Synopsis of the thesis on

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

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By

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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 °C 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 28° 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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