in cultured cells is a useful system for research on differentiation to a whole plant from a single cell. Distinct phases can be physiologically distinguished in the differentiation of embryos from single cells (Nomura and Komamine, 1985). The suspension liquid culture system allows the study of different physiological and biochemical characteristics such as growth parameters, protein and DNA synthesis, plant growth regulators effect, nutrient uptake and maturation capacity, somatic embryo morphology and extracellular proteins (Salaj et al, 2007). Recently suspension cultures have also been involved in genetic transformation studies (Wenck et al, 1999).

The first phase of somatic embryogenesis is the process in which single cells divide to form embryogenic cell clusters. Exogenous auxin is required for the progression of this phase which can develop to embryos when they are transferred to the embryo-inducing medium without any trigger. This fact suggests that determination for embryogenesis occurs in the first phase. The second phase is the process in which the embryogenic cell clusters formed in the first phase develop to embryos. Development of embryos in this phase is strongly inhibited by exogenous

auxin (Nomura and Komamine, 1985). Various factors are known to influence somatic embryogenesis which includes age of the explant, storage of the explant, season of obtaining tissue from the plant source, role of auxins etc.

There are only a few reports on somatic embryogenesis in *J.curcas* (Jha et al, 2007; Kalimuthu et al, 2007). Jha et al, 2007 have reported that the type and concentration of the plant growth regulators were the strong determining factors for induction of somatic embryogenesis in *J.curcas*. Nodular embryogenic calli was observed on MS+9.3µM Kn. However, highest frequency of globular embryos was observed in the combination of 2.3µM Kn and 1.0µM IBA. Kalimuthu et al, 2007 mentioned the induction of somatic embryo on medium containing 8.87 µM kn however, further development of the embryo has not been mentioned. Soomro and Memon (2007) reported callus and cell suspension culture of *Jatropha curcas.* In the present study a systematic effort has been made to establish a protocol for somatic embryogenesis in *J.curcas* and understand the role of auxins in it. A protocol for liquid suspension culture system for *Jatropha curcas* has also been developed here.

3.2 Materials and Method

3.2.1 Plant Material

Seeds from mature and immature fruits of *Jatropha curcas* plants (1.5-2 years old) were collected and their cotyledons used as explants. Seeds were kept under running tap water for 30 minutes followed by wash with a mild detergent. They were again kept under running tap water for 30 minutes. They were treated with 70% ethanol for 30 seconds followed by three rinses with distilled water. Surface sterilization was done with 0.1% mercuric chloride for 2 minutes. Traces of mercuric chloride were removed by giving five rinses with sterile distilled water. Surface sterilized seeds were de-coated under aseptic conditions and inoculated on the respective medium.

3.2.2 Culture Media

Murashige and Skoogs (MS) salts with 0.4 mg/l, 0.8 mg/l and 1.2 mg/l 2, 4-Dichlorpphenoxyacetic acid (2, 4-D) was used for inducing callus for suspension culture studies.

MS salts supplemented with various concentration of 2,4-D (2.4 mg/l, 3.6mg/l and 4.0 mg/l and 0.4 mg/l+2% coconut water (CW), 0.8 mg/l+2% CW and 1.2 mg/l+2% CW and 0.4 mg/l+10% CW, 0.8 mg/l+10% CW and 1.2 mg/l+10% CW) and NAA (5mg/l,10mg/l and 15mg/l) were used for callus initiation and for direct somatic embryogenesis studies. The medium was fortified with 3% sucrose and agar (0.8%) was used as a solidifying agent wherever required. The-pH of the media was adjusted to 5.8 before addition of agar and was autoclaved at 121°C for 20 min. Cultures were maintained at 24±2°C under 14 hour photoperiod.

3.2.3 Optimizing liquid suspension culture system

The friable callus formed was transferred to MS liquid medium and kept on shaker at 110 rpm at 25°C. The cells were harvested at regular interval of 3 days and cell counting was done. The following protocol was followed for staining and counting of cells:

3.2.4 Staining of cells in suspension cultures

1 mL of cells from suspension culture was taken asceptically in a sterile tube and centrifuged at 5000 rpm for 10 min. The cells from the pellet were stained with Evan's blue dye and observed under 40 X objective of light microscope.

3.2.5 Cell count

The number of cells per mL were counted using a hemocytometer

3.2.6 Auxin extraction and estimation

Auxin was determined by Gordon and Weber, 1951. Tissue of specific stages (0.5gm) was ground in a mortar with pestle, together with 0.01M EDTA and 0.02M Sodium diethyldithiocarbamide in 80% ethanol. This was transferred to test tube, kept on ice bath for 15-20 minutes with intermittent shaking (Sinha and Basu, 1981). This mixture was centrifuged at 5,000 g for 20 minutes at 4 °C. Auxin was estimated in the supernatant at 530 nm.

3.3 Results and Discussion

Somatic embryogenesis acts as a powerful tool for genetic improvement of any plant species because of its single cell origin. Somatic embryogenesis offers promise for rapid multiplication and genetic transformation of plants (Finer, 1988). Somatic embryogenesis can be initiated by two mechanisms: directly on explanted tissue where plants are genetically identical (clonation) and indirectly from unorganized tissues (callus). In the current investigation we report both direct and indirect somatic embryogenesis. The experiments were carried out on solidified media as well as liquid medium for suspension cultures. The advantage of liquid suspension culture system is that the growth of plant cells is more rapid than in callus culture and is also more readily controlled because the culture medium can be easily amended or changed. In the current study the roles of a few factors that have an influence on the performance of explants in culture conditions have been discussed. Age of seeds is known to be an important parameter influencing the outcome of cells in culture. Here, immature and mature seeds were used to obtain first cotyledonary leaves which then served as explants. It was observed that mature seeds could give rise to cotyledonary leaves upon initiation in callus inducing media, whereas immature seeds failed to do the same. Similar reports were made by Malik et al, 2004 (*Triticum aestivum*) and by Dhavala et al, 2009 (*Solanum trilobatum).* As seeds served to be the initial source for explant, seed viability will have a bearing on the outcome of the experiments. It was observed that seeds stored at room temperature did not germinate and their cotyledons were degenerated. Unviable seeds were treated with GA10 ppm to break seed dormancy however; it did not help. Hence for all experiments seeds after collection were stored at -20°C until use. Andersone and Ievinsh, 2002 have reported the effect of cold storage on in vitro development of bud in *Pinus sylvestris* where they have shown that cold storage affects oxidative metabolism, thereby affecting the morphogenesis during *in vitro* studies.

3.3.1 Effect of Season on Somatic embryogenesis

Seasonal cues like environmental conditions also have an effect on the performance of explants in culture conditions. In this study, embryogenic calli formation was found to be higher in the months of January to April (figure 3.3 and 3.4). This is the season when fruit maturation initiates as October and November are the flowering months. In monsoon the explants in culture are less

responsive and more contamination is encountered, which is in agreement with reports of Bisht et al, 2010 for bamboo explants. Hohtola (1988) has reported higher viability and growth of explants in June and July as it represents the normal active growth period in *Pinus sylvestris*. In its study it is also commented that the season dependent metabolism is retained in tissue culture conditions and consequently, it is hard to break down the dormant state. The same difficulty may explain the lack of organogenesis in *J.curcas* as it would be difficult to switch off the callusing type of growth in cultures.

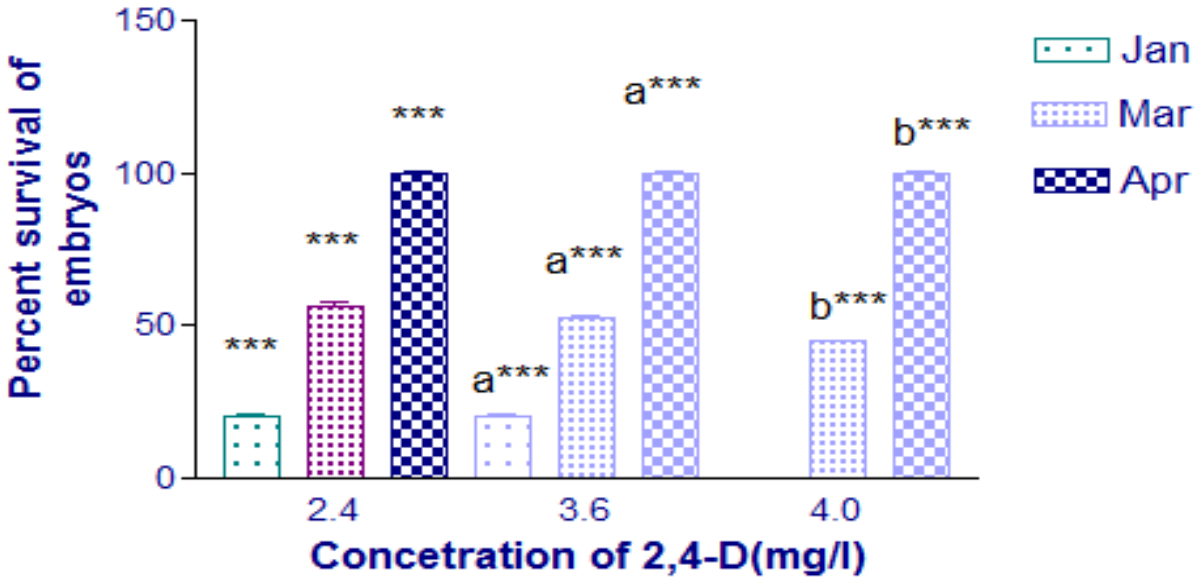

Figure 3.3: Percent Embryogenesis in MS medium supplemented with 2, 4-D

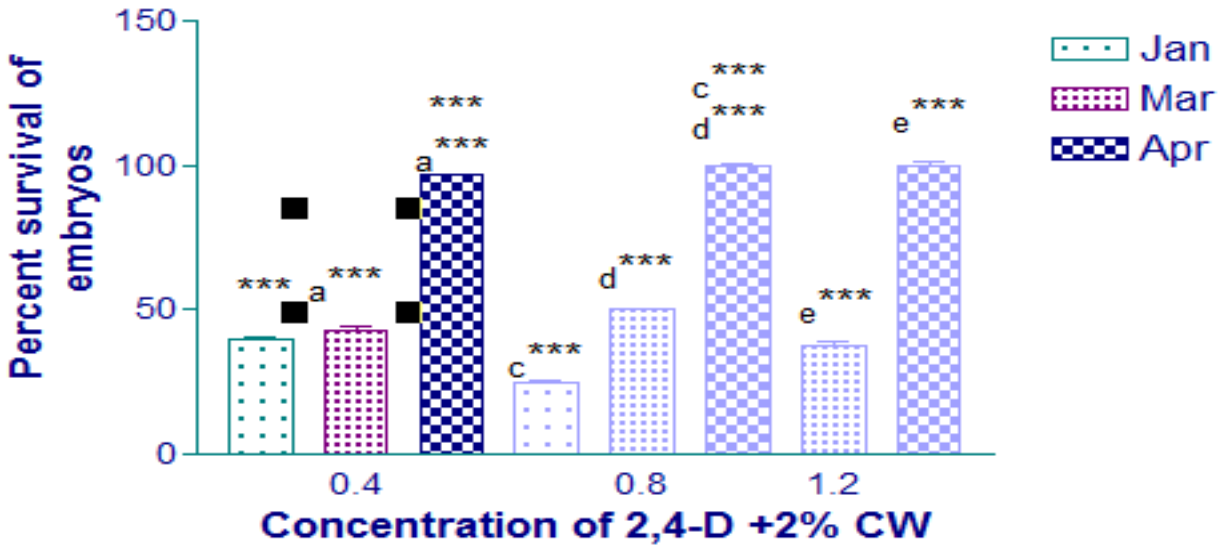

Figure 3.4: Embryogenesis in MS medium supplemented with 2, 4-D

3.3.2 Role of Auxins

The role of auxins in morphogenesis is evident from the fact that the cells which respond to auxin revert to a dedifferentiated state and begin to divide. It was reported by Lo Schiavo et al, 1989 that auxins cause DNA to become more methylated than usual and they suggested that this might be necessary for the re-programming of differentiated cells. Tissue–specific programmes specifically associated with differentiation would become eradicated by hyper methylation, with perhaps a small fraction of the cells reaching an ultimate state of differentiation in which they become capable of morphogenesis or embryogenesis. In the present study MS medium was supplemented with two plant growth regulators (auxins) namely 2, 4-D and NAA at different concentrations. MS fortified with different levels of 2, 4-D (0.4, 0.8, 1.2, 2.4, 3.6 and 4.0mg/l) has been used for inducing embryogenic calli in *J curcas*. MS with 0.4, 0.8 and 1.2 mg/l 2, 4-D was supplemented with 2% and 10% coconut water respectively. It has been reported that the culture of explants in medium containing 2,4-D, increases the endogenous auxin levels in the responsive explants and this acts as a crucial signal determining embryogenic fate of cultured cells (Jimenez, 2005). Higher endogenous IAA concentrations have been shown in different species/explants as being associated with an increased embryogenic response. (Pasternak et al,

2002). Auxins could hence play a determining role in somatic embryo development and maturation in *Jatropha curcas*. Here we report studies on somatic embryogenesis by solid and liquid culture conditions and the role of auxin.

3.3.3 Liquid suspension culture studies

In this study, the cotyledonary leaves were initiated on MS supplemented with varied concentration of 2, 4-D (0.4, 0.8, 1.2 mg/l) and NAA (5mg/l, 10 mg/l, 15 mg/l) for a period of 30 days followed by their transfer to no auxin media. It has been reported that the presence of auxin is critical for embryo initiation and lowering of auxin concentration or its complete absence fosters maturation (Halperin and Wetherell, 1964). Hence in order to obtain somatic embryos one month old calli from explanted cotyledon were inoculated into suspension culture having only MS salts with no plant growth hormone. Cells in suspension were sub cultured at a regular interval of one week and observed microscopically at an interval of three days under 40 x. Cells were kept in suspension for a period of 30 days. Total cell count was performed and embryogenic structures were looked for by double staining (Acetocarmine+Evan's blue) using hemocytometer.

Greenish white callus started appearing at the edges of cotyledonary leaves and also at the midrib and on small veinlets. It turned to be dense mass of greenish white friable calli. After 30 days in culture, these calli were transferred to embryo inducing medium which was MS basal (liquid). It has been reported that 1.0 gram of tissue is required for initiation of suspension culture (Salaj et al, 2007).

In the current study it was observed that 350-700 mg of tissue shows good growth response in suspension culture. Once in suspension culture, it was observed that the cell number was initially low for first 4-5 days. It could be attributed to acclimation of cells in new medium. As shown in table-3.1, the cell number increased within 10-12 days and then a gradual decrease was observed after 20 days. Similar reports have been cited by Soomro et al, 2007.

Initially cell suspension culture comprised of single cells. These cells were round and densely cytoplasmic with distinct nuclei. Some large and highly vacuolated cells with sparse cytoplasm were also observed. Early embryogenic structures like globular, heart shaped and torpedo shaped

cells were seen albeit a little late. This could be because of lingering effect of 2, 4-D on the cells as they were in prolonged 2, 4-D exposure. At four days in suspension, generally elongated cells were seen. Globular shaped cells were observed at 17th day in suspension culture. Heart shaped and torpedo shaped cells could be observed between 18-25 days time. The presence of these structures confirmed that embryogenesis had been induced in the cells in liquid suspension (figure 3.5).

Table-3.1 Cell count after transfer of calli (30 days old) from MS+2, 4-D to MSB (liquid)

Media with 2,4-D (mg/l)	**After 5 days Cells/ml**	**After 10 days Cells/ml**	**After 20 days Cells/ml**	**After 30 days Cells/ml**	**After 34 days Cells/ml**
0.8	200	1213	750	212.5	150
1.2	1250	2475	2060	1625	500

Similar observations were reported with somatic embryogenesis from cotyledons of Soybean (Santos et al, 2006). The structure of somatic embryos has not been affected by culture in liquid medium. Somatic embryos in liquid medium showed similar morphology as in tissues cultured on solidified media. However, when plated on solid media, these cell aggregates failed to grow. Similar studies have been reported in Pine, wherein proliferation of embryogenic tissues occurs in liquid medium; however, the maturation is less frequent (Salaj et al, 2007). While no such cells were seen in media supplemented with various concentration of NAA. Hence, NAA as a plant growth regulator to induce somatic embryogenesis in *Jatropha curcas*, could be a less preferred substitute as compared to 2, 4-D.

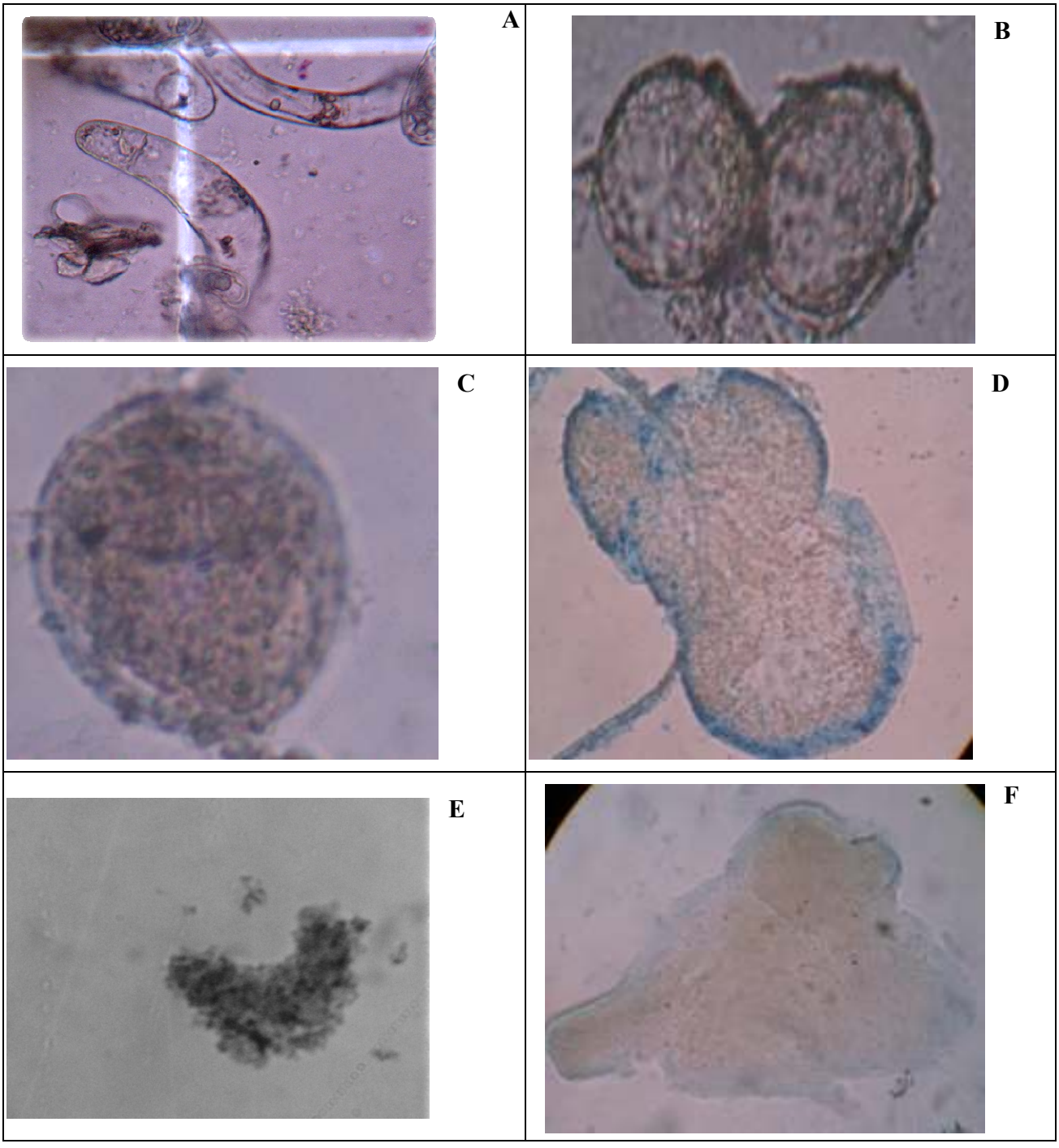

Figure 3.5: A-F: Cells seen in suspension culture;A- Elongated cells B-Round cells,C-Globular embryos,D- Cell aggregate with a globular embryo,E- Heart shaped cells ,F-Torpedo shaped cells

3.3.4 Solid media studies

Second approach was short time auxin pulse treatment wherein the cotyledonary leaves were initiated on MS +2, 4-D (0.4, 0.8, 1.2 mg/l + 2% coconut water and 0.4, 0.8 and 1.2 mg/l +10% coconut water and 2.4, 3.6 and 4.0 mg/l) and MS+ NAA for a period of seven days or fourteen days followed by their transfer to no auxin medium. In the current study different stages of somatic embryos were seen (figure 3.6).

Figure 3.6: G-J: Stages in direct somatic embryogenesis as observed on solid media: G-Globular embryos seen at the edge, H: Small nodular embryos seen at the edge and veinlets, I- Heart shaped embryo, J- Direct somatic embryogenesis

Nomura and Komamine (1986) have reported that there are atleast two stages in somatic embryogenesis, stages requiring and inhibited by auxins. Hence, auxin levels were checked on day 4, day 7 and day 14. On day 4 pre-embryogenic masses were not observed whereas on day 7 pool of embryogenic and non-embyrogenic structures were observed. On day 14 most of the calli had turned embryogenic. Hence, day 4 calli was considered as non-embryogenic calli (NE) and day 7 and day 14 had both embryogenic (E) and non-embryogenic characteristics. In this set of experiment the cotyledons were cultured in MS+2, 4-D and MS + NAA for a period of 7 days and fourteen days respectively. Total auxin levels were estimated in E and NE calli (figure 3.7, 3.8, 3.9).

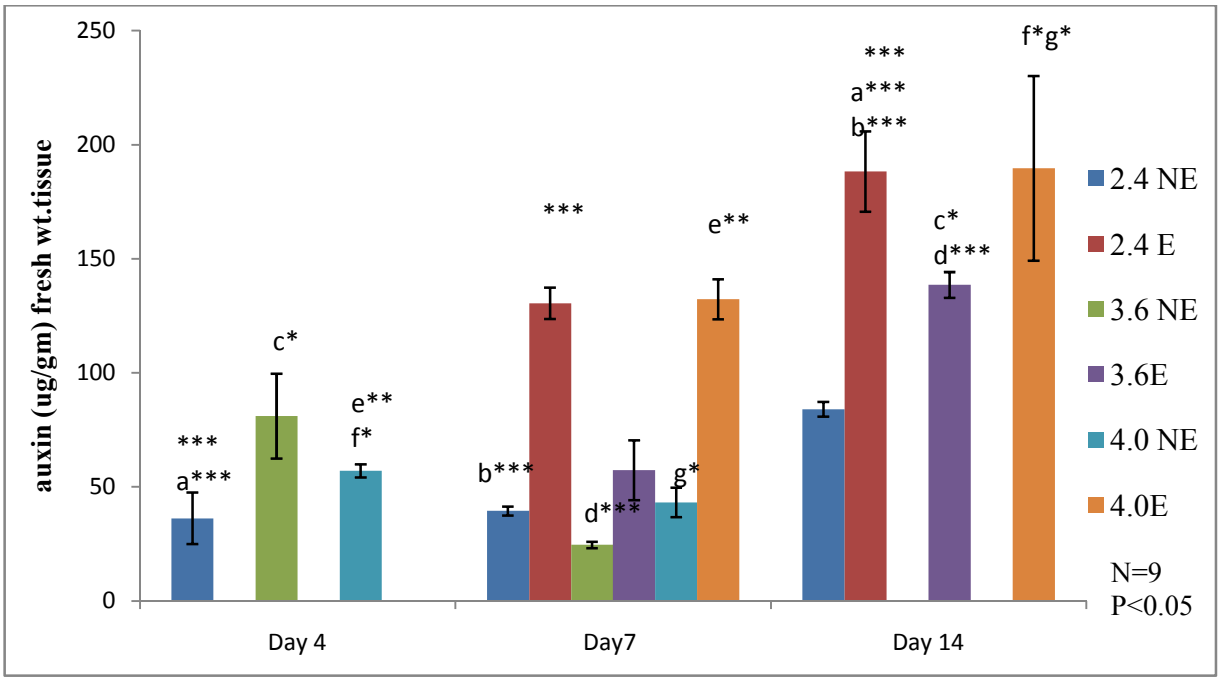

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Figure 3.7: Comparative auxin levels in MS +2.4, 3.6 and 4.0mg/l 2, 4-D

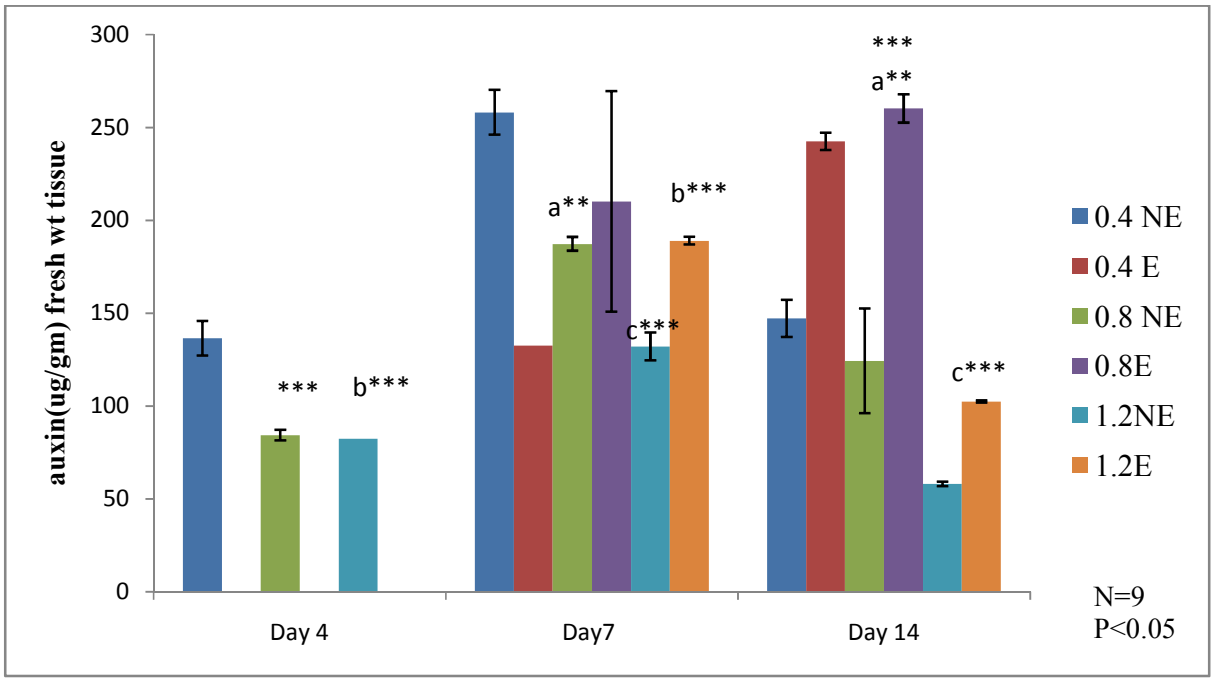

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Figure 3.8: Comparative auxin levels in MS +0.4, 0.8 and 1.2mg/l 2, 4-D+(10%)CW

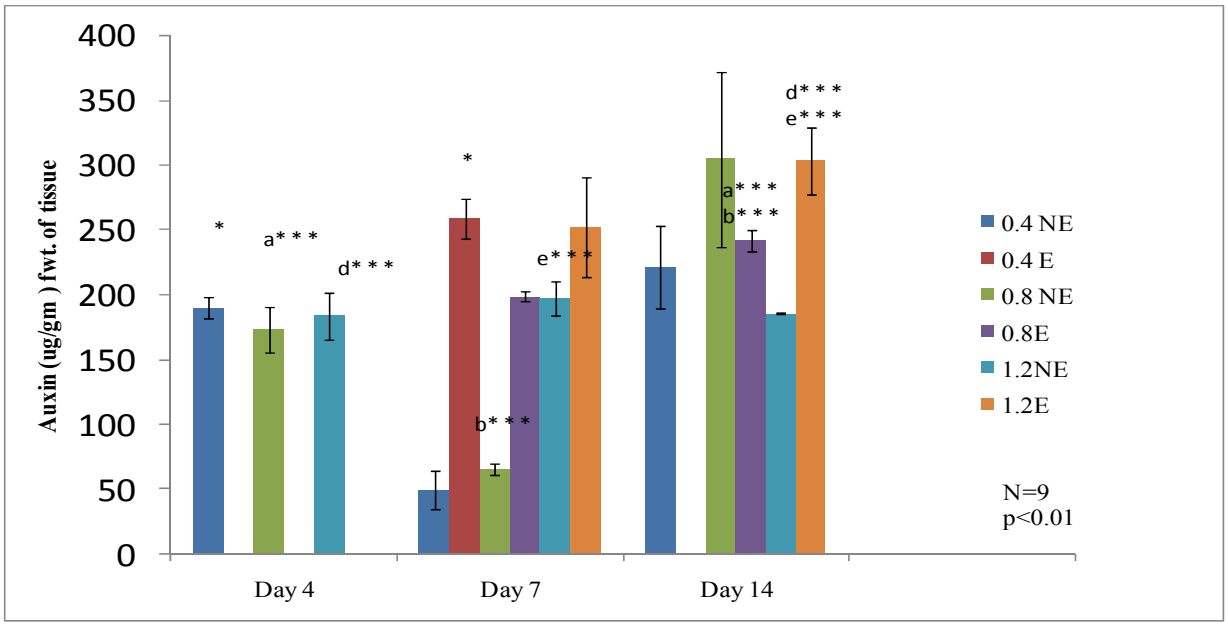

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Figure 3.9: Comparative auxin levels in MS +0.4, 0.8 and 1.2mg/l 2, 4-D+ (2%) CW

It was observed that MS fortified with higher levels of 2, 4-D devoid of coconut water could trigger higher embryo formation. Lower levels of 2, 4-D in MS medium could trigger embryogenesis only if supplemented with coconut water. Total auxin levels were estimated and it was found that a significant increase is seen in total auxin in embryogenic(E) calli(7 days and 14 days) as compared to non-embryogenic calli (NE)(4 days) in various media combinations studied. MS supplemented with 1.2 mg/l 2, 4-D + 10% CW is the only combination observed so far which shows a decrease in total auxin on day 14 in E calli (figure 3.7). Each media was observed for the number of embryos formed. It could be concluded from the present study that 2, 4-D alone at higher concentration (4.0 mg/l) is a preferred medium to induce embryogenesis in *J. curcas* as compared to lower 2, 4-D levels with coconut water. This could be due to the fact that coconut water is an undefined medium and hence may interfere with reproducibility of procedure.

This is the first report of liquid suspension culture studies of *Jatropha curcas* and direct as well as indirect somatic embryogenesis.

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Generation of DGAT2 Transformants of *J.curcas*

4.0 Introduction

Genetic transformation for improving traits is another valuable method for the development of *J.curcas* variety. Recent advances in genetic transformation and the availability of gene databank has made it possible to transfer the gene of interest to the recipient species to produce transgenic progeny with desired characteristics. This may help surpass certain existing limitations of the breeding programmes and also may help reduce the time required to generate those improved varieties.

J.curcas is still in its infancy with respect to genetic improvement. The application of metabolic engineering and RNAi technology may show their potential in basic research and as tools of modern plant breeding. Research and development should aim at metabolic engineering of the oil bio-synthesis pathway. There is a possibility to increase the yield of oil from *Jatropha* seeds through genetic transformation (Mukherjee et al, 2011).

Jatropha seeds contain 22-44% oil in its seeds. Main constituent of oil is TAG (Triacylglycerol). DGAT (Diacyl glycerol acyltransferase) is the enzyme that catalyzes the committed step of TAG synthesis from DAG via the Kennedy pathway. A number of studies with both mammalian (Mayorek et al, 1989; Tijburg et al, 1989) and plant (Ichihara et al, 1988; Perry and Harwood, 1993a, b; Settlage et al, 1995; Perry et al, 1999) systems have suggested that DGAT may catalyze a rate limiting reaction in TAG bio-assembly. Hence increasing the amount of this enzyme may increase TAG biosynthesis thus leading to increase in oil content per seed. Similar work has been done in *Arabidopsis* where seed specific over-expression of an *Arabidopsis* cDNA encoding DGAT resulted in an enhancement of seed oil content and seed weight (Jako et al, 2001). He et al, 2004 have reported the cloning and characterization of a cDNA encoding DGAT from castor bean. Increase in levels of DGAT enzyme can be brought about by increased

expression of the DGAT gene. This can be done by cloning cDNA copy of DGAT gene or using DGAT gene whose sequence is highly similar to that of *Jatropha curcas* DGAT and cloning this gene into a binary vector and its transformation into *Jatropha* followed by its expression under a seed specific strong promoter.

Of the available transformation methods, *Agrobacterium* mediated transformation is the most frequently used method for the development of transgenic plants. In the current study an attempt to obtain genetically transformed *Jatropha curcas* is made. DGAT gene from *Vernicia fordii* is used for the purpose of genetic transformation. *V.fordii* belongs to same family (Euphorbiaceae) as *Jatropha curcas* and shows 84% identity as found using blast search. DGAT2 construct used in this study was kindly provided by Prof. Jay Shockey from Southern Regional Research Centre, USDA-ARS, New Orleans, USA.

4.1 Literature Studies

Diminishing petroleum reserves and environmental awareness has led to an increased demand for oil plants and their derivatives as substitutes for petrochemicals in industrial applications such as biofuels, biolubricants, nylon precursors etc. However, oil plants have certain limitations such as low yields, toxicity, limited geographical cultivation area and susceptibility to insect pests. Fortunately, genetic engineering offers a novel opportunity to overcome these limitations. Recent studies have envisaged the potential of TAG synthesis and accumulation in oil seed crops as the main lipids store in plants are TAG's. Three key steps are involved in the production of storage oils. First is the fatty acid synthesis in plastids, second is fatty acid modification by enzymes located in endoplasmic reticulum (ER) and thirdly packaging of the fatty acids in TAG's which subsequently accumulate in oil bodies that bud off from the ER. Formation of TAG can be achieved in several ways as shown in figure 4.1

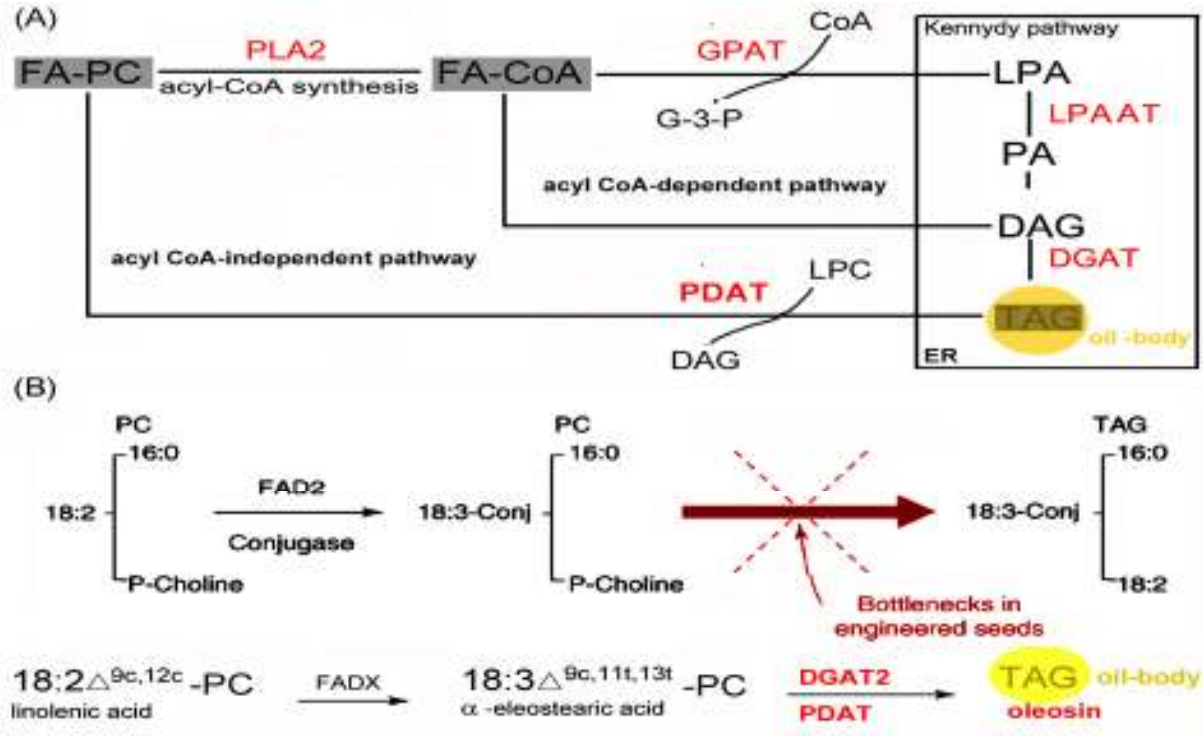


Figure 4.1 (A) The general pathway of TAG synthesis and accumulation. The first is classical Kennedy pathway catalyzed by enzyme GPAT, LPAAT and DGAT. The second is acyl-CoA independent pathway catalyzed by enzyme PDAT. The fatty acids flux between FA-PC, FA-CoA and TAG determine the final TAG level. (B) The bottlenecks in engineering plants to accumulate TAG enriched novel fatty acids are the low production of TAG but high FA-PC (Chen et al, 2008).

The first is the classical Kennedy pathway where acyl-CoA acts as a donor and diacylglycerol (DAG) as an acceptor. This reaction is catalysed by acyl-CoA diacylglycerol acyltransferase (DGAT) and transfers an acyl group from acyl-coA to sn-3 of DAG and forms TAG. The second is acyl-CoA independent pathway, which uses phosphatidylcholine (PC) as acyl donor and DAG

as acceptor, catalyzed by an enzyme called phospholipids: diacylglycerol acyltransferase (PDAT), which can transfer the sn-2 acyl chain from PC (phosphotidylcholine) to DAG, forming lyso-PC and TAG. Also the acyl-CoA independent pathway can be catalyzed by DGAT, using two molecules of DAG to produce TAG and monoacylglycerol (MAG) (Chen et al, 2008). Many studies have been reported on various enzymes involved in TAG synthesis like phospholipase A2 (PLA2), G-3-P acyltransferase (GPAT), phospholopid:diacylglycerol acyltransfersae (PDAT), lyso phosphatidic acid acyltransferase (LPAAT) and diacylglycerol acyltransferase (DGAT). Beisson et al, 2007 have identified atleast eight GPAT genes in *Arabidopsis thaliana*; however none of them have been implicated to have a promising role in TAG biosynthesis. PDAT has been studied by Dahlqvist et al, 2000. It catalyses the acyl transfer from PC to sn-1, 2-DAG to yield TAG however, it is not found to have any major role in TAG synthesis.

A correlation between DGAT transcript level and oil accumulation has been reported in common oilseeds as well as in plant species that accumulate unusual fatty acids. The level of DGAT expression has been shown to be directly related to TAG accumulation in mammals. Role of DGAT in pheromone synthesis in insects is also reported. With the extensive progress in genomics, transgenics and protein purification, genes encoding two major types of DGAT were characterized in eukaryotes and designated *DGAT1* and DGAT2 (Liu et al, 2012). Shockey et al, 2006 studied *Vernicia fordii* DGAT enzymes and reported that DGAT enzyme activity is encoded by at least two classes of genes in eukaryotic cells. DGAT1 proteins are; 500 amino acids in length with 10 predicted transmembrane domains (TMDs), whereas DGAT2 proteins are; 320 amino acids long with two predicted TMDs (figure 4.2). DGAT1 plays a more constitutive role in TAG metabolism in various tung tissues, whereas DGAT2 functions more directly in the production of seed-specific TAGs. Sub-cellular localization studies indicated that both the enzymes are localized to specific sub-cellular regions of the endoplasmic reticulum and these regions are not shared by these two enzymes.

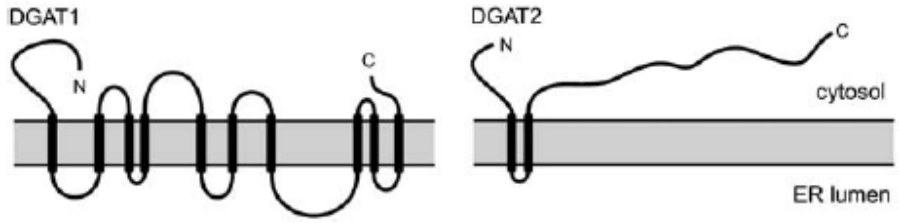


Figure 4.2: **Predicted topological maps of DGAT1 and DGAT2 of *Vernicia fordii* by Shockey et al, 2002 Jain et al, 2000 have reported an increase in oil content of *Arabidopsis* seeds from 8 to 29% in selected transgenic lines by expressing a plant plastidal and a bacterial GPAT gene directly and after modification.**

Genetic engineering of any crop species through genetic transformation requires a rapid and reproducible regeneration protocol. The potential of *Jatropha curcas* is well known and it is also known that it requires genetic engineering to gain commercial success and hence a few scientists have reported genetic transformation in this crop. First report on genetic transformation of *J.curcas* was given by Li et al, 2007 using *Agrobacterium tumefaciens* mediated transformation of cotyledon explants. Kumar et al, 2010 reported genetic transformation of *Jatropha* using in vitro leaf explants via *Agrobacterium tumefaciens* infection. In this study an attempt has been made at transformation of *J.curcas* via *Agrobacterium tumefaciens* carrying the gene of interest (DGAT2) via somatic embryogenesis and leaf mediated regeneration.

4.2 Materials and Method

4.2.1Cultures and Maintenance:

1) *E. coli* DH5α was used for transformation of ligation mixtures and maintaining recombinant plasmids.
2) *E. coli* DH5α bearing helper plasmid pRK2013 was used as a helper strain for mobilizing the donor plasmid contained in another E. coli DH5α strain
3) *Agrobacterium tumefaciens* LBA4404 was used as a recipient in the triparental mating.

Culture Maintenance:

• *E. coli* DH5α strains were maintained on Luria agar plates.

• *E. coli* DH5α bearing helper plasmid pRK2013 was maintained on Luria agar plates containing 50 μg/ml kanamycin as the plasmid pRK2013 has a kanamycin resistance gene.

• *Agrobacterium tumefaciens* LBA4404 was maintained on AB Medium containing 5 μg/ml rifampicin & 10 μg/ml tetracycline as the strain LBA 4404 is resistant to rifampicin & tetracycline antibiotics.

• *E.coli* DH5α clones harbouring pE172 recombinant plasmid were maintained on LA plates with 50 μg/ml kanamycin. pE172 was used for transformation of somatic embryos as its under seed specific promoter. pE309 was used for transformation of leaf discs as its under constitutive promoter. pE172 and pE309 were kind gifts from Prof. Jay Shockey, Southern Regional Research Centre, USDA-ARS, New Orleans, USA.

4.2.2 Media and Antibiotics:

Media:

Luria Agar (LA):

Caesin enzymic hydrolysate 10g/l

Yeast extract 5g/l

Sodium Chloride 5g/l

Agar 15g/l

Luria Broth (LB):

Caesin enzymic hydrolysate 10g/l

Yeast extract 5g/l

Sodium Chloride 5g/l

4.2.3 Antibiotic Stocks:

Kanamycin – 100 mg/ml

Tetracycline- 50 mg/ml (Dissolved in absolute ethanol)

Rifampicin - 50 mg/ml (Dissolved in 100% methanol)

Final Concentration of Antibiotics in Media:

Kanamycin - 50 µg /ml

Tetracycline- 12-15 µg /ml

Rifampicin - 5 µg /ml

4.2.4 Plasmid Extraction by Alkaline Lysis Method:

Plasmid extraction was done by Alkaline Lysis method of Sambrook et al, (1989) (Miniprep).

Reagents & Composition:

• ALS-I Tris-Cl 25mM (pH 8.0), EDTA 10mM (pH 8.0), glucose 50mM

• ALS-II 0.2N NaOH, 1% (w/v) SDS

• ALS-III 3M Potassium acetate, 5M glacial acetic acid
• Isopropylalcohol and 70% alcohol

Procedure:
1) A single colony of transformed bacteria was inoculated in 5 ml of LB with proper antibiotic. The culture was incubated overnight at 37°C under vigorous shaking condition on shaker.
2) Next day the culture was centrifuged at 10,000 rpm for 10 min.
3) The supernatant was discarded & 100 µl of ice cold ALS-I was added to resuspend the pellet by vortexing.
4) 150 µl ALS-II was added & the contents of the tube were mixed by inverting 5 times, the tube was stored on ice for 10min.
5) 200 µl ALS-III was added & mixed by inverting the tubes 5 times, tube was stored on ice for 15-20 min
6) Centrifugation done at 12,000 rpm for 10 min, the supernatant was transferred to a fresh microfuge tube and equal volume of Isopropylalcohol was added. The tube was incubated at -20°C overnight.
7) Centrifugation was done at 12,000 rpm for 10min.The supernatant was carefully discarded & 200 µl 70% alcohol was added & inverted gently 2 times & centrifuged for 2 min, then the alcohol was removed carefully.
8) The microfuge tube was kept in an inverted position to completely remove last traces of alcohol. 20 µl Triple distilled water was added and kept at room temperature for 15 min for DNA to dissolve. The purity of the plasmid preparation was checked by agarose gel electrophoresis.

4.2.5 Competent Cell Preparation:

The protocol from Sambrook et al, (1989) was used for preparation of competent *E. coli* with an efficiency of ~106 transformed colonies/ µg of supercoiled plasmid DNA.

Reagents & Composition:

$MgCl_2$-$CaCl_2$ solution: 80 mM $MgCl_2$, 20 mM $CaCl_2$ $CaCl_2$: 0.1M

Procedure:

1) A single bacterial colony was inoculated in 5 ml of LB. The culture was incubated overnight at 37°C with vigorous shaking.

2) 1 ml of overnight grown culture was transferred in 100 ml LB and incubated at 37°C with vigorous shaking till the OD600 reaches 0.4. (To ensure the culture doesn't grow to a higher density measure the OD600 of culture every 15-20min)

3) The bacterial cells were transferred to sterile, disposable, ice-cold 50 ml polypropylene tubes. The cultures were cooled to 0°C for 10 min.

4) Cells were recovered by centrifuging at 4500 rpm for 10 min at 4°C.

5) The medium was decanted from cell pellet & tubes were placed in an inverted position for 1 min to allow last traces of the media to drain away.

6) Pellet was resuspended by swirling or gentle vortexing in 30 ml of ice-cold $MgCl_2$-$CaCl_2$ solution.

7) Cells were recovered by centrifuging at 4500 rpm for 10min at 4°C.

8) Medium was decanted from cell pellet; tubes were placed in an inverted position for 1 min to allow last traces of the media to drain away. Pellet from each tube was resuspended by swirling or gentle vortexing in 1ml of 0.1 M $CaCl_2$ and 1ml of 40% glycerol. The cells were directly used for transformation or dispensed into aliquots & frozen at -80°C.

Transformation using $CaCl_2$:

1) 100-200 µl $CaCl_2$ treated cells were transferred to a sterile chilled polypropylene tube. Upto 5 µL of DNA sample or ligation system was added to the tube and the contents mixed by swirling gently. The tube was stored on ice for 30 min.

2) The tube was transferred to a rack and kept in preheated 42°C water bath and incubated exactly for 90 sec without shaking.
3) The tube was rapidly transferred to an ice bath to chill for 2 min.
4) 400-800 µl of LB medium was added accordingly to the tube and incubated for 45 min at 37°C.
5) Appropriate volume of cells was plated onto pre-warmed LB plates with appropriate antibiotic.
6) The plates were incubated at 37 °C for 16-18 hours.

4.2.6 Agarose gel electrophoresis

Requirements:
TAE (Running buffer)
• Tris base 242g
• Glacial acetic acid 57.10ml
• 0.5M EDTA (pH 8.0) 100ml
• D/W 1000ml
50X TAE was diluted to 1X prior to use.
0.8% Agarose

4.2.7 Triparental Mating

Strains involved:
1) *Agrobacterium tumefaciens* LBA4404 (Recipient strain) - It was grown at 30°C on AB minimal medium with rifampicin & tetracycline.
2) *E. coli* DH5α harboring recombinant plasmid to be mobilized (Donor strain) - It was grown at 37°C on LB with 50µg/ml Kanamycin.

3) *E. coli* DH5α bearing helper plasmid pRK2013 (Conjugal helper strain). This plasmid when introduced into an E. coli strain harboring plasmid (Donor), mobilizes that plasmid into *Agrobacterium tumefaciens*. E. coli DH5α harboring pE172 was grown at 37°C on LB medium with 50µg/ml Kanamycin. This plasmid cannot multiply in *Agrobacterium tumefaciens* due to the lack of wide host range replicon.

Procedure

1) Four days before performing the triparental mating, *A. tumefaciens* was streaked to obtain a single colony on Luria agar plates which contained rifampicin & tetracycline and was incubated at 30 °C.

2) One day before, *E. coli* DH5α harboring pRK2013 & *E. coli* DH5α harboring the plasmid to be mobilized were streaked to obtain a single colony on LB agar with 50µg/ml of Kanamycin.

3) On the day of the triparental mating, a plate of Luria agar without any antibiotic was prepared.

4) One colony each from *E. coli* DH5α bearing pRK2013, E. coli DH5α harboring the plasmid which has to be mobilized and *A. tumefaciens* was patched separately on Luria agar plate very close to each other. With a sterile loop, all the three bacterial strains were mixed very well and the plate was left at 30 °C for 12-18 hrs.

5) On the second day from the procedure, six culture tubes which contained 0.9ml LB were autoclaved and kept ready. Six plates containing Luria agar plate with relevant antibiotics were poured and kept ready (In the medium there was an antibiotic selection for both the donor and the recipient so that the donor and the recipient that had mobilized into the *A. tumefaciens* can be maintained).

6) After mating, the bacteria on the Luria agar plate were scrapped and suspended in 1ml LB. A serial dilution was performed by transferring 0.1ml of bacterial suspension into 0.9ml of LB. Likewise 4-5 dilutions were made up to 10-4/ 10-5.

7) 100 µl of each dilution was added to Luria agar medium with appropriate antibiotics and was spread uniformly. The plates were incubated for 4-5days.

4.2.8 Ketolactose Test (Bernaerts and Ley, 1963)

Reagents:

Benedict's reagent [100 ml]: 17.3g Sodium citrate, 10.0g Na2CO3, 1.73g CuSO4.5H2O

Procedure:

1) *Agrobacterium tumefaciens* colony was patched on Luria agar plate and was allowed to grow for 12-16 hours.

2) On the next day, the plate was flooded with Benedict's reagent.

3) Within five minutes, due to the formation of ketolactose, a yellow colored zone was observed which confirmed the presence of *A. tumefaciens.*

4.2.9 Vector Map:

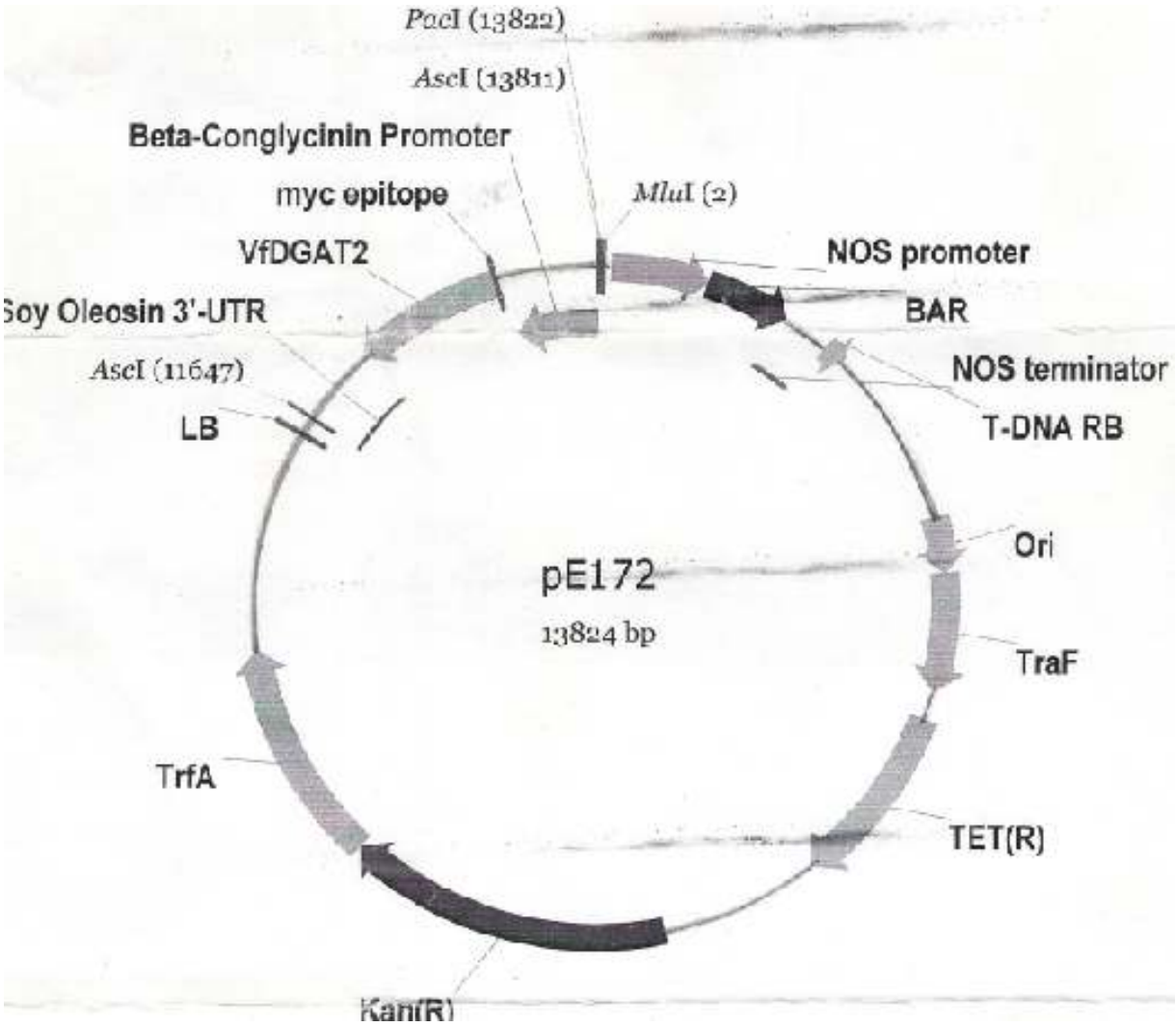


Figure 4.3: Map of pE172

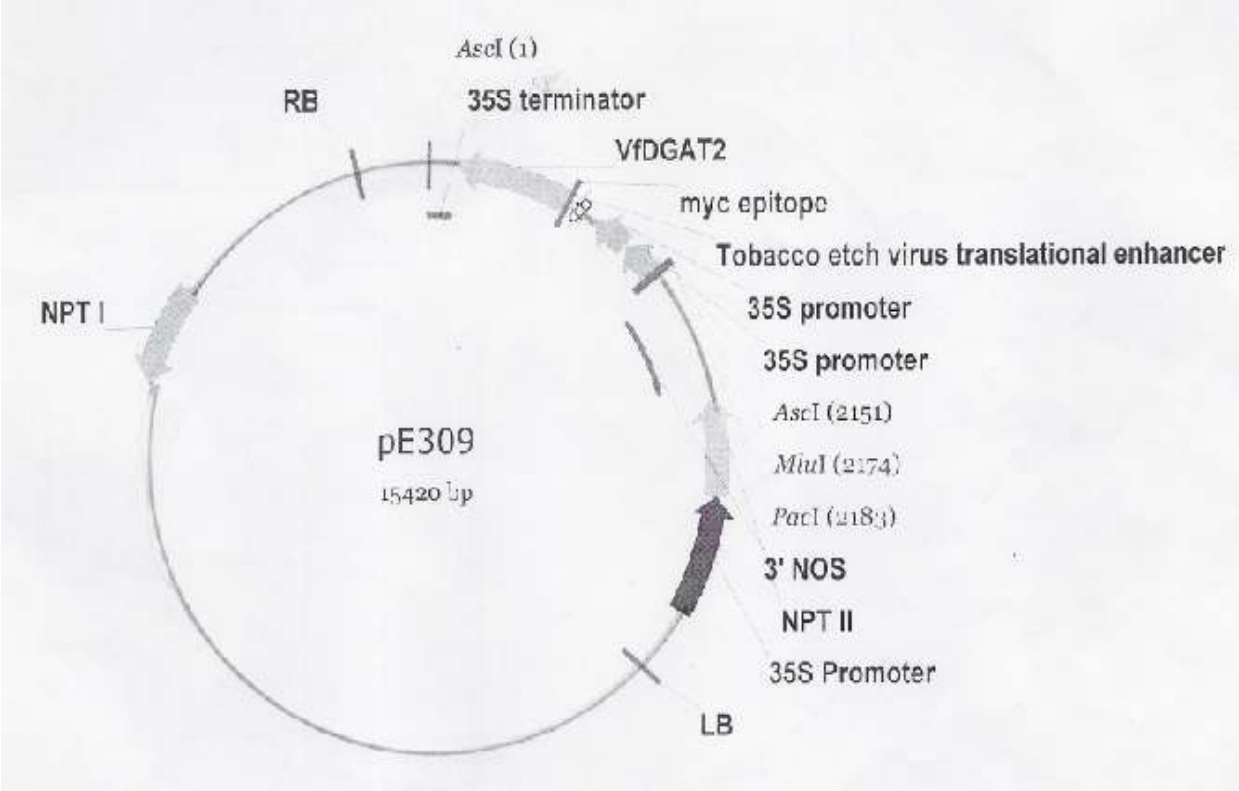


Figure 4.4: Map of pE309

4.3 Co-cultivation

4.3.1 Preparation of *Agrobacterium* suspension for co-cultivation

A single colony of *Agrobacterium* strain containing the gene of interest was incubated in 25 ml of YEP medium and grown overnight on a shaker at 200 rpm at 28°C to an OD at 600 of 1.4–1.6. The bacterial culture was centrifuged at 5,000 rpm and the pellet was resuspended in 25 ml of liquid suspension medium containing MS salts (Murashige and Skoog, 1962), 100 mg/l myo-inositol, 30 g/l glucose and 100 ml acetosyringone. The suspension was kept at 28°C for 5 hours

and used for explant infection. Different explants as explained above were infected with *A. tumefaciens* suspension culture for 20 min at room temperature under continuous shaking, blot dried on sterile filter paper and finally transferred on to regeneration medium. Regeneration medium used was with and without supplementation of acetosyringone. Explants were co-cultivated for different duration on this medium. Explants submerged in bacteria-free MS served as control.

4.4 Explants and Media Composition:

4.4.1 *Agrobacterium* mediated transformation of somatic embryos of *J.curcas*

Seeds from mature fruits of *Jatropha curcas* plants (1.5-2 years old) were collected and their cotyledons used as explants. Seeds were kept under running tap water for 30 minutes followed by wash with a mild detergent. They were again kept under running tap water for 30 minutes. They were treated with 70% ethanol for 30 seconds followed by three rinses with distilled water. Surface sterilization was done with 0.1% mercuric chloride for 2 minutes. Traces of mercuric chloride were removed by giving five rinses with sterile distilled water. Surface sterilized seeds were de-coated under aseptic conditions and inoculated on MS medium supplemented with 4.0 mg/l 2,4-D. The medium was fortified with 3% sucrose and agar (0.8%) was used as a solidifying agent. The pH of the media was adjusted to 5.8 before addition of agar and was autoclaved at 121°C for 20 min. Cultures were maintained at 24±2°C for 14 hour photoperiod.

4.4.2 *Agrobacterium* mediated transformation of leaf discs of *J.curcas*

Young leaves at 3rd and 4th node from the apex were collected from 1.5-2 year old plants grown on campus. The explants were thoroughly washed with tap water for nearly half an hour followed by washing with soap solution. The explants were surface sterilized with 0.1% mercuric chloride for 2 to 3 minutes followed by five rinses with sterile distilled water. Leaves

were then excised into small pieces of 1x1cm and inoculated on Murashige & Skoog (MS) basal medium. A week after inoculation on MS basal medium, the explants were cultured on (4.5 µM-27.0 µM) BAP and (3.5 µM-7.5 µM) IBA.

4.5 Determinaiton of phytotoxic level of selective and bactericidal antibiotics:

Leaf explants and somatic embryos were inoculated on MS medium containing different levels of kanamycin (30, 40, 60, 80 and 100mg/L) respectively for selection of transformants. In a separate study different levels of cefotaxime (50mg/L, 100mg/L,150mg/L and 200mg/L) were tested for controlling *Agrobacterium* infection after transformation.

4.6 Evaluation of factors influencing transformation:

A range of parameters were evaluated and each experiment included three replicates of ten explants each. These parameters included length of pre-culture period (1, 4, 5, 6, 7 days) of explants in regeneration medium prior to *Agrobacterium* infection, effect of acetosyringone, effect of wounding, length of co-cultivation medium (3, 4,5, 6 days).

4.7 Results and Discussion

Binary vector pE172 contains DGAT2 gene under a seed specific promoter and binary vector pE309 contains DGAT2 gene under a constitutive promoter were used in this study. In this thesis an attempt has been made to study the over-expression of DGAT2 in oil seed crop *J.curcas*. For this two different regeneration systems have been taken into consideration. Over-expression studies of DGAT2 under beta congylcinin promoter (seed specific) were done with somatic embryos (detailed investigation reported in chapter three of this thesis). Over-expression studies of DGAT2 under a constitutive promoter (35 S) were attempted with leaf discs as explants (chapter two covers regeneration studies from leaf discs).

4.7.1 Tranformation of *Agrobacterium* with DGAT2 by Triparental Mating:

Triparental mating was carried out using *E coli* DH5α with pE172 (Kan^R) as the donor strain, *E coli* DH5α with pRK2013 (Kan^R) as the helper strain and *Agrobacterium-LBA4404* (Rif^R, Tet^R) as the recipient. Similar experiments were carried out with pE309 (Kan^R) as the donor strain. Figure 4.3 shows the growth of transformants as observed on Luria Agar plates with Kanamycin, Rifampicin and Tetracycline.

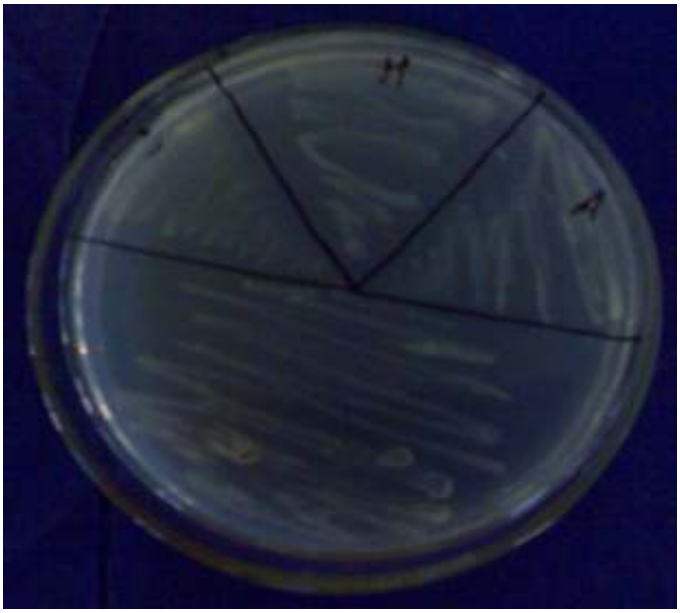
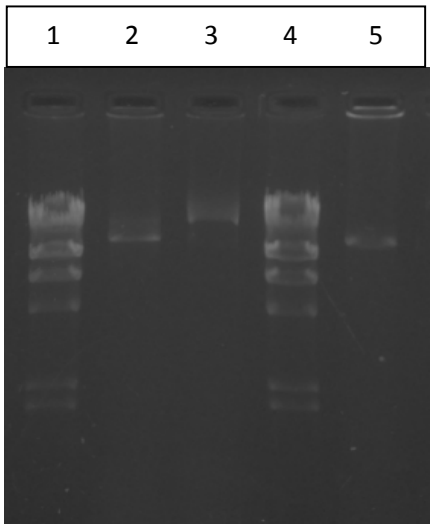


Figure 4.5: Growth of transformants was observed after plating on Luria Agar plates containing tetracyclin(10 µg/ml),rifampicin(5 µg/ml) and kanamycin(50 µg/ml).

4.7.2 Confirmation of *Agrobacterium* transformants using restriction digestion

Agrobacterium containing pE172 was digested with single cutter Bam HI (figure 4.4) and *Agrobacterium* containing pE309 was digested with single cutter Eco RI (figure 4.5).



Lane1: Lamda *Hin*dIII Marker

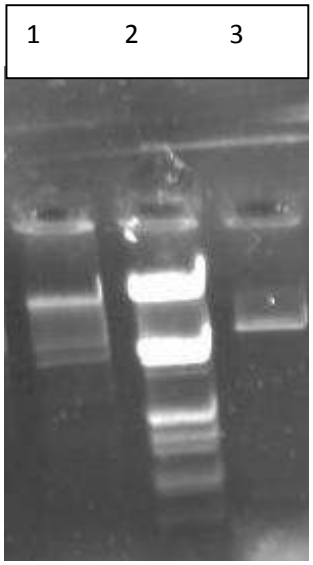
Lane 2: Digested DGAT2 in Donor (pE172)

Lane 3: Undigested DGAT2

Lane 4: Lamda *Hin*dIII Marker

Lane 5: Digested DGAT2 in *Agrobacterium* transformants.

Figure 4.6 A: **Conformation of transformation (pE172)**



Lane 1: Undigested pE309

Lane 2: Lamda *Hin*dIII Marker

Lane 3: Digested DGAT2 in *Agrobacterium* transformants (pE309)

Figure 4.6 B: **Conformation of transformation (pE309)**

In species where low transformation efficiencies are expected, the study of the effect of several factors by comparing the percentage of recovered transformed plants may prove unsuccessful because limited numbers of transformants are produced. Therefore, it becomes important to evaluate the influence of diverse factors on the efficiency of T-DNA transfer. However, these studies can be used as a guide, and only major differences between tested parameters should be considered (Suma et al, 2008). Some others have also reported that these parameters are known to influence the transformation efficiency and the optimized conditions are host species dependent (Xing et al, 2007).

4.7.3 *Agrobacterium* infection of leaf explants

Axenic leaf discs and somatic embryos were immersed in Murashige and Skoog broth seeded with transformed *Agrobacterium.* Fifteen and twenty minutes of immersion time of explants in

Agrobacterium culture was studied and it was found that twenty minutes is more effective period for immersion as compared to fifteen minutes. Figure 4.7 shows transformed leaf discs with *Agrobacterium* containing pE309. Figure 4.8 shows transformed somatic embryos. As seen in figure 4.7 *Agrobacterium* infection is obsserved on the edges of leaf discs. These leaf discs are then transferred to primary selection medium which is MS+ Kan for both the explant types. The surviving explants are then treated as putative transformants. Figure 4.8 C shows embryos after primary selection on MS + kanamycin. These putative transformants would now be subjected to PCR analysis using DGAT2 specific primers. Overall transformation efficiency with leaf discs was found to be 3.3% and further survival of explants was also hampered. While somatic embryos showed a little higher transformation efficiency of 10.08%, the problem encountered here too was further survival of putative transformants and regeneration of survived putative transformants. Kumar et al, 2010 have reported 18.3% of transformation efficiency of leaf explants of *J.curcas* as measured by the surviving shoots on selective medium. Li et al, 2007 have reported 13% transformation efficiency of *J.curcas* cotyledon discs as explants. However, this is the first report of *Agrobacterium* mediated transformation in somatic embryos of *J.curcas*. Xing et al, 2007 have reported to have achieved more than 25% of transformation frequency of embryogenic calli of sweet potato in suspension cultures. It should be noted that solid media cultures and liquid suspension media are both different systems for achieving regeneration and hence cannot be compared.

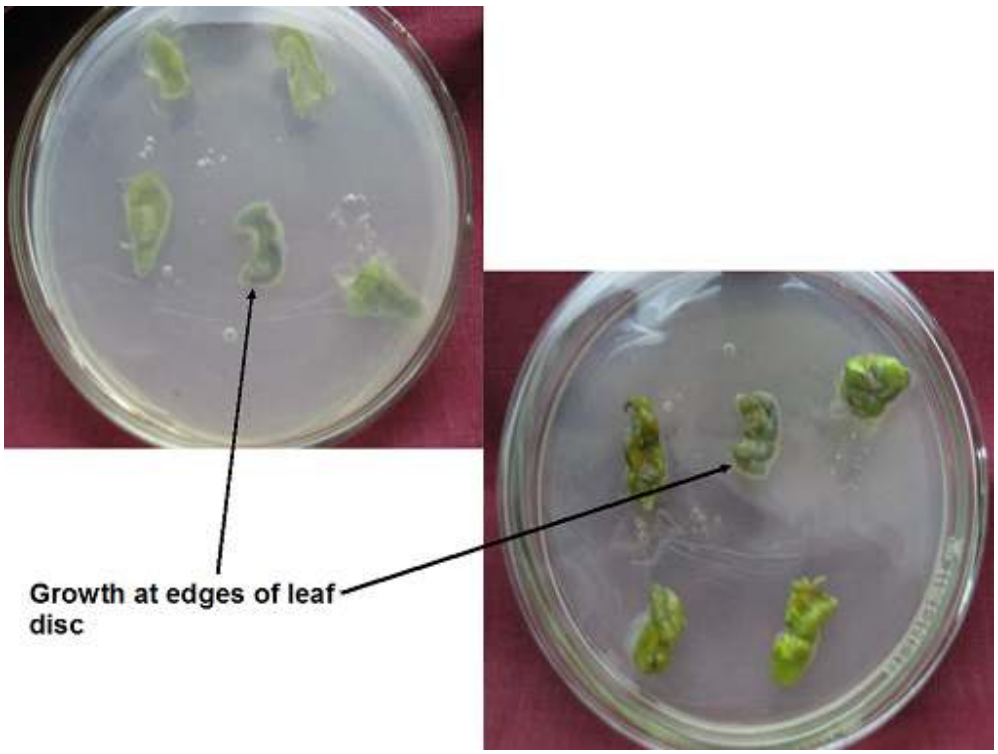


Figure 4.7: **Co-cultivation of transformed *Agrobacterium* and *Jatropha* leaf discs**

Figure 4.8 A: Somatic embryos on the edges of cotyledonary leaf discs

Figure 4.8 B: Co-cultivation of somatic embryos on MS medium

Figure 4.8 C: Transformed embryos on MS+ Kan

4.7.4 Evaluation of selective and bactericidal antibiotics on leaf discs and somatic embryos

These antibiotics are detrimental to tissue proliferation and growth at higher concentrations and fail to suppress bacterial growth at low concentrations (Sathyanarayana et al, 2012). The effect of increasing levels of kanamycin (20, 40, 60, 80 and 100 mg/L) was checked on leaf explants and somatic embryos. At higher concentration of kanamycin (80 and 100 mg/L), 100% bleaching of explants was observed in a week's duration. The phytotoxic effect of kanamycin was seen at 40 and 60mg/L as explants started bleaching after 10 to 12 days in culture resulting in total loss of chlorophyll pigmentation. Hence, in the subsequent experiments 20mg/L of kanamycin was used. However, Vadawale et al, 2012 reported 60mg/L as a suitable concentration of kanamycin for selection of putative transformants in *Arachis hypogea*. In a separate study, increasing levels of cefotaxime (50mg/L, 100mg/L, 150 mg/L and 200mg/L) were used. 200mg/L was found to be effective concentration.

4.8 Optimization of factors influencing transformation

4.8.1 Explant Pre-culture Period

Preculturing explants prior to inoculation and co-cultivation with *Agrobacterium* has been shown to improve genetic transformation frequencies in many plants like *Zingiber officinale* Rosc (Suma et al, 2008), *Lathyrus sativus* (Barik et al, 2005), *Ipomoea batatas* (L) Lam (Xing et al, 2007) and microalgae like *Chlorella* sp. and *Nannochloropsis* sp. (San et al, 2011). It was observed in the present study that one day pre-cultured explants gave similar transformation efficiency as seven days pre-cultured explants. Pre-cultivation allowed proliferation of the plant cells to provide a large population of competent cells as potential targets for transformation (Xing et al, 2007). Hence, for both leaf explants and somatic embryos seven days of pre-culture was preferred. More so in case of somatic embryos as seven days pre-culture would enable to

generate a few somatic embryos which would then be susceptible for *Agrobacterium* infection during co-cultivation.

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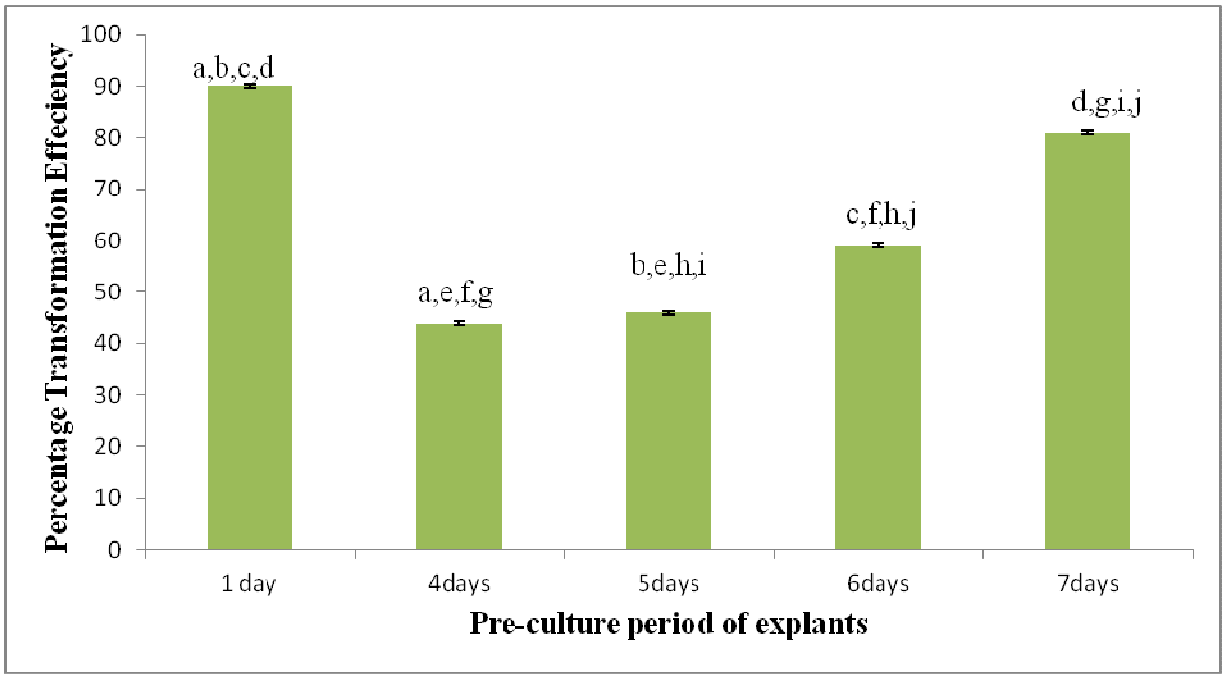

Figure 4.9: Effect of pre-culture period variation on transformation efficiency, N=30

a,b,c,d,e,f,h,i,j at p<0.0001 and g at p<0.005

4.8.2 Wounding Effect:

Wounding helps in the secretion of various phenolic compounds from the wound sites of dicotyledonous plant explants, a process which subsequently increases the transformation efficiency (Barik et al, 2005). Wounding can be through needle pricking or glass beads. There are generally two types of responses to wounding. In some plants it is found that the phenolic

compounds secreted from the wounded sites inhibit transformation efficiency whereas in some plant species it is known to improve transformation efficiency. In the present study effect of hand pricking with the help of a needle was studied. As shown in figure 4.10 needle pricking reduced the transformation efficiency. The explants so treated also showed browning. Kumar et al, 2010 have reported similar observation in *J.curcas.* Wounding increases the levels of secondary metabolites which might be responsible for tissue browning.

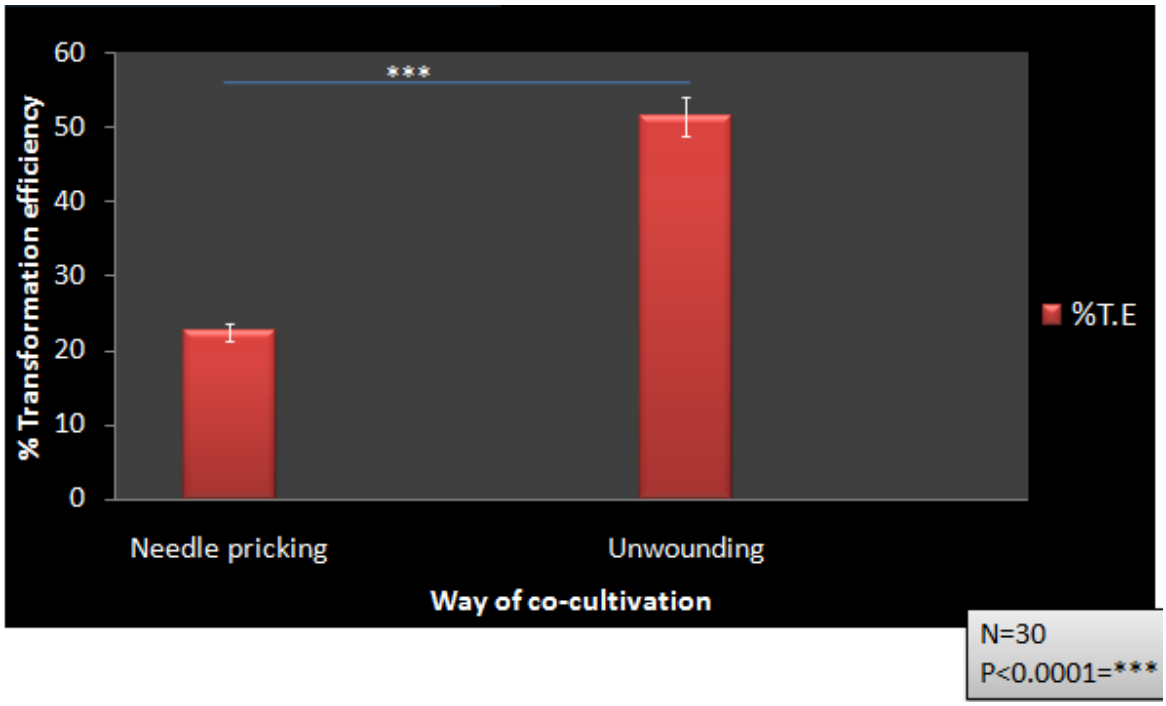

Figure 4.10: Effect of wounding on transformation efficiency

4.8.3 Effect of acetosyringone:

Phenolic compounds like acetosyringone are known to increase the transformation efficiency of *Agrobacterium* (Bolton et al, 1986). 20mg/L of acetosyringone was used in the present study (Li

et al, 2007; Kumar et al, 2010). As shown in figure 4.11 nearly 50% increase in transformation efficiency was observed with the use of acetosyringone.

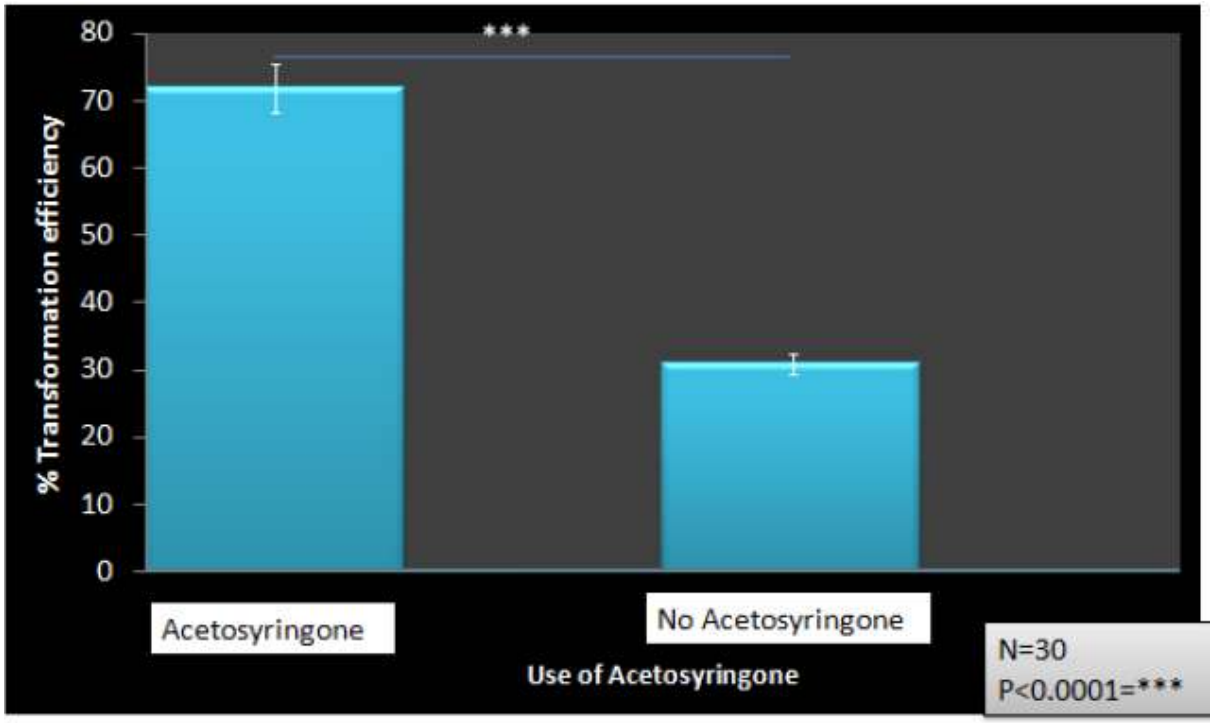

Figure 4.11: Effect of acetosyringone on transformation efficiency

4.8.4 Effect of co-cultivation:

Co-cultivation leads to induction of virulence and gene transfer. Co-cultivation period has great influence on transformation. Too short co-cultivation period is not favorable for transformation. However, too long co-cultivation period results in overgrowing *Agrobacterium* and therefore is harmful to plant cells as longer period may cause necrosis and cell death (Xing et al, 2007). Co-cultivation for 3-6 days is generally suitable for *Agrobacterium* mediated transformation (McHughn et al, 1993). In the present study, co-cultivation for 4 days yielded maximum

transformation efficiency (figure 4.12). At this time point higher number of callus forming discs and lower number of necrotic discs were observed. Co-cultivation for more than 5 days encouraged an overgrowth of bacteria with a concomitant decrease in transformation efficiency and difficulty in removal of *Agrobacterium*. Similar results have been reported for *J.curcas* by Kumar et al, 2010 whereas Li et al, 2007 have reported 3 days of co-cultivation in *Jatropha curcas*.

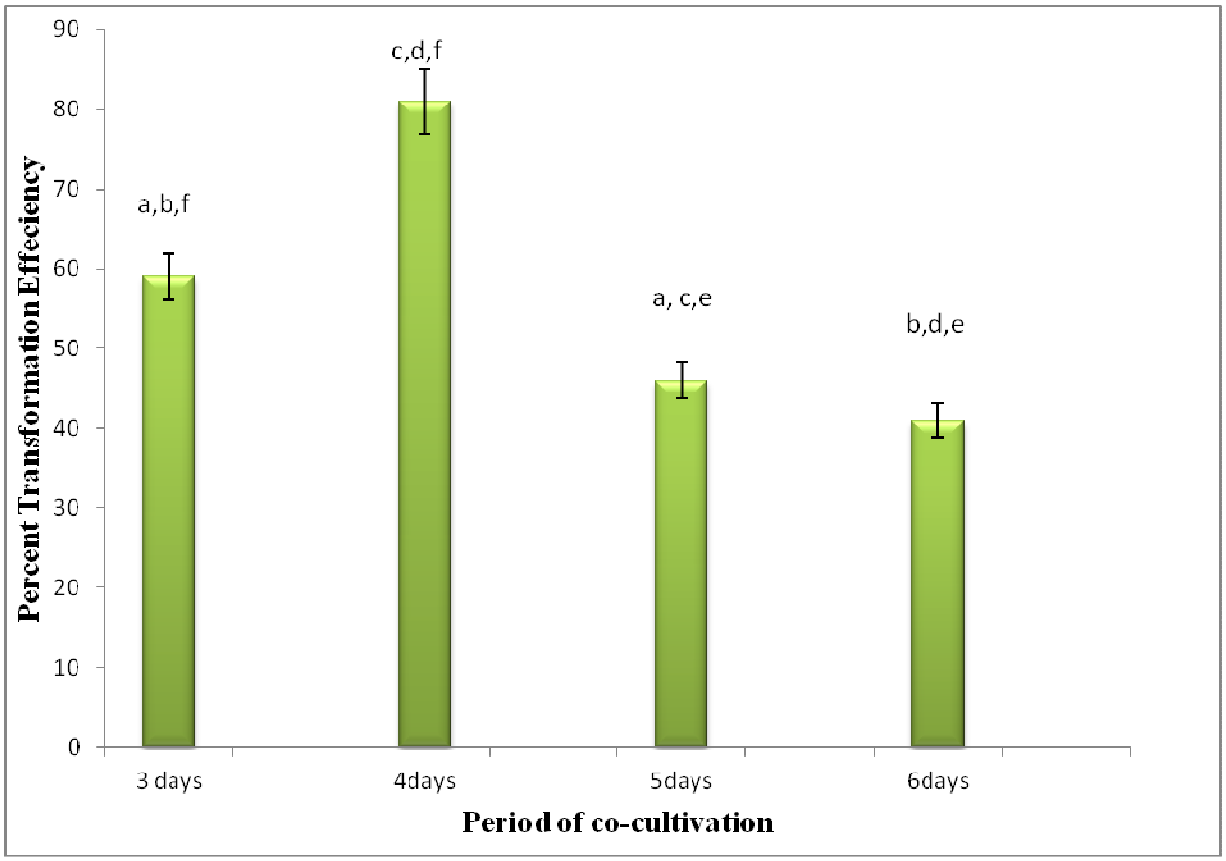

Figure 4.12: Effect of period of co-cultivation on transformation efficiency N=30, p<0.0001

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Study of Genetic Diversity of *J.curcas* by RAPD

5.0 Introduction

The genetic difference between two individuals of a species is the basis of evolution and adaptation. Conservation and sustainable use of biological diversity is important. The International Union for the Conservation of Nature and Natural resources (IUCN) identifies three levels of biological diversity that are equally important to conserve: ecosystem, species and genetic diversity (McNeely et al, 1990). Plant genetic resources comprise the present genetic variation that is potentially useful for the future of mankind. Plant genetic resources should hence be studied and conserved with the ultimate aim of eventually being a source of potentially useful genetic variation. There is virtually no information with regard to the number of introductions and the genetic diversity of *J.curcas* populations grown in India. Several researchers have attempted to define the origin of *J.curcas*, but the source remains controversial (Dehgan and Webster, 1979; Heller, 1996). Three distinct varieties are reported viz., the Cape Verde variety that has spread all over the world, the Nicaraguan variety with few but larger fruits and a non-toxic Mexican variety devoid of phorbol esters. There are no named varieties of *J.curcas* in India with the exception of the variety SDAUJ1 (Chatrapathi) that was released during the year 2006 based on selection from local germplasm (Basha and Sujatha, 2007). Hence the need for studies on the varieties of *J.curcas* using molecular markers becomes imperative. In our state of Gujarat too numerous studies on *J.curcas* plantations have been done but molecular studies have not been reported. The conservation and sustainable use of plant genetic resources require accurate identification of their accession (Arif et al, 2010). In this study an attempt has been made to study the genetic diversity existing in *J.curcas*. The study of genetic diversity with the help of molecular markers can be broadly classified into phenotypic markers, biochemical

markers and molecular markers. Of these, molecular markers are more promising as any change in the protein sequence would be brought about by a mutation in its DNA sequence. DNA markers are not typically influenced by environmental conditions and therefore can be used to help describe patterns of genetic variation among plant populations and to identify duplicated accessions within germplasm collections (Ganesh Ram et al, 2008). Molecular markers can be studied by techniques based on polymerase chain reaction (PCR) and non-polymerase chain reaction. RFLP (Restriction Fragment Length Polymorphism) dominates the non-PCR based techniques. This technique is time consuming and highly expensive. Among the PCR based techniques, RAPD is generally a preferred method. This is more so when the genome to be studied is unknown. The advantage it has over other molecular techniques is that it is less time consuming, more cost effective and the starting material (genomic DNA) requirement is low. Reports have shown that RAPD analysis can be used to detect variation within a restricted range, to identify suitable parents for linkage map construction, and for gene tagging for drought resistance (Virk et al, 1995)

The prerequisite for RAPD is good quality DNA. DNA isolation from plant source is at times difficult owing to the large concentration of polysaccharide, protein, pigment or phenolic compounds. Some plant taxa may not permit optimal DNA yield. Closely related species of the same genus also may require different isolation protocols. Thus, an efficient protocol for isolation of DNA as well as the optimization of the PCR conditions is required (Subramanyam et al, 2009).

Study of Genetic Diversity of *J.curcas* by RAPD

5.1 Literature Studies

Detection and analysis of genetic variation can help us to understand the molecular basis of various biological phenomena in plants. Since the entire plant kingdom cannot be covered under sequencing projects, molecular markers and their correlation to phenotypes provide us with requisite landmarks for elucidation of genetic variation. Genetic markers can be classified into three types: morphological trait based markers, protein based (biochemical) markers and DNA based (molecular) markers. Traditionally, diversity within and between populations was determined by assessing differences in morphology. Its advantages are being readily available and non requirement of sophisticated equipment. However these attributes are subject to change due to environmental factors and vary at different time points. Biochemical markers also have similar limitation of being influenced by environment. Genetic or DNA based marker techniques such as RFLP (restriction fragment length polymorphism), RAPD (random amplified polymorphic DNA), SSR (simple sequence repeats) and AFLP (amplified fragment length polymorphism) are routinely being used in ecological, evolutionary, taxonomical, phylogenic and genetic studies of plant sciences. These techniques are well established and their advantages as well as limitations have been realized (Agarwal et al, 2008).

An ideal molecular marker technique should have the following criteria: (i) highly polymorphic in nature as it is polymorphism that is investigated in genetic diversity studies (ii) co-dominant in nature as it allows determination of homozygous and heterozygous states of diploid organisms (iii) frequently occurring in the genome (iv) neutral in behavior, easy, cheap and (v) highly reproducible (Kumar et al, 2009). Genomic abundance, level of polymorphism detected, locus specificity, reproducibility, cost etc. are important aspects known to influence various techniques. Table 5.1 enlists these important features regarding the techniques most frequently used (Agarwal et al, 2008).

Table 5.1 Comparision of various aspects of frequently used molecular maker techniques

	Abundance	Reproducibility	Degree of Polymorphism	Locus specificity	Technical requirements	Quantity of DNA required	Major application
RFLP	High	High	Medium	Yes	High	High	Physical mapping
RAPD	High	Low	Medium	No	Low	Low	Gene tagging
SSR	Medium	Medium	Medium	No	Medium	Low	Genetic diversity
SSCP	Low	Medium	Low	Yes	Medium	Low	SNP mapping
CAPS	Low	High	Low	Yes	High	Low	Allelic diversity
SCAR	Low	High	Medium	Yes	Medium	Low	Gene tagging & Physical mapping
AFLP	High	High	Medium	No	Medium	Medium	Gene tagging
IRAP/ REMAP	High	High	Medium	Yes	High	Low	Genetic diversity
RAMPO	Medium	Medium	Medium	Yes	High	Low	Genetic diversity

RFLP restriction fragment length polymorphism, RAPD random amplified polymorphic DNA, SSR simple sequence repeats, SSCP single strand conformational polymorphism, CAPS cleaved amplified polymorphic sequence, SCAR sequence characterized amplified region, AFLP amplified fragment length polymorphism, IRAP/REMAP inter-retrotransposon amplified polymorphism/retrotransposon-microsatellite amplified polymorphism.

Genetic diversity assessment with molecular markers is important for efficient management and conservation of plant genetic resources in gene banks. Very little information regarding the genetic diversity and number of introductions of *J.curcas* populations grown in India is available. Keeping in view the commercial applications of *J.curcas* it becomes imperative to use high quality planting material for all future plantations. Genetic diversity studies of *J.curcas* in India are limited to the accession available around in here. Exception to this is the study reported by Basha and Sujatha, 2007 where they have incorporated a non-toxic accession from Mexico along with 43 accessions from different regions from India. Montes et al, 2008 reported study of accessions from 30 countries. Senthil Kumar et al, 2009 reported the use of accessions from India and Zimbabwe.

Very little analysis of genetic polymorphism in *J.curcas* has been performed so far. Protein based isozyme markers have been reported to be used to determine the genetic relatedness of the members of the genus *Jatropha* and *Ricinus* sp. (Sujatha et al, 2008). Gupta et al, 2008 used RAPD (Random Amplification of Polymorphic DNA) and ISSR (Inter Simple Sequence Repeat) markers to study different accessions of four geographical locations of India and divided them into four populations. ISSR markers have also been reported to be used to study inter and intra population variability in *J.curcas* (Basha and Sujatha, 2007; Senthil Kumar et al, 2009). Ganesh Ram et al, 2008 assessed the genetic diversity of 12 *Jatropha* species using RAPD. Irrespective of the geographical locations of different accessions and primers, it was observed that all accessions from India clustered together. Diversity analysis with local germplasm showed a narrow genetic base in India (Basha and Sujatha, 2007; Ganesh Ram et al, 2008).This indicates the need of widening the genetic base of *J.curcas* through introduction of accessions with broader geographical background and creation of variation through mutation and hybridization techniques (Mukherjee et al, 2011). Gupta et al, 2008 reported 40%-100% polymorphism using RAPD and approximately similar percentage polymorphism using ISSR markers in 13 *Jatropha* accessions from different geographical locations of India. Senthil Kumar et al, 2009 reported

nearly 100% polymorphism using RAPD and ISSR markers in eight *Jatropha* species and three *Jatropha curcas* accessions. RAPD analysis in *J.curcas* shows a narrow genetic base (Basha and Sujatha 2007; Ganesh Ram et al, 2008).

5.2 Materials and Method

5.2.1 Plant material

Fresh and young plant tissue material from leaves and petioles were used to extract DNA using available protocols. Standardization of protocol was done using plant species grown in Department of Biochemistry, The M.S. University of Baroda, Vadodara. They were rinsed with distilled water and blotted gently on filter paper.

5.2.2 Reagents

DNA Extraction buffer A

2%	CTAB (cetyltrimethylammonium bromide)
100mM	Tris-HCl
20mM	EDTA
1.4M	NaCl
4%	PVP (polyvinyl pyrrolidone)

DNA Extraction buffer B

100mM Tris-HCL

50mM EDTA

100 mM NaCl.

5M NaCl

3M sodium acetate (pH 5.2)

5M Potassium acetate

5 M potassium acetate, 60.0 ml

Glacial acetic acid, 11.5 ml

H2O, 28.5 ml

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. Store the solution at 4°C and transfer it to an ice bucket just before use.

Solutions and buffers were autoclaved at 121°C at 15 psi pressure and stored at RT (Room Temperature).

5.2.3 Chemicals

Chloroform: isoamyl alcohol (24:1)

80% ethanol

5.2.4 DNA Isolation Protocol (Keb--Llanes et al, 2002)

1. Grind 0.3 g of leaf tissue to a fine power using a mortar, pestle, and liquid nitrogen. Transfer the powder to an Eppendorf tube.

2. Add 300 µL buffer A, 900 µL buffer B, and 100 µL SDS.

3. Vortex the mixture. Incubate in a water bath at 65ºC for 10 min.

4. Add 410 µL cold potassium acetate. Mix thoroughly. Centrifuge at 15,300 g for 15 min at 4ºC.

5. Transfer 1 mL of the supernatant to a clean Eppendorf tube. Add 540 µL cold isopropanol. Incubate on ice for 20 min.

6. Centrifuge at 9600 g for 10 min. Discard the supernatant. Wash the pellet with 500 µL 70% ethanol and let dry.

7. Resuspend pellet in 600 µL buffer TE. Add 60 µL 3 M sodium acetate (pH 5.2) and 360 µL cold isopropanol. Incubate on ice for 20 min.

8. Centrifuge at 9600 g for 10 min. Repeat steps 5-7 twice.

9. Resuspend the pellet in 50 µL buffer TE.

5.2.5 Agarose Gel Electrophoresis

0.8% agarose gel electrophoresis used to separate, analyze and quantitate nucleic acids and buffer used will be 1x TAE (Tris-Acetate EDTA) buffer.

5.2.6 Spectrophotometric determination of DNA concentration

DNA quantification is a very important step for many downstream applications such as cloning , RAPD etc. DNA yield and purity were checked spectrophotometrically by measuring absorbance at 260 and 280 nm. Nucleic acid concentration was calculated using the following formula:

A260 x 50µg/ml x dilution factor (995/5). This will provide the concentration of the stock DNA (µg/ml).

5.2.7 Restriction Digestion

In existing protocol extracted DNA sample showed higher absorbance at A260/280nm than expected. So in order to ensure good quality DNA, RE (Restriction enzyme) digestion was performed. In this method HindIII was used by varying its concentrations 2, 4, 6 and 8 Units respectively along with control (without adding enzyme) in the corresponding buffer at 37˚C for 3 h. Digested DNA along with control was analyzed by running the samples on 1% agarose gel.

5.2.8 DNA Amplification

37 decamer primers from Operon Technologies Inc., (USA) and Integrated DNA Technology ( ) were initially screened for their repeateable amplification with *Jatropha* accessions. Amplification was carried out in 25 µl reaction volumes containing 1X Assay buffer (50 mM KCl, 2.5mM of each dNTP, 0.8 µM primers, 1.5 U of Taq DNA polymerase (Banglore Genei Pvt. Ltd. India) and 20 ng of template DNA. PCR conditions were as shown below. Amplification products were separated on 1.8% agarose gel and stained with ethidium bromide and photographed under UV light.

94°C	94°C	38°C	72°C	94°C	45°C	72°C	72°C	4°C
5 min	45 sec	1 min	1.5min	45 sec	1 min	1 min	10 min	store
10 cycles				35 cycles				

5.3 Results and discussion

5.3.1 Standardization of DNA isolation from *J.curcas*

Molecular aspects of biological studies are highly valued and the first approach to such studies is extraction of nucleic acids. Lots of limitations in genetic material extractions are solved by some changes in compound and pH of functional buffers, so that extracted DNA is much more quantified and also better qualified (Alaey et al, 2005). It is also important to use a method which can be done acceptably and economically too. DNA isolation from plant source is at times difficult owing to the large concentration of polysaccharide, protein, pigment or phenolic compounds. As mentioned earlier some plant taxa may not permit optimal DNA yield. Closely related species of the same genus also may require different isolation protocols. Thus, an efficient protocol for isolation of DNA as well as the optimization of the PCR conditions is required (Subramanyam et al, 2009). *Jatropha curcas* like other Euphorbiaceae family members contain exceptionally high amounts of polysaccharides, polyphenols, tannins and other secondary metabolites such as alkaloids, flavanoids, phenols, terpenes etc. which might interfere with successful DNA isolation. Subramanyam et al, 2009 reported certain problems encountered during the isolation and purification of DNA especially from *Jatropha curcas* which included degradation of DNA due to endonucleases, co-isolation of highly viscous polysaccharides, inhibitor compounds like polyphenols and other secondary metabolites that interfere with enzymatic reactions. Many a times, RNA co-precipitates with DNA resulting in many problems including suppression of PCR amplification during RAPD analysis.

Keeping in view the above mentioned problems associated with successful DNA isolation from *J.curcas*, protocol given by Kebb Llanes et al, 2002 was used in the current study. It is generally observed that plant DNA being difficult to isolate due to above mentioned reasons, requires strong treatments with chemicals like liquid nitrogen or cetyltrimethylammonium bromide (CTAB). However, the problem with liquid nitrogen is its toxic nature and non-availability in some remote areas. On the contrary CTAB is not only easily available but also yields good quantity of DNA. In the present study, certain modifications to the existing protocol (Kebb Llanes et al, 2002) were required to be made as lot of polysaccharide co-precipitated with DNA. The first study undertaken was to check the required levels of CTAB (2%-as mentioned in actual protocol, 3% and 4%) and its effect on DNA yield as shown in table 5.2. For all the optimization studies young leaves and petioles of *J.curcas* were used as samples.

Table 5.2 Effect of varying percentage of Cetyl Trimethyl Aammonium Bromide on genomic DNA yield

Samples	2% CTAB	3% CTAB	4%CTAB
$A_{260/280}$ Mean±SEM	2.600±0.4933	2.233±0.2186	2.300±0.2082
DNA (µg/g fresh weight of tissue)	143	182	253

It could be observed from table 5.2 that higher levels of CTAB yields higher DNA, however, fire type bands were seen at higher levels of CTAB (3% and 4%).

Hence, it was desirable to use CTAB at 2% level in the extraction buffer. Incorporation of PVP (polyvinylpyrrolidone), beta-mercaptoethanol and ascorbic acid in extraction buffer A as mentioned in the reported protocol did not help in obtaining good quality DNA. When a buffer devoid of these was used and by introduction of a new Chlorofom:Isoamyalcohol step (24:1) a better yield and quality of DNA was obtained.

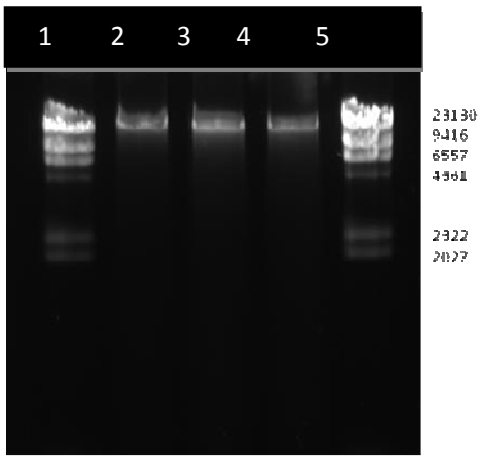


Figure 5.1: Electrophoresis of Genomic DNA of *J.curcas,* Lane1&5-λ *Hin*dIII marker, Lane 2, 3&4-Genomic DNA

Figure 5.1 shows DNA as observed on agarose gel electrophoresis along with λ HindIII marker.

It was observed that when fresh samples were used for DNA isolation no phenolic exudates were seen and hence a transparent pellet of DNA was observed after ethanol wash. However, in stored samples (-80°C) pigmentation was observed and also secondary metabolite contamination was seen. This was overcome by addition of pinch of PVP (44,000 Dalton) and ascorbic acid during crushing of tissue with mortar and pestle. The purity and clean nature of DNA samples could be confirmed through complete digestion by the restriction enzyme HindIII after incubating the reaction tubes at 37 °C for 1.5 h (Figure 5.2).

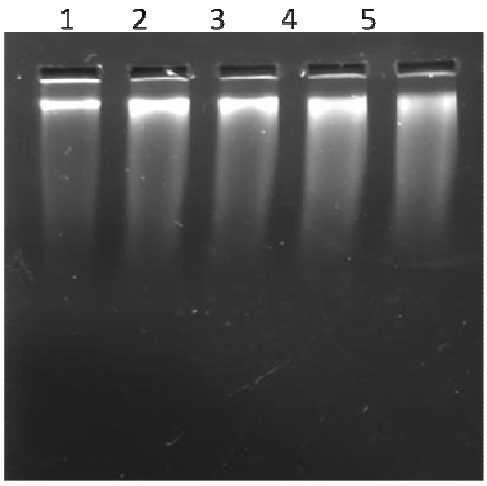


Figure 5.2: Electrophoresis of *J.curcas* DNA digested with *Hin*dIII on 1% agarose gel,Lane1-Undigested DNA, Lane2-5 DNA digested with 2,4,6 and 8 U of *Hin*dIII

This indicated that the isolated DNA was amenable to further processing in cloning experiments as well as DNA fingerprinting. Similar results have been reported by Khanuja et al, 1999 for plants producing large amount of secondary metabolites and essential oils, Pamidimarri et al, 2009 for *Jatropha curcas*, Doulis et al, 2000 for Cupressus sempervirens L.

The second crucial step for effective DNA isolation is the heat shock treatment. In the protocol used heat treatment was at 65°C for 10 minutes. However, this did not yield good results; hence varied time of heat treatment was studied (10, 30, 50, 70, 90, 110, 120 minutes) (figure 5.3). As seen is table 5.3, DNA yields increase even after an hour of treatment and best results were found at 110 minutes of treatment. Though it could be argued that increasing the time duration of heat treatment would lead to a lengthy procedure, during the course of the study, it was observed that heat treatment duration of 60 minutes yielded good quality DNA suitable for many

downstream processes. Pamidimarri et al, 2009 reported 90 minutes of heat treatment gave best results (132.5±7.8 µg/g of tissue). Overall higher DNA yield (from 150 µg/gm of tissue onwards) was obtained in the present study. This far exceeds the reported range (70-120 µg/ gm tissue) in the actual protocol (Kebb Llanes et al, 2002). Yield of DNA in the present study was higher when compared to other reported work (85.95-105.35 µg/ gm tissue by Suramanyam et al, 2009; 57-132 µg/ gm tissue by Pamidimarri et al, 2009). However, Dhakshanamoorthy and Selvaraj, 2009 have reported (2360 ±52 µg/ gm tissue) DNA from *J.curcas* using modified CTAB protocol.

Table 5.3 Variation in duration of heat treatment in leaf samples

Time of incubation at 65°C (minutes)	$A_{260/280}$ Mean±SEM	DNA yield (µg/g of tissue)
10	2.400±0.100	152
30	1.867±0.066	315
50	1.700±0.0577	326
60	1.820 ±0.066	347
70	1.900±0.0577	282
90	1.567±0.133	448
110	1.900±0.100	744
120	1.733±0.333	447

Values are represented as Mean ± SEM (N=9)

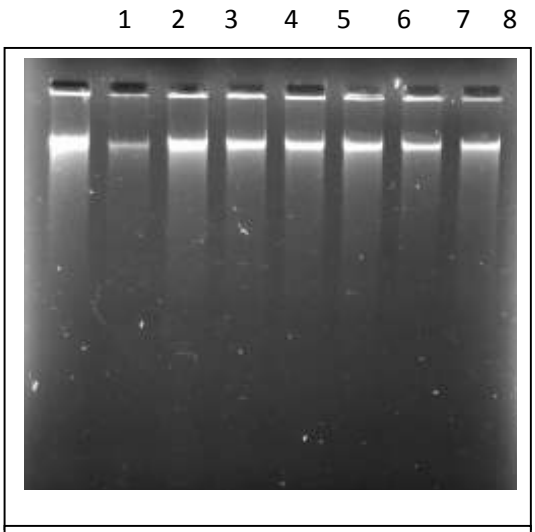


Figure 5.3: Electrophoretic separation of genomic DNA extracted from leaf using different incubation time at 65°C lane no. 1(10'), lane no.2(30'), lane no.3(50'), lane no.4(60'), lane no.5(70'), lane no.6(90'), lane no.7(110'), lane

Table 5.4 Variation in heat treatment attempted in petiole samples

Time of incubation at 65°C (Minutes)	$A_{260/280}$ Mean±SEM	DNA yield (µgDNA/gm tissue
10	1.767±0.1764	207
30	1.800±0.0577	419
50	1.767±0.0333	207
60	1.867±0.066	330
70	2.100±0.4041	215
90	1.767±0.0333	304
110	1.867±0.0333	541
120	1.933±0.0881	207

Values are represented as Mean ± SEM (N=9)

One of the other problems experienced with DNA isolation was increased protein co-precipitation as the reported protocol does not encompass any phenol chloroform treatment. Protein co-precipitation has also been reported by Dhakshanamoorthy and Selvaraj, 2009 where they have stated that photosynthetically active tissue contains phenolic compounds that oxidize during extraction and irreversibly interact with proteins and nucleic acids to form a gelatinous matrix. This matrix might inhibit proper extraction and amplification. Phenol is known to degrade protein present in the sample. However, it is also seen to be a deterrent as it not only is

toxic in nature but residual phenol in the extract also hampers yield of DNA. When phenol was used in the current study, it did not help in eradication of protein that was contaminating the DNA sample. This problem was not overcome even by the use of beta-mercaptoethanol as mentioned in the reported protocol. Hence, the phenol treatment step was dropped and DNA extraction was attempted using chloroform: isoamyl alcohol (24:1). Treatment with chloroform:isoamylalcohol (24:1) was introduced to remove extra proteins present in the sample. Pamidimarri et al, 2009 emphasized on the use of phenol for removal of proteins as it was found to affect the A260/280. However, in the present study A260/280 in the range of 1.7 to 2.0 was obtained even in the absence of phenol in extraction procedure.

The protocol mentions the use of TE along with salt and isopropanol for DNA precipitation. However, residual EDTA of the TE buffer could affect the further series of reactions like PCR amplification and hence, TE was omitted from the treatment and only salt and isopropanol were used for salt–DNA complex precipitation. This also did not affect the yield of isolated DNA. This modified protocol could be used for different tissues like nodes, leaves and petioles. Of all the samples tried it could be concluded that petiole proved to be a better sample for obtaining high quality and quantity of DNA suitable for further downstream processing. Since in this study young leaf samples were incorporated, many a times pigmentation was seen even in the DNA so obtained. On the other hand, nodes being hardy in nature, may at times, lead to lower levels of DNA. The modified protocol for successful DNA isolation from various plant samples is thus outlined below:

Chop 0.3 g of leaf tissue using fine blade and grind petiole samples to a fine power using pre-cooled (-20°C) mortar and pestle . Transfer the powder to microfuge tube. Add 300 µl buffer A, 900 µl buffer B, and 100 µl SDS.

Vortex the mixture. Incubate in a water bath at 65ºC for 60 minutes.

Add 410 µL cold potassium acetate. Mix thoroughly. Centrifuge at 12,000 rpm for 25 min at 4ºC.

Transfer 1 ml of the supernatant to a clean microfuge tube. Add equal amount of chloroform: isoamyl alcohol. Invert it gently and centrifuge at 10,000 rpmfor 10 min at 4ºC

Transfer 1 ml of the supernatant to a clean Eppendorf tube. Add 540 µL cold isopropanol. Incubate in -20 ºC for 1 hour

Centrifuge at 10,000rpm for 10 min. Discard the supernatant. Wash the pellet with 500 µl 80% ethanol.

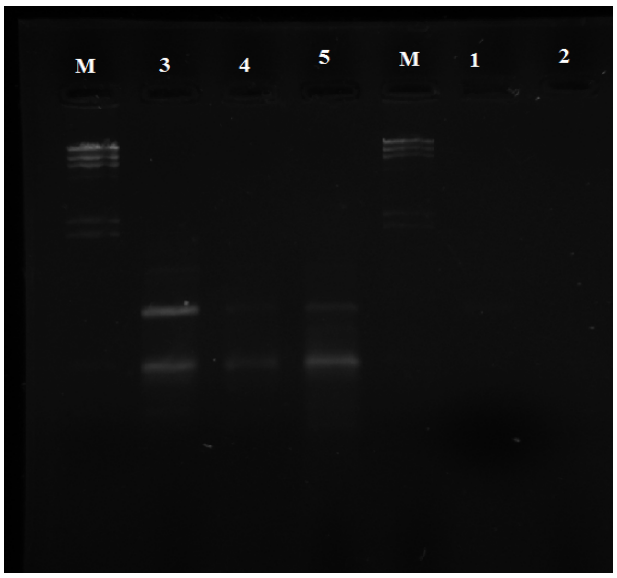
Add 60 µl 3 M sodium acetate (pH 5.2) and 360 µl cold isopropanol. Incubate in -20 ºC for 1 hr. Centrifuge at 12,000rpm for 10 min. Resuspend the pellet in 20-25 µl TDW. Quantify the DNA spectrophotometrically at 260 nm.

5.3.2 Optimization of RAPD- PCR parameters

Parameters for random amplification of polymorphic DNA from *J.curcas* were studied and optimized. Parameters studied were variation in annealing temperature; optimal concentration of template DNA, optimal primer concentration, MgCl2 concentration, number of PCR cycles etc. Artifactual non-genetic variation in analysis can be considerable if primer-template concentration and annealing temperature are not carefully optimized (Caetano-Anolles, 1993). RAPD in *J.curcas* has been reported by many others with different goals (Basha and Sujatha, 2007 for development of population (inter and intra population) specific SCAR markers; Basha and Sujatha, 2009 for genetic analysis of *Jatropha* species and interspecific hybrids using nuclear and organelle specific markers; Gupta et al, 2008 for comparative analysis of genetic diversity among *Jatropha curcas* genotypes using both ISSR and RAPD; Ganesh Ram et al, 2008 for studying genetic diversity among *Jatropha* species using RAPD markers; Pamidimarri et al,

2009 for molecular characterization of *Jatropha* resources through ISSR; Dakshanamoorthy and Selvaraj, 2009 for extraction of Genomic DNA from *Jatropha sp.* using modified CTAB method. Each of these studies has a lot of variation in the PCR conditions, DNA concentration, primer levels etc. in their reports. In the present study varied concentrations of DNA (20ng, 40ng, 60ng, 80ng, 100ng, 120ng, 140ng, 160ng, 180ng, and 200ng) were tried to achieve a amplifiable PCR reaction. Out of these 20ng of DNA was suitable for PCR analysis.

In most cases 1.5mM concentration is available along with reaction buffer which is sufficient for Taq DNA polymerase to work (Padmalatha and Prasad, 2006; Basha & Sujatha, 2007). However, in certain instances 3mM of $MgCl_2$ is incorporated (Pamidimarri et al, 2009). In a few instances 2mM of $MgCl_2$ is also reported to be added (Jubera et al, 2009). In the present study, 1mM-3mM of $MgCl_2$ was included in the reaction mix. As shown in figure 2.6, 2mM, 2.5mM and 3mM when included in the reaction mix gave a amplification product on agarose gel electrophoresis.



M – Molecular wt marker ($\lambda_{HindIII}$)

3- 2mM $MgCl_2$

4- 2.5mM $MgCl_2$

5- 3mM $MgCl_2$

1- 1mM $MgCl_2$

2- 1.5mM $MgCl_2$

Figure 5.4 Amplification of *J.curcas* by RAPD in varying $MgCl_2$

Table 5.5 Different *Jatropha* genotypes with their oil content

Sample Code	Sample Name	Seed oil%
A-2	JCP-4, Pantnagar	31.09
A-3	PJA-1, Hyderabad	32.32
A-5	TNAU, Mettupalayam	28.27
A-7	TFRI, Jabalpur	22.94
A-8	JIP-12, Jammu	26.12
A-9	MSU, Vadodara	36.36

Table 5.5 enlists the six different genotypes used in the present study along with their oil content. Out of 37 primers used to study the genetic diversity of 6 *Jatropha curcas* accessions, 7 primers could generate reproducible amplification products. Rest of the primers resulted either in no amplification or smeared products. These primers yielded 95 amplified bands/fragments. The number of amplified fragments ranged from 1 (RAN-10, RFU-10) to 7 (RAN-3) with an average of 9.42 bands per primer (table 5.6). The size of amplified fragments ranged from 190-3000bp (figure 5.5 – 5.11). Similar results have been reported by Ikbal et al, 2010 in *Jatropha curcas* genotypes from different states of India. Ganesh Ram et al, 2008 have reported the size of amplified products in the range of 200-2400bp for different *Jatropha* genotypes. Similar work has been documented by Gupta et al, 2008 in different *Jatropha curcas* genotypes. As shown in table 5.6, in the current study, of the 95 bands scored 66 were polymorphic whereas 28 were monomorphic. Table 5.7 shows eleven unique alleles detected with a total of five primers in five genotypes. The putatively similar bands originating for RAPDs in different individuals may not necessarily be homologous, although they may share the same size in base pairs (Gupta et al, 2008).

The pairwise comparison of the RAPD profiles based on both shared and unique amplification products was made to generate a similarity matrix. As shown in table 5.8 Jaccard's similarity coefficient varied from (0.34 – 0.66). This narrow range of similarity co-efficient value suggests a close genetic population. This could be due to the fact that *Jatropha* is not a cultivated variety and has been propagated randomly throughout India. The highest value of similarity coefficient (0.66) was detected between JIP-12 and JCP-4 and PJA-1 respectively. The lowest value of similarity coefficient (0.345) was detected between accessions from Jammu (JIP-12) and TNAU (Mettupalayam). This also explains the geographical conditions playing a decisive role as the climatic conditions of both the places are different. Cluster analysis based on Jaccard's similarity coefficient generated a dendogram (figure 5.12) which depicts the overall genetic relationship among the genotypes studied. Two distinct clusters could be observed. Genotypes JIP-12 and

TNAU which show a low similarity coefficient are also placed in different clusters. Ecological and geographical differentiation are two important factors which influence breeding and sampling strategies of tree crops which further help in understanding the population structure. Variation in genetic diversity within species is usually related with geographic range, mode of reproduction, mating system, seed dispersal and fecundity (Ikbal et al, 2010). The genetic diversity observed between the genotypes in the present investigation could be due to all the above mentioned factors. Similar conclusions have been drawn by Ikbal et al, 2010, Gupta et al, 2008.

Table 5.6 Amplified DNA bands and polymorphism generated in *J.curcas* genotypes

Primer (Sequence 5'- 3')	Total Bands	Polymorphic Bands	Monomorphic Bands
RAN-3 GGC ACG TAA C	18	15	3
RAN-10 GTG CCC GAT G	9	8	1
RAN-14 TCG CCG CTT A	9	2	7
RFU-6 CCT GGG CTA C	9	3	6
RFU-10 CCT GGG TGA C	7	4	3
RBA-13 CCG GCC ATA C	10	4	6
RBA-12 CCG GCC TTA A	4	2	2
Total	66	38	28
Mean	9.42	5.42	4

Table 5.7 Primers showing amplification of unique alleles of different genotypes of *J.curcas*

Primer	Total Bands	No. of unique alleles	Allele size (bp)	Genotypes
RAN-3	18	5	190, 210 & 450	JCP-4
RAN-10	9	2	220 & 700	TFRI
RAN-14	9	1	230	JCP-4
RAN-3	18	1	320	MSU
RBA-13	10	1	570	MSU
RAN-13	18	1	580	TFRI
RAN-14	9	1	750	PJA-1
RFU-10	7	1	1000	JIP-12

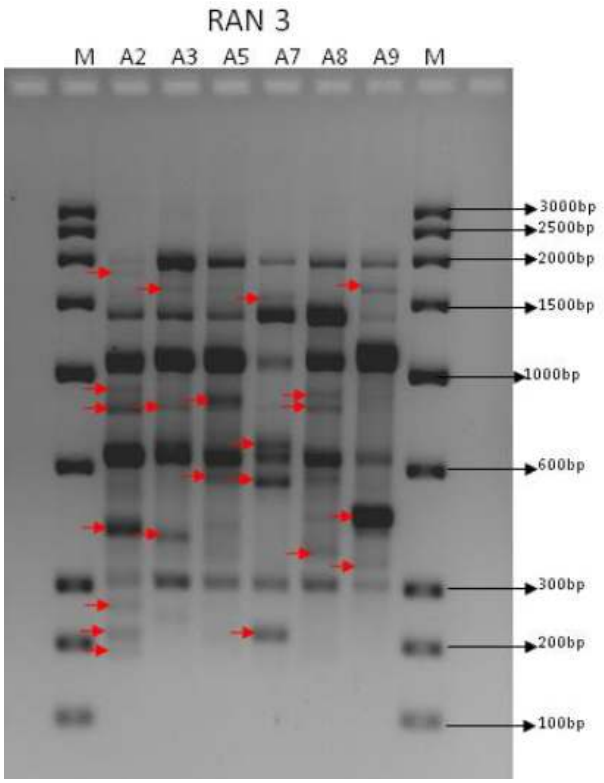


Figure 5.5: RAPD profile generated with RAN3

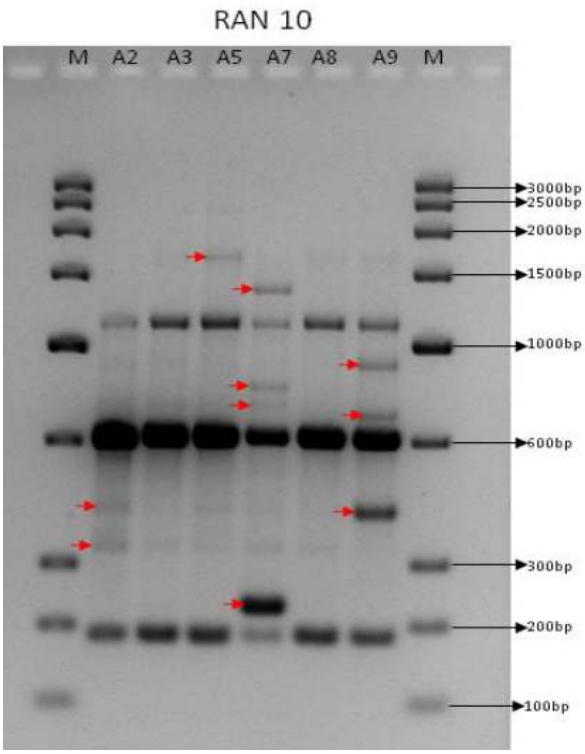


Figure 5.6: RAPD profile generated with RAN10

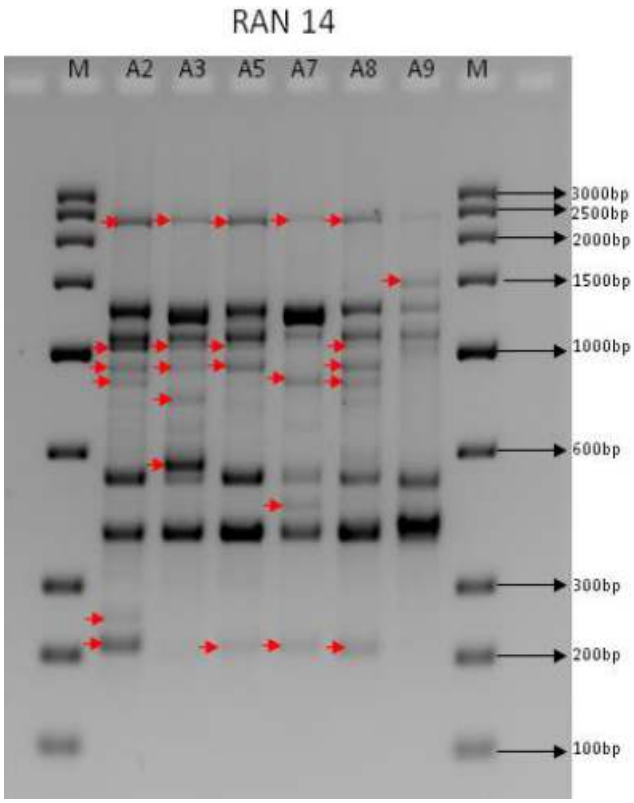


Figure 5.7: RAPD profile with RAN14

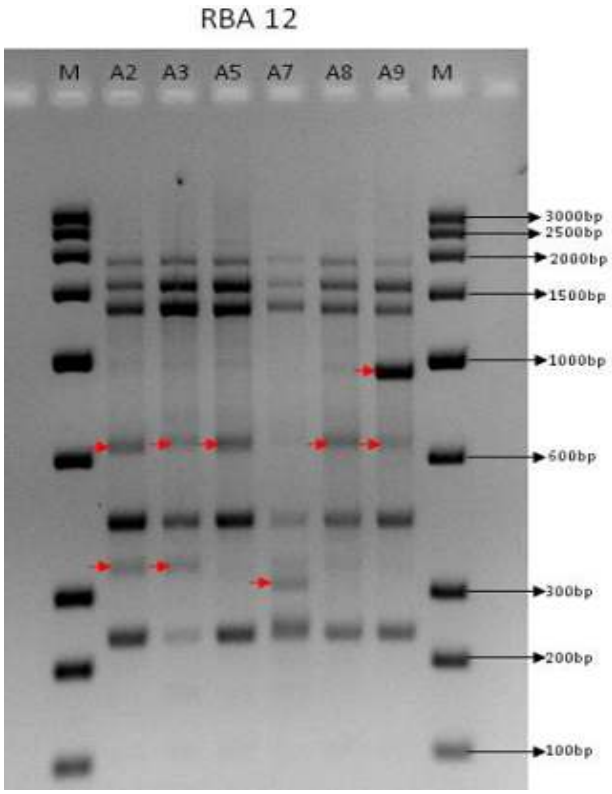


Figure 5.8: RAPD profile with RBA14

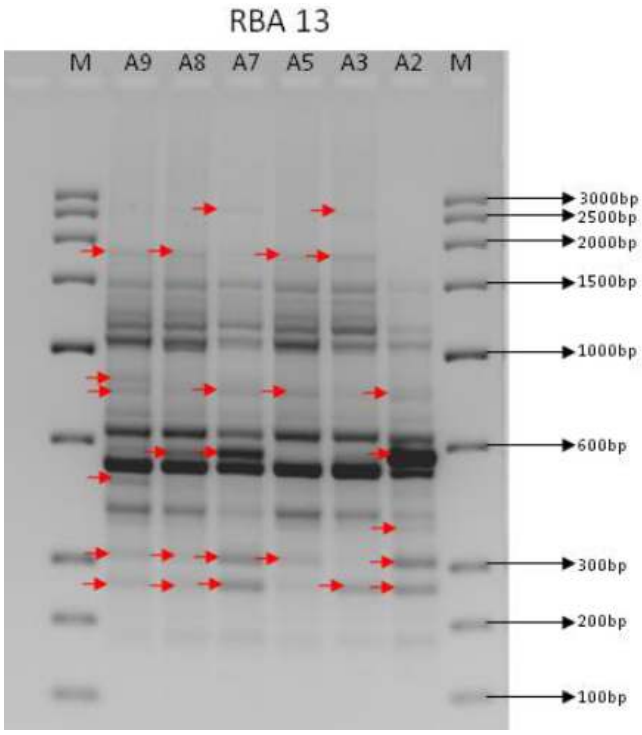


Figure 5.9: RAPD profile with RBA13

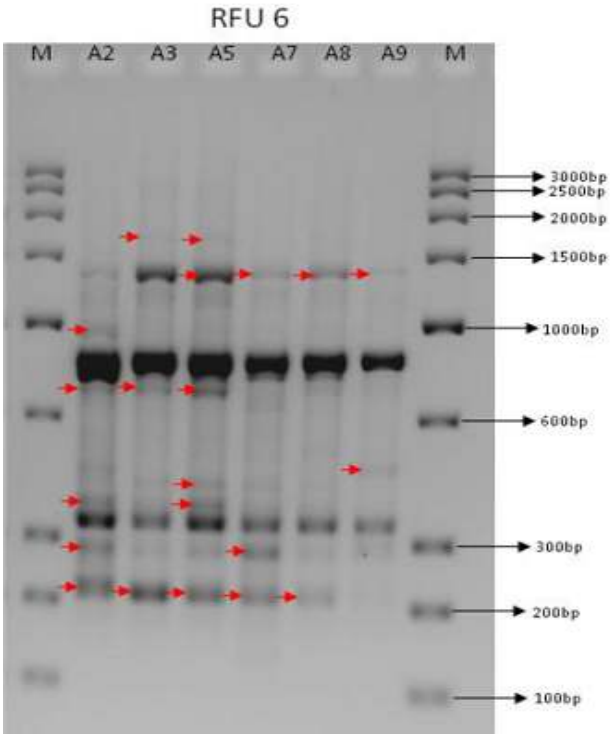


Figure 5.10: RAPD profile with RFU6

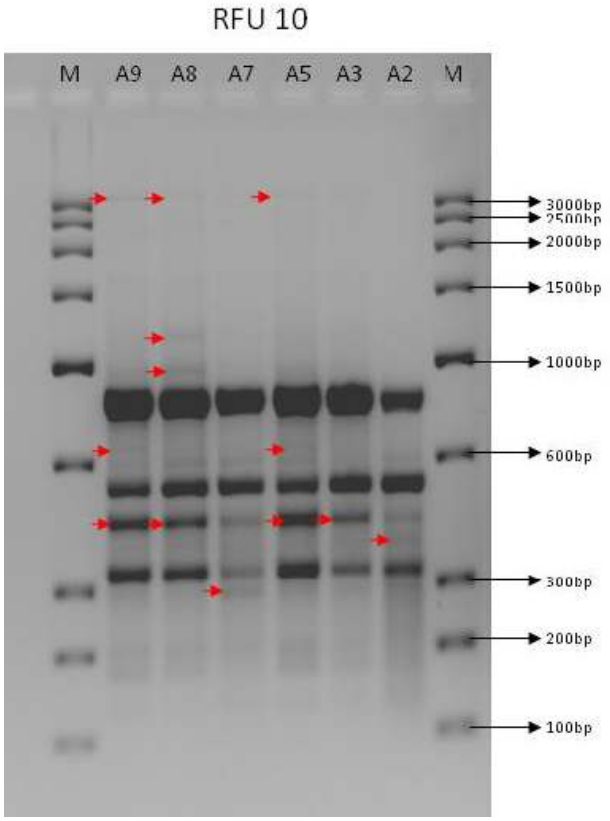


Figure 5.11: RAPD profile with RFU10

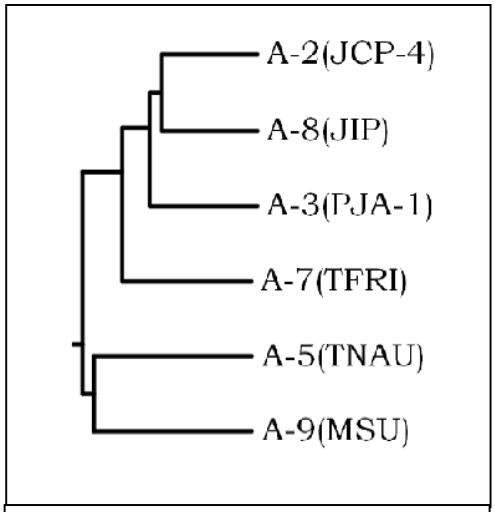


Figure 5.12 Phylogenetic tree analysis

Table 5.8 Jaccard's similarity matrix of 7 *Jatropha curcas* accessions

	JCP-4	PJA-1	TNAU	TFRI	JIP-12	MSU
JCP-4	1	0.585	0.439	0.566	0.667	0.418
PJA-1		1	0.414	0.509	0.667	0.368
TNAU			1	0.397	0.345	0.434
TTFRI				1	0.549	0.375
JIP-12					1	0.423
MSU						1

Table 5.9 Distance matrix based on Jaccard coefficient

	JCP-4	PJA-1	TNAU	TFRI	JIP-12	MSU
JCP-4	0	0.415	0.561	0.434	0.333	0.582
PJA-1		0	0.586	0.491	0.333	0.632
TNAU			0	0.603	0.655	0.566
TFRI				0	0.451	0.625
JIP-12					0	0.577
MSU						0

Table 5.10 Distance matrix based on RMSD coefficient

	JCP-4	PJA-1	TNAU	TFRI	JIP-12	MSU
JCP-4	0	0.549	0.662	0.561	0.468	0.662
PJA-1		0	0.682	0.608	0.468	0.702
TNAU			0	0.692	0.721	0.641
TFRI				0	0.561	0.692
JIP-12					0	0.641
MSU						0

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ORIGINAL PAPER

GA application induces alteration in sex ratio and cell death in *Jatropha curcas*

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Abstract *Jatropha curcas* L. is a commercially important plant with biodiesel and medicinal potential. It is a monoecious plant with staminate and pistillate flowers in same inflorescence with number of staminate flowers being higher than pistillate ones resulting in very low fruit yield. Altering sex ratio to increase the number of female flowers would lead to better yield. Phytohormones are most important factors known to alter sex ratio in plants. The mechanism by which phytohormones alter sex ratio differs in different plant species. Among phytohormones, GA plays an important role in sex alteration. In this study, we report the effect of exogenous application of GA on sex modification in *J. curcas*. There was considerable increase in number of female flowers by application of GA. At lower concentrations (10 and 100 ppm), increase in number of female flowers and fruit yield was proportionate to the concentration of hormone used but at higher concentration (1,000 ppm) though there was an increase in number of female flowers, fruit yield decreased. This was due to an increased peduncle length and enhanced cell death as a consequence of endogenous release of hydrogen peroxide in response to increased GA, resulting in withering of fruits.

Keywords Gibberellin (GA) · ppm—parts per million · Flower sex ratio · Hydrogen peroxide · Fruit fall · Cell death

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Introduction

Jatropha curcas is a plant with tremendous untapped potential as a source of biodiesel. It is a monoecious shrub with staminate (male) and pistillate (female) flowers being present on same inflorescence. A typical inflorescence has more male flowers as compared to female flowers, average male to female flower ratio being 29:1 (Solomon Raju and Ezradanam 2002). A change in flower sex ratio towards a greater number of female flowers could result into greater seed yield and hence greater oil yield. This would improve *J. curcas* potential as a biofuel crop. Various factors regulate floral sex expression in plants (Irish and Nelson 1989; Dellaporta and Calderon-Urrea 1994; Stehlik et al. 2008). Among these factors, phytohormones play a very important role (Ghosh and Basu 1983; Durand and Durand 1991). Of various phytohormones, role of Auxin, Ethylene and Gibbrellin (GA) in regulation of flower sex expression has been widely studied and is documented in recent studies (Tanimoto 2007; Thomas 2008; Salman-Minkov et al. 2008). Effect of exogenous phytohormones on flower sex ratio depends on species of plant. GA has been shown to promote female flowers in maize and castor bean, on the contrary it increases masculine features in hemp, spinach and cucumber (Lazarte and Garrison 1980; Pharis and King 1985). In some plant species such as Arabidopsis and Tomato, GA deficiency leads to male sterility because of abnormal anther development (Nester and Zeevaart 1988; Goto and Pharis 1999).

Though most studies have reported an increase in fruit yield in response to GA, Almeida et al. (2004), have reported decreased fruit yield in response to GA in oranges. They have attributed this to increased fruit withering as a result of endogenous release of hydrogen peroxide (H_2O_2) at the peduncle. GA has been shown to be responsible for

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inducing cell death by enhancing the ethylene biosynthetic pathway (Steffens and Sauter 2005). Programmed Cell Death (PCD) is a genetically defined process and is an integral part of plant ontogenesis. It is associated with morphological and biochemical changes (Jong et al. 2002). PCD plays key role in embryo development, formation and maturation of many cell types and tissues, and plant reaction/adaptation to the environmental conditions. It also occurs during terminal differentiation, formation of vessel members and tracheary elements, lysigenous aerenchyma formation, and aleurone degradation (Alan 2001; Gunawardena 2008).

Here, we report the role of GA in inducing sex alteration and cell death in *J. curcas*. GA at lower concentration alters sex ratio of flowers in favor of female flowers, resulting in high fruit yield. However, at higher concentration, increase in number of female flowers did not translate into fruit yield due to H_2O_2 mediated cell death.

Materials and methods

Experimental plants

Fifteen months old *J curcas* plants of Vishwamitri Railway Colony plantation, Vadodara, India, were selected for experiments. Gibberellic acid (GA) at three different concentrations 10 ppm, 100 ppm and 1,000 ppm were used. Solutions were prepared by dissolving GA in small volume of isopropyl alcohol and final volume was made up with demineralized water after adjusting pH to 7.5–7.8. A few drops of surfactant were added to the solution. Plants sprayed with demineralized water containing only surfactant were considered as control. The selected time of spray was early morning hours and spraying was initiated from the time foliar bud emerged. Each inflorescence received three sprays of equal volume of solution at an interval of 5 days. Ten plants were taken per treatment. Total number of flowers and sex ratio were calculated 1 week after the last spray whereas fruit yield was measured 1 month after the last spray. Test and control plants were tagged with appropriate labels to follow flower development till about one and half months. Fruit yield was observed for 3 months at an interval of 1 month. After each treatment, dried seeds were collected and weighed (Results reported are average of 20 seeds of each group).

Determination of H_2O_2

H_2O_2 extraction and estimation from plant tissues was carried out as described by Zhou et al. (2006). Fresh leaves (0.5 gm) were frozen in liquid nitrogen and ground to powder in a mortar with pestle, along with 5 ml of 5% TCA and 0.15 gm activated charcoal. This mixture was then centrifuged at 10,000 g for 20 min at 4°C. The supernatant was recovered and its pH was adjusted to 8.4 with 17 M ammonia solution before subjecting to filtration. The resultant filtrate was used to determine H_2O_2 levels. The reaction mixture containing 1 ml filtrate and 1 ml colorimetric reagent was incubated for 10 min at 30°C. In a similar way, 1 ml of distilled water was used as a blank instead of filtrate. Absorbance at 505 nm was determined spectrophotometrically.

Cell death study

Thin section of stem (peduncle part) was stained with Evans blue stain and observed under 40× objective of the light microscope. Evans blue stained only dead cells which appeared blue in color while live cells reduced the stain to a colorless form.

Statistical analysis

All the values are reported as mean ± S.E.M. The statistical significance was obtained by using Student's t-test.

Results and discussion

GA treatment, in a concentration dependent manner, resulted in an increase in total number of flowers and

Table 1 Effect of GA on flower sex ratio in *Jatropha curcas*

GA treatment	Number of plants treated	Number of inflorescences observed	Total flowers in inflorescences	Total male flowers	Total female flowers	Ratio female: male flower
Control	10	50	4220.9 ± 0.378	4040 ± 8.432	172.1 ± 0.481	1:24
10 ppm	10	50	5023.7 ± 0.471***	4791 ± 91.62***	232.7 ± 0.472***	1:21
100 ppm	10	50	6234.2 ± 0.489***	5846 ± 19.65***	388.9 ± 0.526***	1:15
1,000 ppm	10	50	6683.9 ± 0.481***	6193 ± 33.90***	490.1 ± 0.566***	1:13

Equal volume of GA was sprayed at three different concentrations 10, 100 and 1,000 ppm. Three such sprays were given at an interval of 5 days each. Numbers of flowers are calculated after the end of third spray. Values are mean ± SE, *** indicates significantly different at $P < 0.001$ as compared to the corresponding control

Table 2 Effect of GA on time scale of inflorescence development in *Jatropha curcas*

GA treatment	Duration (in days) for inflorescence development					
	Initial appearance of floral bud	Distinct appearance of flower bud	Distinct male and female flower bud	Opening of male flowers	Opening of female flowers	Complete bloom
Control	9.00 ± 0.577	6.333 ± 0.333	4.667 ± 0.333	6.160 ± 0.333	9.467 ± 0.333	36.20 ± 1.068
10 ppm	5.60 ± 0.509***	5.80 ± 0.583***	3.40 ± 0.748***	6.40 ± 0.748***	6.60 ± 0.812***	27.80 ± 0.812***
100 ppm	5.00 ± 0.447***	4.80 ± 0.374***	3.40 ± 0.509***	6.10 ± 0.871***	5.60 ± 1.02***	24.90 ± 1.02***
1,000 ppm	4.20 ± 0.374***	3.60 ± 0.374***	3.20 ± 0.583***	5.60 ± 1.020***	5.20 ± 1.249***	21.80 ± 1.249***

Emergence of floral bud was considered as day zero, Values are mean ± SE, *** indicates significantly different at $P < 0.001$ as compared to the corresponding control

Table 3 Effect of GA on peduncle length in *Jatropha curcas*

Treatment	Female flower (cm)	Male flower (cm)
Control	0.47 ± 0.18	0.55 ± 0.08
10 ppm	1.25 ± 0.14*	0.75 ± 0.14
100 ppm	3.25 ± 0.14***	1.16 ± 0.08*
1,000 ppm	3.90 ± 0.20***	1.41 ± 0.22*

Length of peduncle in flower starts to increase immediately after first spray. The length of peduncle was measured after the end of third spray. Values reported are mean ± SE; *, *** indicates significantly different at $P < 0.05$ and $P < 0.001$, respectively as compared to control ($n = 20$)

increased ratio of female: male flowers in all groups, highest effect being observed in plants treated with GA at 1,000 ppm (Table 1). The total number of flowers increased by 58% on 1,000 ppm GA treatment. Number of female flowers increased by almost three fold (Table 1) and this reflected in an appreciable and desirable increase in ratio of female: male flowers from 1:24 to 1:13. The role of GA in regulation of flowering has been well studied and molecular events involved have been deciphered. GA regulates the development of flowers by activation of LFY and AP1 genes. GA activates floral meristem LFY signal which up regulates AP1 promoter, responsible for flowering (Jack 2004). Thus, from this study it can be concluded that GA can be used as a potent phytohormone to increase flowering and seed yield from Jatropha plants.

Apart from increasing total number of flowers and female: male ratio, GA also hastened the process of flower development. Application of GA at 1,000 ppm, decreased

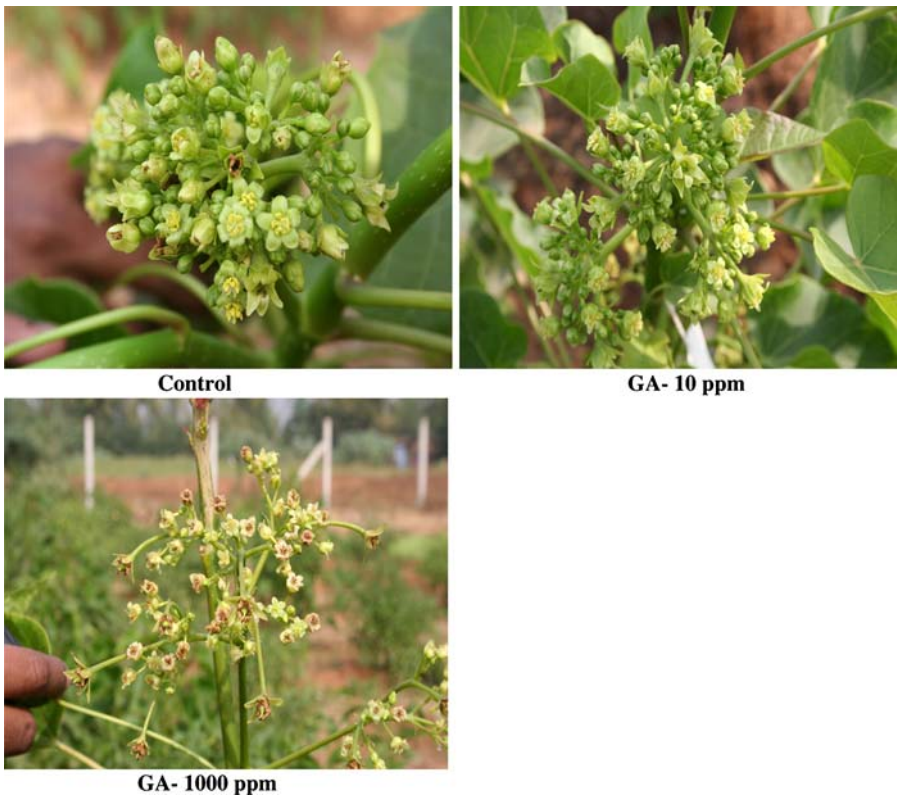

Fig. 1 Effect of GA on peduncle length in *Jatropha curcas*

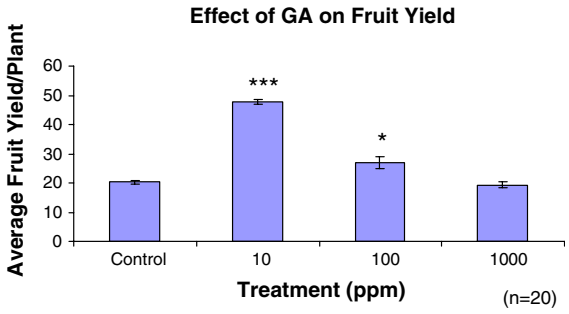

Fig. 2 Effect of GA on Fruit yield in *Jatropha curcas*. Numbers of fruits are calculated 1 month after third GA spray. Values represented is the mean of 20 replicates and bars indicate SE, *, *** indicates significantly different at $P < 0.05$, and $P < 0.001$, respectively as compared to control ($n = 20$)

Table 4 Effect of GA on endogenous release of Hydrogen peroxide in *Jatropha curcas*

Treatment	Hydrogen peroxide (μM/gm)
Control	8.738 ± 0.3287
10 ppm	10.61 ± 0.5916
100 ppm	13.86 ± 0.7699*
1,000 ppm	77.87 ± 1.267***

Hydrogen peroxide is determined by Zhou et al. (2006). Values are mean ± SE; *, *** indicates significantly different at $P < 0.05$ and $P < 0.001$, respectively as compared to the corresponding control ($n = 4$)

duration of flower development from 36 to 21 days as shown in Table 2. In GA treated plants, initial three stages of flower development were faster when compared to control. Time required for the entire process of flower development was shortened by nearly 12 days. Thus, GA not only increased flowering but also enhanced the rate of flower development.

Other obvious morphological change brought about by GA application was a pronounced increase in length of peduncle. In plants treated with 100 and 1,000 ppm GA, the length of peduncle of female flowers increased by 7 and 8 folds, respectively (Table 3); whereas the increase in peduncle length of male flowers was only 2 and 3 fold. This showed that GA effect on peduncle length was more prominent in female flowers compared to male flowers. GA at a lower concentration (10 ppm) showed negligible effect on peduncle length (Fig. 1). However, increase in length of peduncle was accompanied by greater withering of immature fruits. It was observed that there was higher fruit fall in plants treated with 100 and 1,000 ppm of GA as compared to 10 ppm. As a consequence when fruit yield was calculated, the highest yield was from plants treated with 10 ppm GA (Fig. 2). It has been reported that fruit fall is a consequence of senescence and programmed cell death and one of the important initiators of senescence is endogenous release of H_2O_2 (Strother 1988). There have been several reports which have demonstrated that GA treatment leads to release of endogenous hydrogen peroxide, a factor responsible for cell death (Bethke et al. 1999; Steffens and Sauter 2005). Bethke et al. (1999) have demonstrated the role of GA in accelerating ethylene induced cell death through generation of H_2O_2. However, the common signaling pathway between GA and ethylene leading to cell death has not been elucidated. Jong et al. (2002) have proposed that ethylene acts as a cofactor for H_2O_2 production by affecting phosphorylating signals which are involved in NADPH oxidase activation and catalase inhibition. Based on this premise, we estimated the levels of endogenous H_2O_2 to correlate PCD and fruit withering (Table 4). Plants treated with 1,000 ppm GA shows 10 times higher endogenous release of H_2O_2 compared to control, while no appreciable change was seen in plants treated with 10 ppm GA. Increased level of H_2O_2 is responsible for metabolic release of reactive oxygen species which leads to senescence resulting in cell death. Evans blue staining of sections from plants treated with 1,000 ppm GA showed an approximate 50% cell death (Fig. 3). H_2O_2 has been implicated in cell death by acting as a diffusible factor responsible for initiation of programmed cell death (Jong et al. 2002).

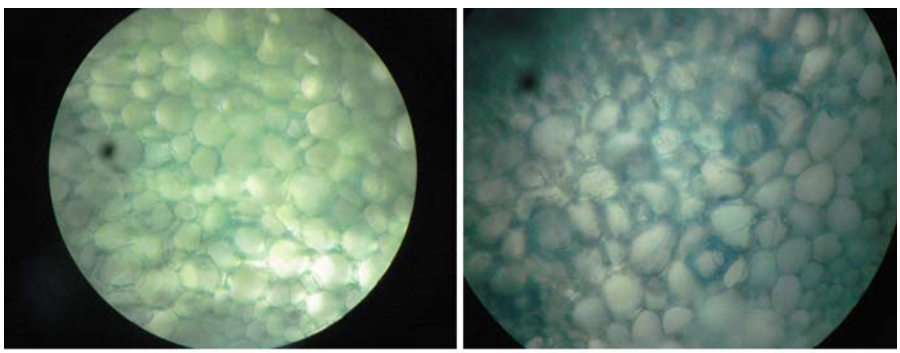

Fig. 3 Effect of GA on cell death in *Jatropha curcas*. Thin section of peduncle was stained with Evans blue stain and observed under 40× objective of light microscope. Evans blue stains only the dead cell which appears blue in color. In the living cells the dye is reduced to a colorless form

In summary, GA treatment of *Jatropha curcas* foliar bud increases the number of female flowers and also hastens the flower development. Higher number of female flowers at 1,000 ppm of GA did not translate into high fruit yield due to increased withering of immature fruits. Withering of fruits could presumably be due to cell death mediated by H_2O_2 release at the zone of abscission and greater peduncle length. If inhibiting release of H_2O_2 is realized, it would translate to an increase in female flowers and hence fruit yield. It would be a huge leap in per hectare production of *Jatropha* ensuring its commercial success. *Jatropha curcas* then can truly be an apt source of biodiesel.

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Comparing potential of GA and 2, 4-D in increasing fruit yield from Jatropha curcas

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ABSTRACT

Jatropha curcas L. has been accepted world wide as a plant with tremendous commercial importance as a source for biodiesel. It is a monoecious plant with staminate and pistillate flowers on same inflorescence. The number of staminate flowers is higher than pistillate ones resulting in very low fruit yield. Altering the sex ratio, to increase number of female flowers would lead to better yield. Phytohormones are one of the most important factors known to alter sex ratio in plants. The mechanism by which phytohormones alter sex ratio differs in different plant species. Earlier studies from our laboratory have shown effect of exogenous application of GA and 2, 4-D on flowering patterns in Jatropha. Here we report a comparative study of the effect of exogenous application of GA and 2, 4-D (50 and 100 ppm) on flower sex modification in Jatropha curcas. There was considerable increase in total number of flowers and female: male flower ratio by application of GA and 2, 4-D. However, at higher concentration of GA a negative result as far as fruit yield is considered was seen. The reasons for this are reported here. 2, 4-D on the other hand increased the ratio of female flowers in a concentration dependent fashion. Of the four treatments reported here, we have observed that 2, 4-D (100 ppm) shows better results than GA and untreated plants. The reasons for this are discussed.

Keywords: Gibberellin (GA), 2, 4-Dichlorophenoxyacetic acid (2, 4-D), ppm - parts per million, Flower sex ratio.

1. INTRODUCTION

India is one of the highest petroleum consuming nations in the world. India's economy has often been unsettled by its need to import petroleum. This coupled with deleterious effects of increased use of fossil fuels on the environment makes it imperative for us to find an environment friendly replacement. Bio-fuels, has been considered as a viable option to replace fully or partially India's dependence on fossil fuels. *Jatropha curcas* is a plant with tremendous untapped potential as a source of biodiesel. It is a monoecious shrub with staminate (male) and pistillate (female) flowers being present on same inflorescence. The inflorescence has more male flowers as compared to female flowers; the average male to female flower ratio being 29:1 [1]. This ratio leads to a very poor seed yield. A change in flower sex ratio towards a greater number of female flowers could increase seed yield and hence oil yield making the plant a commercially viable option. Phytohormones are important factors regulating floral sex expression in plants [2, 3, 4, and 5]. Among phytophormones the role of Auxin, Ethylene and Gibberellin (GA) has been widely studied and is documented in recent studies [6, 7, and 8]. The effect of exogenous phytohormones on flower sex ratio depends on species of plant. GA has been shown to promote female flowers in maize and castor bean, but on contrary it increases masculine features in hemp, spinach and cucumber [9, 10]. In several plant species such as Arabidopsis and Tomato, GA deficiency leads to male sterility because of abnormal anther development [11, 12]. Though most studies have reported an increase in fruit yield in response to GA, Almeida et al. [13], have reported decreased fruit yield in response to GA in oranges.

They have attributed this to increase immature fruit fall as a result of increased length of peduncle. Here, we report the role of GA and 2, 4-D in altering the number of flowers, female: male flower ratio, fruit yield and oil yield. The results reported here would have a significant commercial implication for biodiesel production.

2. MATERIALS AND METHODS

2.1. EXPERIMENTAL PLANTS

Young *Jatropha curcas* plants (15 months old) were selected for experiments. Gibberellic acid (GA) and 2, 4-D (2, 4- Dichlorophenoxyacetic acid) at two different concentrations of 50 ppm and 100 ppm were used. Solutions were prepared by dissolving phytohormone in small volume of isopropyl alcohol and final volume made up with demineralized water after adjusting the pH to 7.5-7.8. A few drops of Tween-20 were added to solution as surfactant. Plants were first sprayed at stage of foliar bud emergence. Plants sprayed with demineralized water containing only the surfactant were treated as control. The selected time of spray was early morning hours. Equal numbers of sprays per inflorescence were given for three times keeping an interval of five days between each spray. Approximately 5-10 ml of solution was used per inflorescence. Five plants were taken per treatment. Total flower sex ratio and flower number was calculated one week after last spray whereas fruit yield was calculated one month after last spray. Peduncle lengths were measured after first spray till fruit developed. Fruit yield were observed three times at interval of one month. Seeds were dried to constant weight. Twenty seeds were pooled together in each group for seed weight determination.

2.2. OIL AND FATTY ACID ANALYSIS

Dry mature seeds were analyzed for oil content after removal of seed coat using the method of Bligh and Dyer, [15]. Fatty acid profile of oil was determined by Gas Chromatography equipped with SS packed column and flame ionization detector.

2.3. STATISTICAL ANALYSIS

The data obtained was subjected to student's t-test. All values reported are mean $\pm$ S.E.

3. RESULTS AND DISCUSSION

Fifteen months old plants were used for this study. The foliar buds of the plants were sprayed thrice at an interval of 5 days with GA and 2, 4-D at two different concentrations (50 and 100 ppm) alone. Observations were made till the fruit development was complete. GA and 2, 4-D treatment resulted in an increase in total number of flowers in a concentration dependent fashion. GA at 50 and 100 ppm resulted in an increase in total number of flowers by 15% and 42 % respectively (Table 1). There was a negligible increase in flower number with 2, 4-D at 50 ppm while plants treated with 2, 4-D 100 ppm showed best result with an increase of 52%. The role of GA in regulation of flowering has been well studied and molecular events involved have been deciphered. It has been shown that GA regulates the development of flowers by activation of LFY and AP1 genes. GA activates the floral meristem LFY signal which up regulates AP1 promoter which results in flowering [16]. Thus from this study, it can be concluded that GA and 2, 4-D can be used as a potent phytohormone to increase yields from Jatropha plants.

The other obvious morphological change brought about by GA application was a pronounced increase in length of peduncle (Figure 1). This effect was more in peduncle of female flowers which showed an increase of 4 and 6 folds in plants treated with 50 and 100 ppm GA as compared to control (Figure 2). The peduncle of male flower also increased in length but increase was only 1 to 2 folds respectively at 50 and 100 ppm

Table 1. Effect of GA and 2, 4-D on flower number and sex ratio in *Jatropha curcas*

Treatment (ppm)	Number of in florescences observed	Total flowers in inflores-cences	Total Male flowers	Total Female flowers	Ratio Female:Male flower	Percent increase in total flowering
Control	20	2050 ±14.23	1970 ± 13.68	80 ± 0.55	1:25	—
GA (50)	20	2358 ± 8.05***	2226 ± 7.48***	132 ± 0.57***	1:17	15 %
GA (100)	20	2919 ± 11.83***	2749 ± 11.12***	171 ± 0.71***	1:16	42 %
2, 4-D (50)	20	2136 ± 4.489***	2025 ± 4.33***	112± 0.21***	1:18	4 %
2, 4-D (100)	20	3116 ± 12.09***	2920 ± 11.24***	196 ± 0.85***	1:15	52 %

Equal volume of GA and 2, 4-D was sprayed at two different concentrations 50 and 100 ppm. Three such sprays were given at an interval of 5 days each. Numbers of flowers are calculated after the end of third spray. Five plants were used in each treatment. Values are mean ± SE, *** indicates significantly different at $p<0.001$ as compared to the corresponding control.

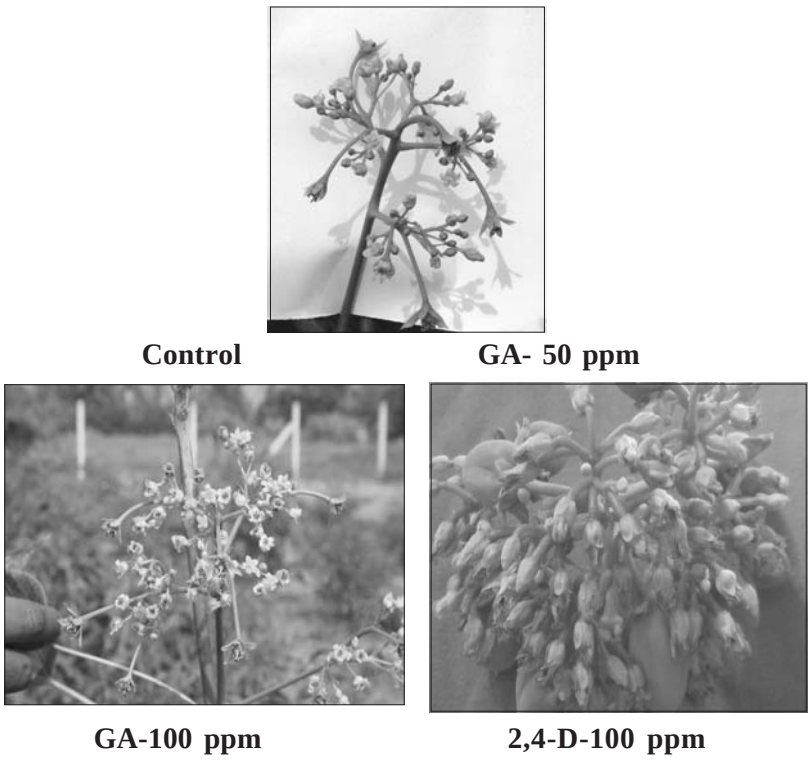
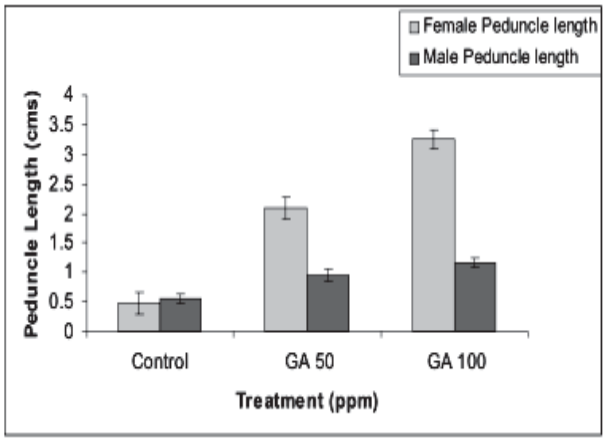


Figure 1. Effect of GA and 2, 4-D on peduncle length in *Jatropha curcas* The length of peduncle was measured after the end of third spray.



n=5

Figure 2. Effect of GA on peduncle length of *Jatropha curcas* Length of peduncle in flower starts to increase immediately after first spray. The length of peduncle was measured after the end of third spray. Values reported are mean ± SE; *, * indicates significantly different at p<0.05 and p<0.001 respectively as compared to control.**

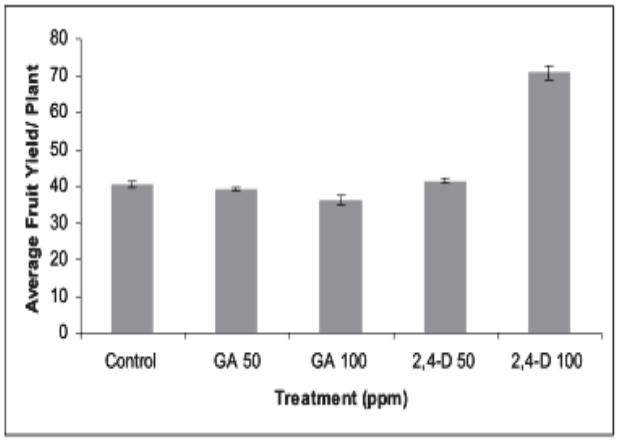


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Figure 3. Effect of GA and 2, 4-D on Fruit yield of *Jatropha curcas* Numbers of fruits are calculated one month after third GA and 2, 4-D spray. Values represented is the mean of 5 replicates and bars indicate SE, **, * indicates significantly different at p<0.01, and p<0.001 respectively as compared to control.**

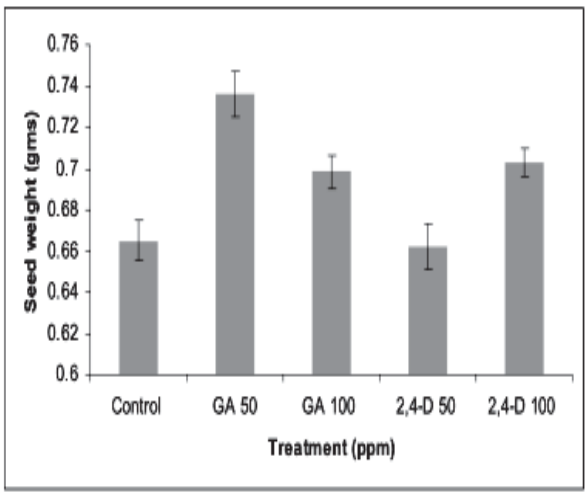
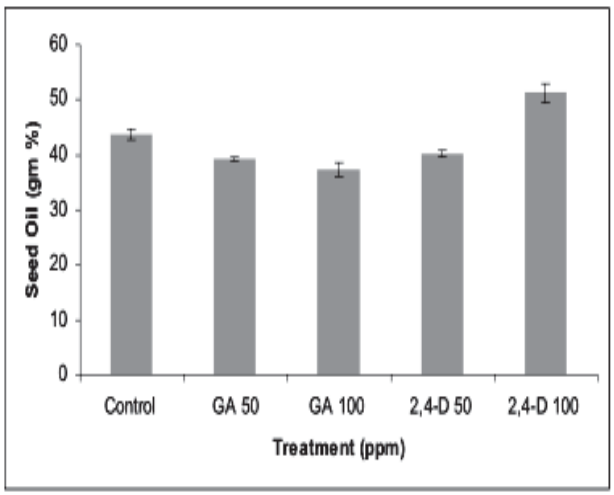


Figure 4. Effect of GA and 2, 4-D on Seed weight of *Jatropha curcas*
Dried seeds of each treatment were weighed (Results reported are average of 20 seeds of each group). Values are mean ± SE; *, ** indicates significantly different at p<0.05 and p<0.001 respectively as compared to the corresponding control.



n=5

Figure 5. Effect of GA and 2, 4-D application on Seed Oil yield of *Jatropha curcas* Dry mature seeds were analyzed for oil content by the method of *Bligh and Dyer* (1959). Values are mean ± SE; *, ** indicates significantly different at p<0.05 and p<0.001 respectively as compared to the corresponding control.

treatments of GA as compared to control. The flip side of an increase in length of peduncle was greater withering of fruits. It was observed that there was higher fruit fall in plants treated with 100 ppm of GA as compared to 50 ppm. Increased length of peduncle leads to weaker peduncle and it causes withering of fruits before it matures. Fruit fall is a process of senescence which is regulated by ethylene. However, there is a good crosstalk between GA and Ethylene which mediates the senescence in plant [17].

A consequence of increase peduncle length was fruit fall. When fruit yield was calculated the highest yield was from plants treated with 100 ppm 2, 4-D as compared to 50 and 100 ppm GA (Figure 3). 2, 4-D 100 ppm shows significant good results as compared to 2, 4-D 50 ppm. Also, there was no significant change in seed weight with GA and 2 4-D 100ppm treatments when compared with control. Seed weight was significantly higher in GA 50 ppm (Figure 4). The oil content in seeds showed appreciable decrease with GA treatment indicating that the fatty acid synthesis pathway is also not up regulated. The oil content in the seeds increases with 2, 4-D 100 ppm (Figure 5), which means that 2, 4-D 100 ppm increases the flux of fatty acid pathway. In conjunction with oil content, the fatty acid profile did not change by phytohormones application (Table 2).

Table 2. Effect of GA and 2, 4-D on Fatty Acids composition of *Jatropha curcas* seed oil

Fatty Acids composition	Control	GA (50 ppm)	GA (100 ppm)	2,4-D (50 ppm)	2,4-D (100 ppm)
Myristic acid	0.29	—	—	0.24	—
Palmitic acid	11.29	10.41	11.62	13.28	12.28
Palmitoleic acid	0.70	0.58	0.68	0.78	0.92
Stearic acid	4.56	3.89	4.23	4.80	4.51
Oleic acid	45.15	45.44	46.39	48.83	49.13
Linoleic acid	37.81	39.35	36.92	31.74	32.75
Linolenic acid	0.20	0.18	0.16	0.19	0.25

Fatty acid analyse from seed oil by Gas Chromatography. Values represents are g (%).

4. CONCLUSION

GA and 2, 4-D treatment on *Jatropha curcas* foliar bud increases the number of female flowers and fruit yield. GA at higher treatment increases female flowers however it did not result in correspondingly high fruit yield due to increased withering of immature fruits. Withering of immature fruits could be due to greater peduncle length. Using inhibitors to decrease peduncle length may help in capitalizing on the increase number of female flowers. 2, 4-D at higher concentration increases the number of flowers and fruit yield. Applying the results of such studies to the field could help to increase the potential of *Jatropha curcas* as a bio-fuel crop.

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Synopsis of the thesis on

Biotechnological approach for Improvement of Oil content in *Jatropha curcas*

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Introduction

Jatropha curcas is a shrub belonging to Euphorbiaceae family. It has been identified as a biofuel crop for its seed oil content and properties. It is a multipurpose crop as it not only serves as a 'crop of choice' for biodiesel production but it also possesses many medicinal values. It also holds promise as an agro-forestry crop since it finds its use in control of floods, soil erosion, nutrient leaching etc. The potential of Jatropha has not been fully realized and still a lot remains to be achieved. *Jatropha curcas* is the most primitive species of the genus and forms artificial and natural hybrid complexes readily and hence poses a problem to the genetic fidelity (Dehgan, 1984; Prabhakaran and Sujatha, 1999). In nature, it propagates through seeds but the seed yields are very low. Limitations like discrepancy in its germplasm, a large variation in oil content (22-44%), unavailability of quality planting material etc makes its large scale cultivation difficult. To make *Jatropha curcas* an economically viable substitute of fossil fuels it is important that elite plant material (one with high oil content) be made available in large numbers for cultivation. Another approach could be to increase seed oil through biotechnological methods. In either case, there is a need to have a successful regeneration protocol. Various attempts have been made towards this (Sujatha and Dhingra, 1993; Sujatha and Mukta, 1996; Sujatha and Reddy, 2000; Sujatha et al., 2005; Deore and Johnson, 2008). A few studies on direct shoot morphogenesis, callus mediated regeneration or somatic embryogenesis in *Jatropha curcas* have been reported (Jha et al., 2007). Though reports show that *Jatropha curcas* can be efficiently propagated the hunt for a perfect protocol continues. In order to undertake regeneration studies the first step would be identification of elite variety of *J.curcas.* One of the methods of identification of elite varieties is to use seed oil as a criterion. Variation in seed oil content of naturally existing varieties could be studied through various molecular marker techniques, one of which is, RAPD (Random Amplification of Polymorphic DNA) (Gupta et al., 2008, Ganesh Ram et al., 2007). Basha and Sujatha 2007 have reported the use of RAPD in studying genetic diversity of *Jatropha* species). RAPD profiles so generated upon comparison would enable in concluding the nature of the oil trait in *J.curcas.* Majority of the oil is present in the form of Triacylglycerol (TAG). The enzyme catalyzing the formation of TAG from DAG (Diacylglycerol) is Diacylglycerol acyl transferase (DGAT). DGAT catalyses the committed step in the biosynthesis of TAG. Genetic manipulation like over-expression of DGAT could increase the accumulation of TAG in seeds.

Similar reports on over-expression studies have been reported in Arabidopsis by Jako et al., 2001.

In light of the above introduction the following objectives were proposed for this study:

Objective 1: Selection of elite varieties of *Jatropha curcas*

- By Seed oil estimation
- By study of genetic diversity using RAPD

Objective 2: In-vitro propagation of elite material

- Through axillary bud proliferation
- Through somatic embryo
- Indirect organogenesis through leaf disc

Objective 3: Target fatty acid biosynthetic pathway to increase oil yield in seeds.

- Standardization of agrobacterium mediated transformation
- Over-expression study of DGAT

1) Selection of elite varieties of *J. curcas*

- **Seed oil estimation:**

The oil content in *Jatropha curcas* seeds have been reported to be highly variable (22-45%). The high oil yielding varieties coexist with the low yielding ones. Identifying high oil yielding varieties would help to propagate such varieties. Seed oil estimation was performed by Soxhlet apparatus and microanalysis method as reported by Bligh and Dyer (1959). Seed samples from various locations in Gujarat were collected and their seed oil was estimated. Variations in seed oil content were observed and very high oil yielding varieties were not obtained. The average seed oil yield was observed to be 35 gm%.

- **Study of genetic diversity of *J.curcas* by RAPD:**

There is virtually no information with regard to the number of introductions and the genetic diversity of *J. curcas* populations grown in India. Several researchers have attempted to define the origin of *J. curcas*, but the source remains controversial (Dehgan and Webster 1979; Heller 1996). Three distinct varieties are reported viz., the Cape Verde variety that has spread all over

the world, the Nicaraguan variety with few but larger fruits and a non-toxic Mexican variety devoid of phorbol esters (Henning 2006). There are no named varieties of *J. curcas* in India with the exception of the variety SDAUJ1 (Chatrapathi) that was released during the year 2006 based on selection from local germplasm (Sujatha et al., 2007). Hence the need for studies on the varieties of *J.curcas* using molecular markers becomes imperative. In our state of Gujarat too numerous studies on *J. curcas* plantations have been done but molecular studies have not been reported. The conservation and sustainable use of plant genetic resources require accurate identification of their accession (Arif et al., 2010). In this study an attempt has been made to study the genetic diversity existing in *J. curcas* accessions of Gujarat region. The study of genetic diversity with the help of molecular markers can be broadly classified into phenotypic markers, biochemical markers and molecular markers. Of these, molecular markers are more promising as any change in the protein sequence would be brought about by a mutation in its DNA sequence. DNA markers are not typically influenced by environmental conditions and therefore can be used to help describe patterns of genetic variation among plant populations and to identify duplicated accessions within germplasm collections (Ganesh Ram et al., 2008). Molecular markers can be studied by techniques based on polymerase chain reaction (PCR) and non-polymerase chain reaction. RFLP (Restriction Fragment Length Polymorphism) dominates the non-PCR based techniques. This technique is time consuming and highly expensive. Among the PCR based techniques, RAPD is generally a preferred method. This is more so when the genome to be studied is unknown. The advantage it has over other molecular techniques is that it is less time consuming, more cost effective and the starting material (genomic DNA) requirement is low. Reports have shown that RAPD analysis can be used to detect variation within a restricted range, to identify suitable parents for linkage map construction, and for gene tagging for drought resistance (Virk et al., 1995)

The prerequisite for RAPD is good quality DNA. DNA isolation from plant source is at times difficult owing to the large concentration of polysaccharide, protein, pigment or phenolic compounds. Some plant taxa may not permit optimal DNA yield. Closely related species of the same genus also may require different isolation protocols. Thus, an efficient protocol for isolation of DNA as well as the optimization of the PCR conditions is required (Subramanya et al., 2009). The available procedures for successful DNA isolation, could be distinguished into, one which uses liquid nitrogen for crushing the material and other that uses detergent (CTAB-

cetyltrimethyl ammonium bromide) to break open the cells. In the current study CTAB method was followed. The method given by Keb Llanes et al., 2002 has been used. Good quality DNA of *J.curcas* was not obtained by following the afore-mentioned protocol and hence certain modifications were made to the existing protocol. The various modifications tried are: Use of varying concentration of CTAB (2%, 3% and 4%); varying duration of heat shock; varying extraction buffer components; introduction of chloroform : isoamyl treatment and use of different tissues. In this study 2% CTAB gave a good yield. Incorporation of PVP (polyvinylpyrrolidone), beta-mercaptoethanol and ascorbic acid in extraction buffer A as mentioned in the reported protocol did not help in obtaining good quality DNA. When a buffer devoid of these was used with introduction of Chlorofom:Isoamyalcohol step (24:1) a better yield and quality of DNA was obtained. Reddy et al., 2009 studied the effect of varied time of heat treatment on total DNA obtained per gm tissue in *Jatropha curcas* and yield of DNA per gm of tissue from different tissues. In this study, too the time duration of heat shock was varied (10, 30, 50, 70, 90, 110, 120 minutes). Increased DNA yield with increasing treatment time till 50 minutes was seen. Three different tissues were used for DNA isolation namely axillary buds, leaves and petioles. It was found that petiole served to be better tissue with high quality DNA. DNA was quantified by taking spectrophotometric reading at 260nm.The DNA yield was found to be nearly 350ug/gm fresh weight of tissue. DNA integrity was checked by observing under UV light after staining with ethidium bromide. Restriction digestion of the genomic DNA was also performed (Doulis et al., 2000).

RAPD has been used in studies reported with *Jatropha curcas* with different goals. In this study, PCR conditions using 10 random primers were tried using the protocol as follows: Initial denaturation -94°C-10', Denaturation -94°C-1',Annealing -35°C-1', Extension-72°C-2', Final extension -72°C -10', Number of cycles: 45. Amplification could be observed even by reducing the number of cycles to 30. Of the primers used; Primer OPL-1 and OPL-3 of operon technologies USA have given amplification product. The protocol for RAPD has been standardized will be used to different accessions of *J. curcas* of Gujarat region. The seed oil yield of these accessions would be studied and then they would be divided into low oil yielding and high oil yielding plants with an attempt to identify polymorphic loci.

2) **Micropropagation of *Jatropha curcas*:**

- **Indirect organogenesis through leaf discs**

Young leaves at 3rd and 4th node from the apex were collected from 1.5-2 year old plants. The explants were thoroughly washed and surface sterilized with 0.1% mercuric chloride for 2 to 3 minutes followed by five rinses with sterile distilled water. Leaves were then excised into small pieces of 1x1cm and inoculated on Murashige & Skoog (MS) medium supplemented with different combination of BAP and IBA for regeneration. A range of combination of BAP and IBA were used for callus initiation. It included 4.5 µM - 27.0 µM BAP and 3.0 µM - 7.5 µM IBA. Earlier studies have shown the importance of BAP and IBA in inducing organogenesis from leaf discs (Sujatha et al, 2005). Curling of enlarged leaf discs was first observed followed by callus appearance at the cut margins. Callus morphology and relative response of callusing was studied for the initial two weeks. Explants on medium supplemented with lower levels of IBA (3.0µM and 5.0 µM) and higher levels of BAP gave maximum callus formation. Similar results were obtained with high levels of IBA in combination with low BAP. Though the media containing highest levels of either BAP or IBA showed massive callus formation no organogenesis was observed. Even on sub-culturing on respective media it continued to callus. In the medium supplemented with higher BAP and IBA callus turned brown and dried after first passage. Combination of 5.0 µM IBA + 27.0 µM BAP in the MS medium favored bud initiation. By reducing IBA (3.0 µM) in combination with all three concentrations of BAP, organogenesis was observed after 45 days in culture. This could be suggestive of the importance of lower levels of IBA in inducing organogenesis. Buds in clusters (3-5) started emerging from various locations of the callus, mostly underneath the explants. First bud initiation was observed in MS medium supplemented with 27.0 µM BAP + 3.0 µM IBA. It was also the most effective combination for shoot bud initiation. After organogenesis has been successfully induced, and shoot bud proliferation observed, increased IBA levels have little influence. The mean length of regenerants was 1.0 cm. MS medium supplemented with 4.5 µM BAP + 3.0 µM IBA and 27.0 µM BAP + 3.0 µM IBA are not significantly different according to DMRT (α=0.05). Hence, it could be concluded that irrespective of BAP levels, low levels of IBA are essential in order to obtain multiple shoot regenerants per explants in *Jatropha curcas.*

- **Organogenesis through axillary buds**

For experiments on culture initiation from axilliary buds, tender, thin, green twigs cut below 2nd and 3rd nodes were taken as explants. Each explant containing one apical bud and 2 to 3 axillary buds were pretreated and surface sterililzed with mercuric chloride. The culture medium comprised of MS medium supplemented with 2.2 μM BAP and 4.9 μM IBA. These cultures were then sub-cultured on MS medium supplemented with 8.9 μM BAP and 2.9 μM IAA (Rajore et al., 2005) with adenine sulphate (100mg/l) and glutamine (100 mg/l) as an addendum. The cultures were incubated at 26 ± 2 ºC under a 16 h photoperiod using cool, white fluorescent lights (30 μmol m^{-2} s^{-1}). Multiple shoots were obtained after two passages. The shoots were then kept on MS basal medium for rooting. Roots were observed after nearly three weeks in rooting medium.

- **Somatic embryogenesis:**

Somatic embryogenesis acts as a powerful tool for genetic improvement of any plant species because of its single cell origin (Bhansali et al, 1991). Somatic embryogenesis can be initiated by two mechanisms: directly on explanted tissue where plants are genetically identical (clonation) and indirectly from unorganized tissues (callus). In the current investigation we report both direct and indirect somatic embryogenesis. The former experiments were carried out on solidified media whereas suspension cultures were used for the later one. The advantage of liquid suspension culture system is that the growth of plant cells is more rapid than in callus culture and is also more readily controlled because the culture medium can be easily amended or changed.

Seeds (both immature and mature) were used as explants. Mature seeds could give rise to cotyledonary leaves upon initiation in callus inducing media, whereas immature seeds failed to do the same. Since seeds serve to be the initial source for explant, seed viability formed to be an important parameter to study. It was observed that upon long term storage at room temperature, seeds lose their viability and would not germinate. They would also show cotyledon degeneration. Treatment of seeds with GA 10ppm was attempted for a period of four hours to break seed dormancy however; it did not show any positive response. As an alternative to this seeds after collection were stored under cooling conditions (-20°C). Seasonal cues like environmental conditions also have an effect on the performance of explants in culture conditions. Callus formation was found to be higher in the months of January to April. This is the season when fruit maturation initiates as October and November are the flowering months. In

monsoon the explants are less responsive and more contamination is encountered, which is in agreement with reports of Bisht et al., 2010 for bamboo explants.

The role of auxins in morphogenesis can be seen as the cells which respond to auxin revert to a dedifferentiated state and begin to divide. In the present study two auxins namely 2, 4-D (2, 4-Dichlorophenoxyacetic acid) and NAA (Naphthalene acetic acid) were incorporated into the MS medium at different concentrations. MS fortified with different levels of 2, 4-D (0.4, 0.8, 1.2, 2.4, 3.6 and 4.0mg/l) was used for inducing embryogenic calli. MS with 0.4, 0.8 and 1.2 mg/l 2, 4-D was supplemented with 2% and 10% coconut water respectively. It has been reported that the culture of explants in medium containing 2,4-D, increases the endogenous auxin levels in the responsive explants and this acts as a crucial signal determining embryogenic fate of cultured cells (Jimenez, 2005). Higher endogenous IAA concentrations have been shown in different species/explants as being associated with an increased embryogenic response (Feher et al., 2003). Hence it is interesting to know the role played by auxin in somatic embryo development and maturation. MS with higher levels of 2, 4-D i.e 2.4, 3.6 and 4.0 mg/l was used without coconut water. MS salts fortified with NAA at a concentration of 5, 10 and 15mg/l (based on grid experiment) were used in this study. The above mentioned combinations could successfully induce friable callus formation.

Two approaches to achieve somatic embryogenesis were used. In the first approach of indirect embryogenesis, the cotyledonary leaves were initiated on MS supplemented with varied 2, 4-D (0.4, 0.8, 1.2 mg/l) and NAA concentrations for a period of 30 days followed by their transfer to no auxin media. In order to obtain somatic embryos one month old calli from explanted cotyledon were inoculated into suspension culture having only MS salts with no plant growth hormone. Greenish white callus started appearing at the edges of cotyledonary leaves and also at the midrib and on small veinlets. After 30 days in culture, friable calli was transferred to liquid culture medium. Once in suspension culture, it was observed that the cell number was initially low for first 4-5 days. The cell number increased within 10-12 days and then a gradual decrease was observed after 20 days. Initially cell suspension culture comprised of single cells. These cells were round and densely cytoplasmic with distinct nuclei. Some large and highly vacuolated cells with sparse cytoplasm were also observed. Early embryogenic structures like globular (17^{th} day), heart shaped and torpedo shaped cells (18-25 days) were seen albeit a little late. A decline in cell number is observed after 30 days in culture. While no such cells were seen in media

supplemented with various concentration of NAA. Hence, NAA as a plant growth regulator to induce somatic embryogenesis could be a less preferred substitute as compared to 2, 4-D. The presence of these structures confirmed that embryogenesis had been induced in the cells in liquid suspension. Similar reports exist in Soybean (Santos et al, 2006

In the second approach of direct embryogenesis short time auxin pulse treatment was used, wherein the cotyledonary leaves were initiated on MS +2, 4-D (0.4, 0.8, 1.2 mg/l + 2% coconut water and 0.4, 0.8 and 1.2 mg/l +10% coconut water and 2.4, 3.6 and 4.0 mg/l) and MS+ NAA for a period of seven days or fourteen days followed by their transfer to no auxin medium. Auxin levels were checked on day 4, 7 and 14. On day 4 pre-embryogenic masses were not observed whereas on day 7 pools of embryogenic and non-embryogenic structures were observed. On day 14 most of the calli had turned embryogenic. MS supplemented with NAA failed to show any embryogenic response in calli so obtained. However, on MS supplemented with 2, 4-D small globular embryos started emerging on the edges of cotyledonary leaves in a linear fashion. Isolated single globular embryos were also seen at certain veinlets. Certain explants showed heart shaped cells at the midrib position. This marks the onset of direct somatic embryogenesis. Along with embryogenic structures rhizogenesis was also observed in some of the explants. Total auxin levels were estimated in E and NE calli. It was observed that MS fortified with higher levels of 2, 4-D devoid of coconut water could trigger higher embryo formation. Lower levels of 2, 4-D in MS medium could trigger embryogenesis only if supplemented with coconut water. Total auxin levels were estimated and it was found that a significant increase is seen in total auxin in embryogenic(E) calli(7 days and 14 days) as compared to non-embryogenic calli (NE)(4 days) in various media combinations studied. It could be concluded from the present study that 2, 4-D alone at higher concentration (4.0 mg/l) is a preferred medium to induce embryogenesis in *J. curcas* as compared to lower 2, 4-D levels with coconut water. This could be due to the fact that coconut water is an undefined medium and hence may interfere with reproducibility of procedure.

For the first time we report liquid suspension culture studies of *Jatropha curcas* and direct as well as indirect somatic embryogenesis. Plant regeneration is yet to be studied.

3) Target fatty acid biosynthetic pathway to increase oil yield in seeds

- **Standardization of agrobacterium mediated transformation**

After standardization of tissue culture of *Jatropha*, the next step was working on transformation of the binary vector pCambia 1305.2 into *E. coli DH5α*. Since the plasmid was obtained in *E coli* as a host, its presence was confirmed by plasmid isolation by alkaline lysis method and the band obtained after agarose gel electrophoresis was compared with λ marker digested with Hind III. Linearization was confirmed by restriction digestion by BamHI. Triparental mating was carried out using *E coli* DH5α with pCambia 1305.2(Kan^R) as the donor strain, *E coli* DH5α with pRK2013 (Kan^R) as the helper strain and *Agrobacterium-LBA4404* (Rif^R,Tet^R) as the recipient. Growth of transformants was observed after plating on Luria Agar plates containing tetracycline (10 μg/ml),rifampicin (5 μg/ml) and kanamycin (50 μg/ml). The transformants were confirmed to be *Agrobacterium* and not *E coli* or other contaminants by the Benedict's test (Bernaerts M.J. and De Ley J., 1963). Confirmation of transformation of plasmid pCambia 1305.2 into *Agrobacterium* was done by its plasmid isolation followed by agarose gel electrophoresis. Axenic leaf discs (obtained from young offshoots from axillary buds and leaf discs from previous inoculation) were immersed in Murashige and Skoog broth seeded with transformed *Agrobacterium.* They were then inoculated in Murashige and Skoog agar and incubated at 28º C in dark for 3 days. Growth was observed at the edges of leaf discs which was confirmed to be *Agrobacterium* by Benedict's test. The infected leaf discs were washed in sterile distilled water following which they were transferred to MS supplemted with 3.0 μM IBA and 27.0 μM BAP medium containing hygromycin(200 μg/ml) and cefotaxime(400 μg/ml). After 5 days of incubation at 25°C, 4 leaf discs out of 10 still remained viable. These were then subcultured on to initiate callus formation.

- **Overexpression study of DGAT**

The expression studies of DGAT are under progress.

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List of Abbreviation

DGAT	Diacylglycerol acyl transferase
2, 4- D	2, 4- Dichlorophenoxyacetic acid
CTAB	Cetyl Trimethyl Ammonium Bromide
IAA	Indole 3-Acetic acid
PVP	Polyvinylpyrrolidone
SDS	Sodium dodecyl sulphate
DNA	Deoxyribonucleic acid
$HgCl_2$	Mercuric Chloride
NaOCl	Sodium Hypochloride
MS	Murashige and Skoog
BAP	N-6 Benzylaminopurine
NAA	Naphthalene Acetic acid
Kn	Kinetin
IBA	Indole-3-Butyric acid
EDTA	Ethylene Diamine Tetracetic Acid
Kan^R	Kanamycin Resistance
Rif^R	Rifamycin Resistance
Tet^R	Tetracycline Resisitance
SE	Somatic Embryogenesis
NE	Non-Embryogenic
E	Embryogenic
CW	Coconut Water

Lists of symbols

Alpha	α
Beta	β
Micro	µ
Degree Celsius	ºC
Percent (age)	%
Gram	g
Hours	h
Kilo	K
Liter	L
Mili	m
Miligram	Mg
Molar	M
Minutes	Min
Second	S
Unit	U
Fresh weight	Fwt