CHAPTER-1

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."*

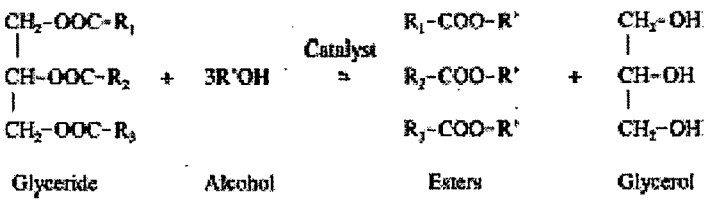
Scientists discovered that a simple chemical process could reduce the viscosity of vegetable oils and it was shown to work well in modern engine. ***This fuel was called Bio-Diesel.*** Since then the technical developments to improve its properties as engine fuel have been completed. Plant oil is highly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

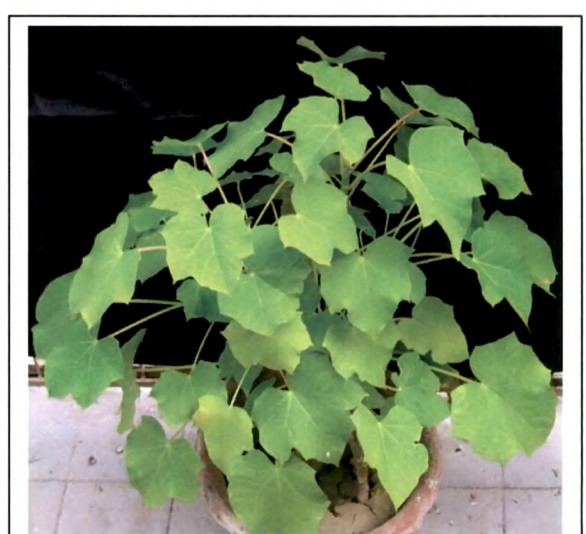


Figure 1.1: Image of *Jatropha curcas*

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%

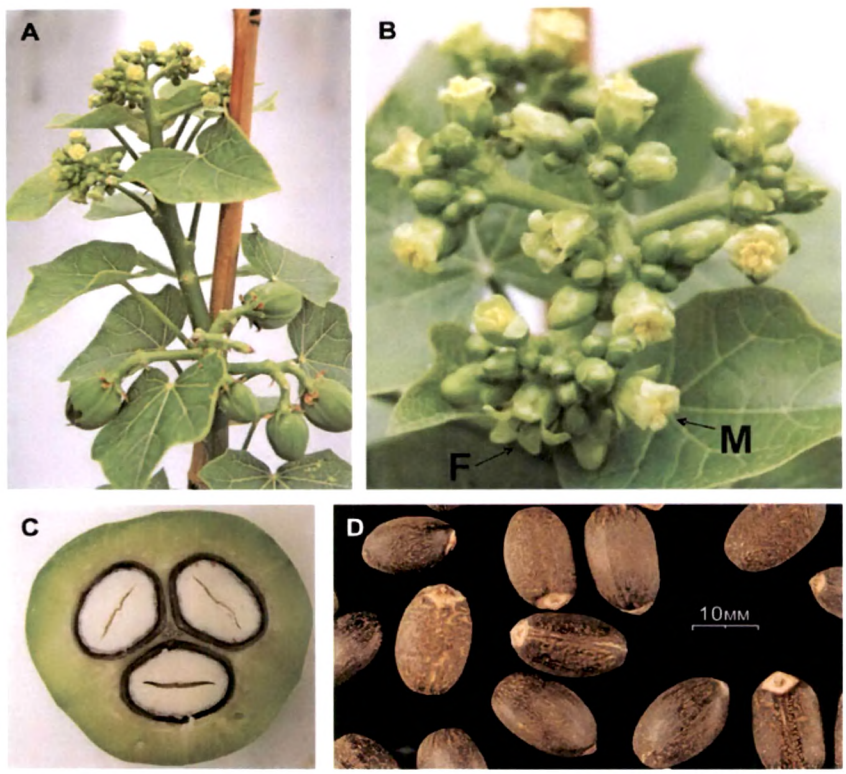


Figure 1.2: Images of *Jatropha curcas* (A) Young *Jatropha curcas* plant with both flowers and developing seedpods. (B) *Jatropha curcas* inflorescence containing both male staminate flowers (M) and female pistillate flowers (F). (C) Cross-section of a *Jatropha curcas* seedpod containing three developing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

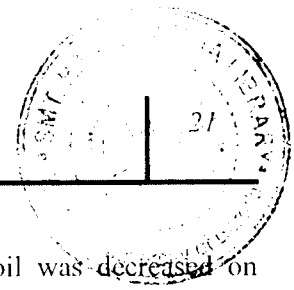
Properties	Diesel	*Jatropha curcas* oil
Density (gm/cc), 30°C	0.836–0.850	0.93292
Kinematic viscosity (cSt), 30°C	4–8	52.76
Cetane No.	40–55	38
Flash point °C	45–60	210
Calorific value, MJ/kg	42–46	38.2
Saponification value	–	198
Iodine No.	–	94

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%.

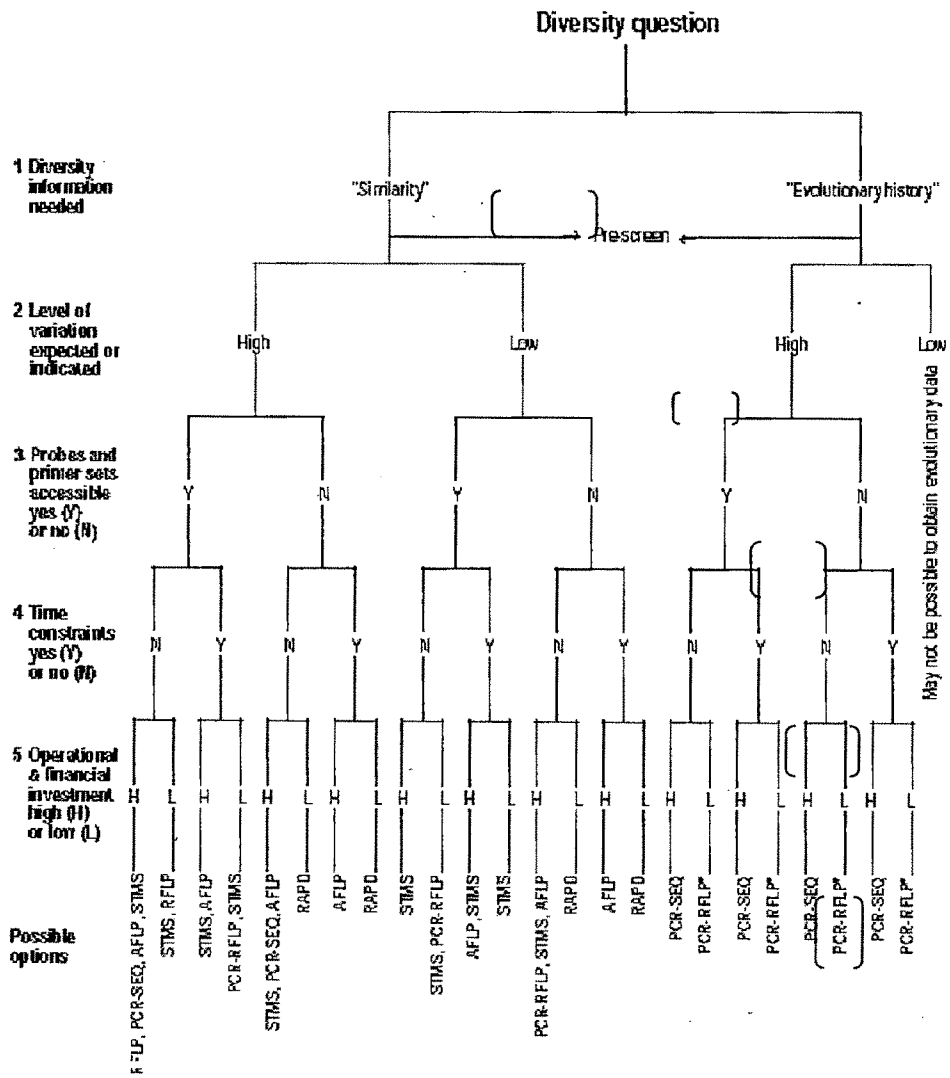
1.9 Existing Genetic variation:

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.

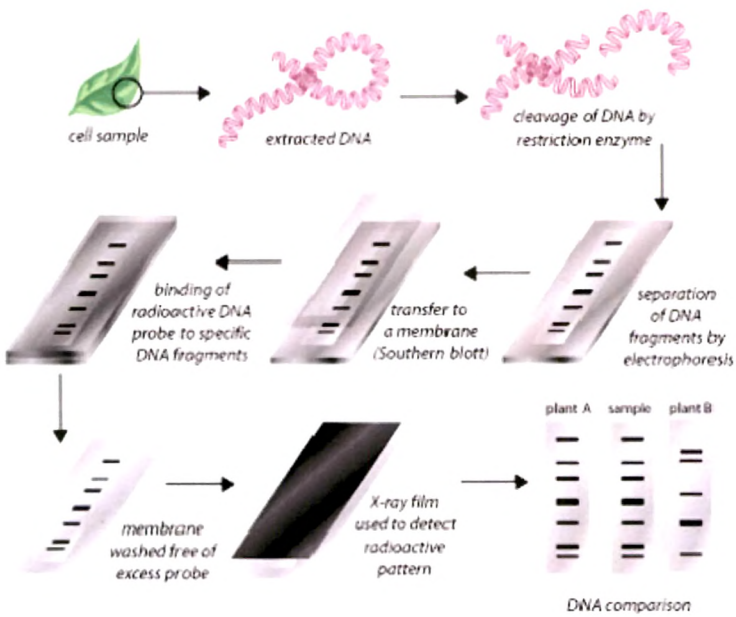


Figure 1.3: Schematic representation of RFLP taken from www.scq.ubc.ca

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.

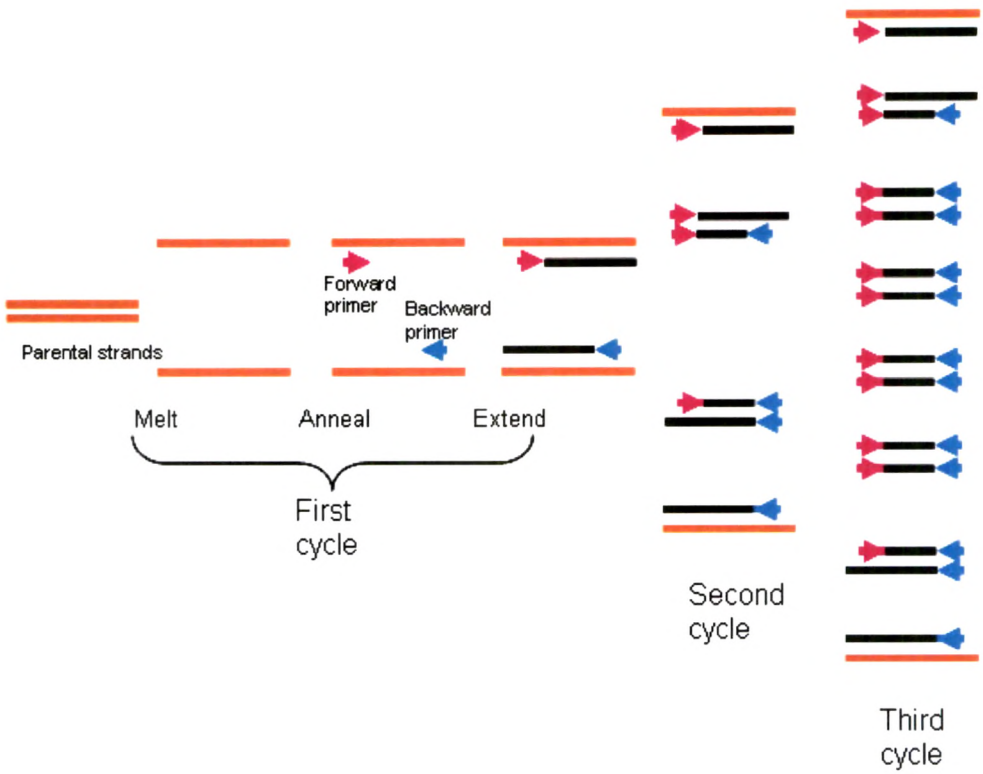


Figure 1.4: A schematic representation of PCR taken from http://biochemistryforlife.wikispaces.com/

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.

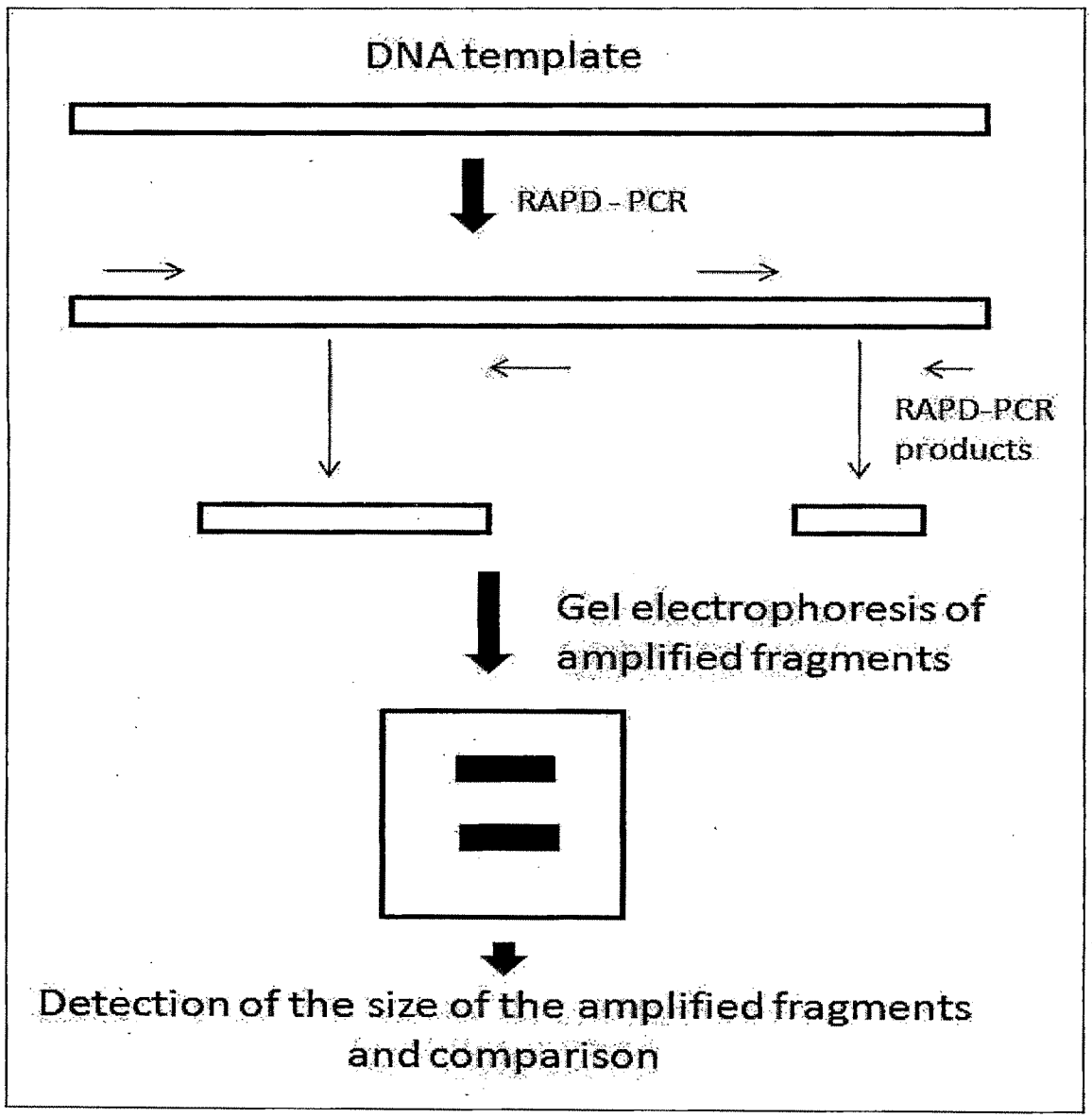


Figure 1.5: Principle of RAPD-PCR technique taken from Arif et al, 2010

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).

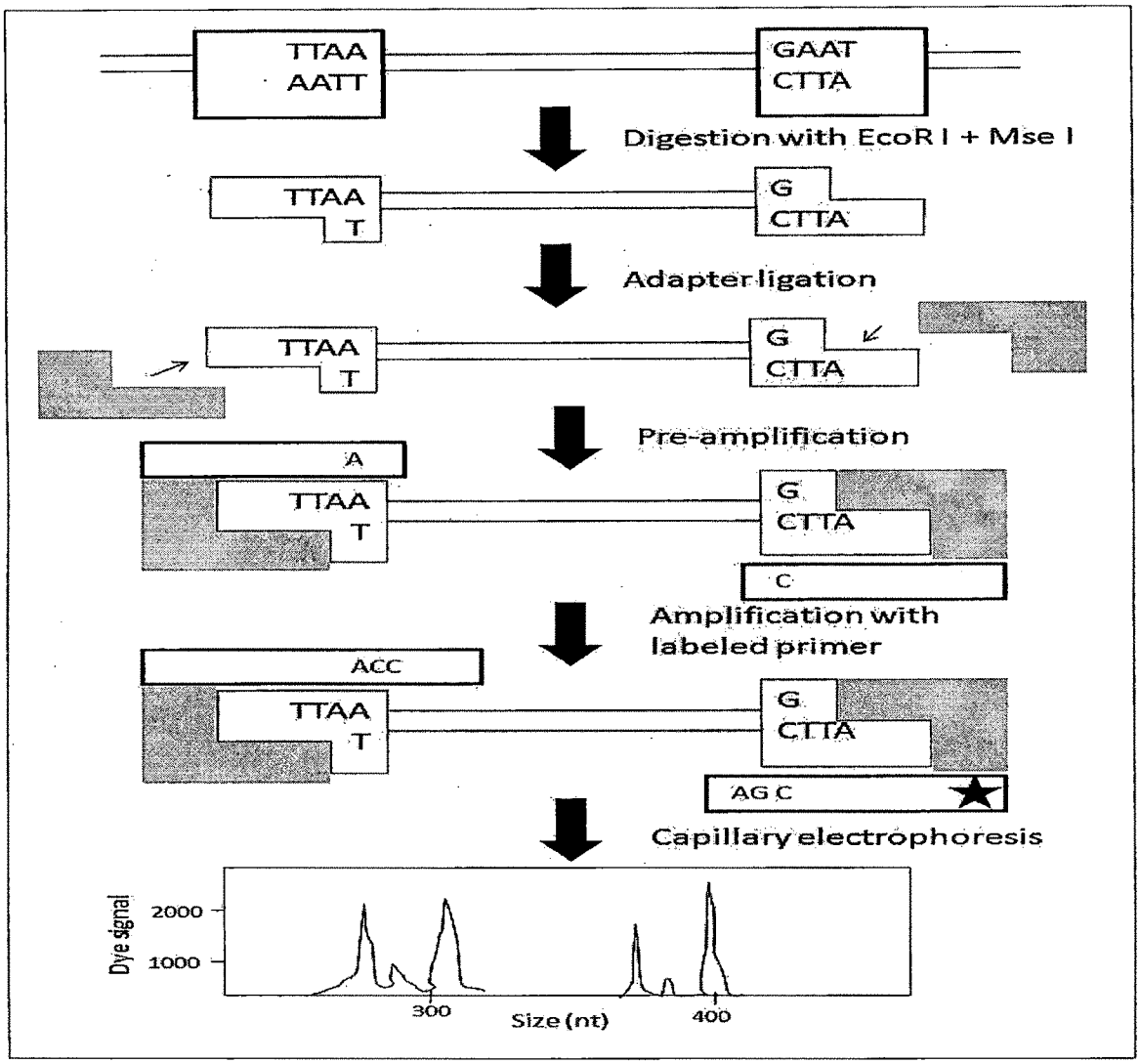


Figure 1.6: A schematic flow chart showing the principle of the AFLP method taken from Arif et al, 2010

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

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).

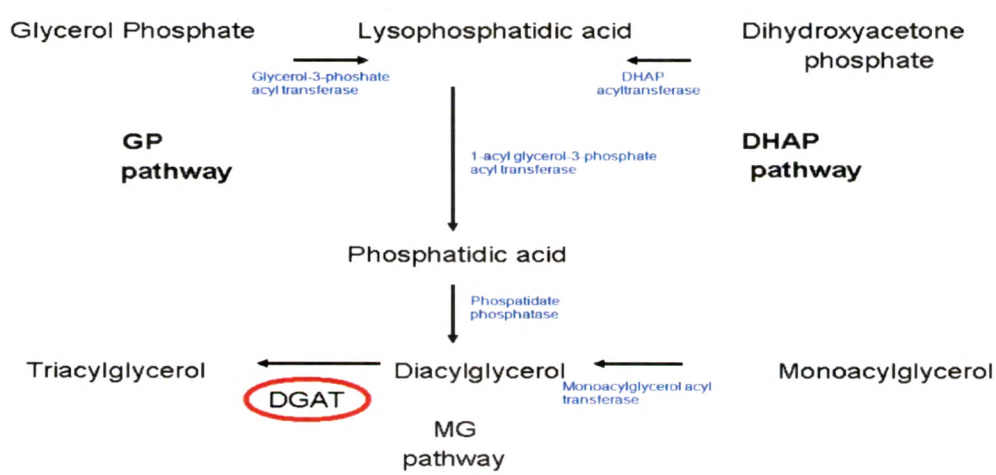


Figure 1.7: Kennedy Pathway

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.

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).

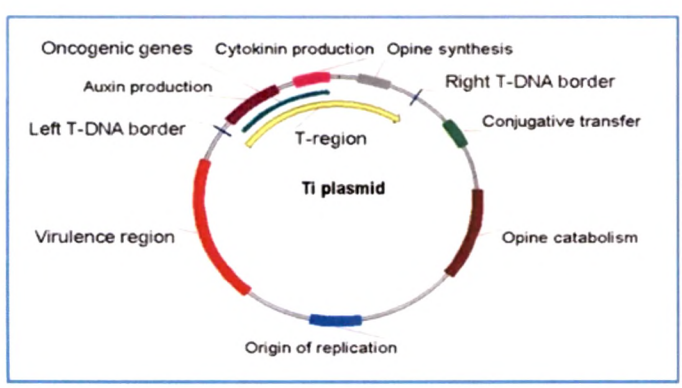


Figure 1.8: Pictorial representation of Ti plasmid

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).

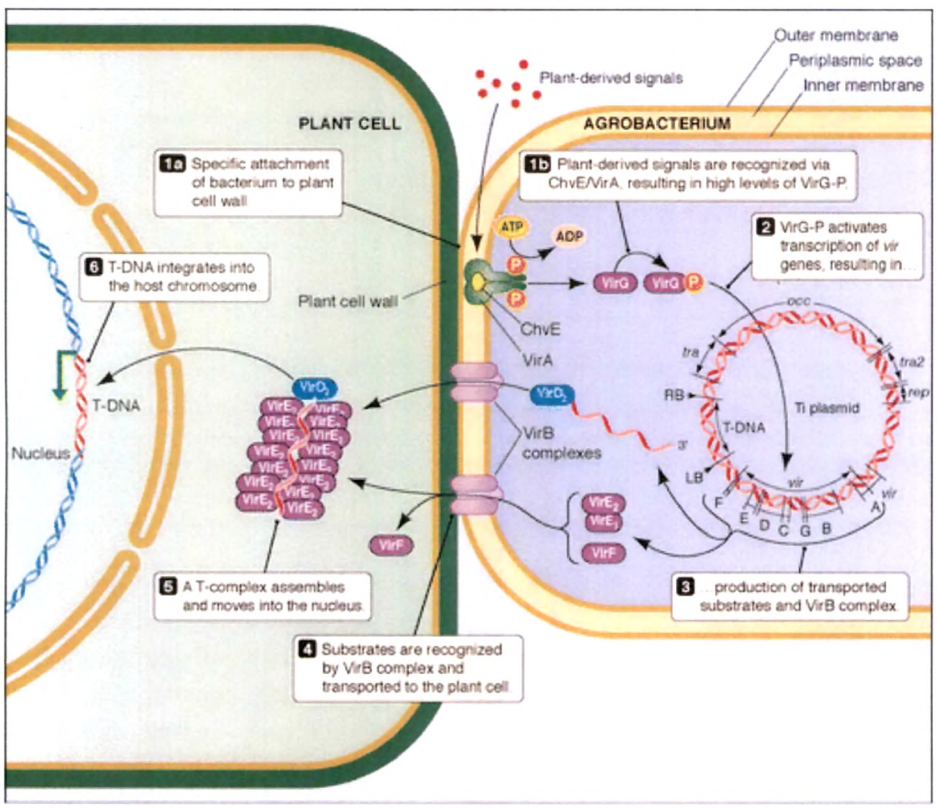
4) T-DNA region which consists of oncogenic genes namely those for auxin and cytokinin production. These phytohormones are responsible for the tumorous growth which results due to a disturbance in the levels of the phytohormones. It also contains gene for opine synthesis. All genes of the T-DNA have 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).



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.

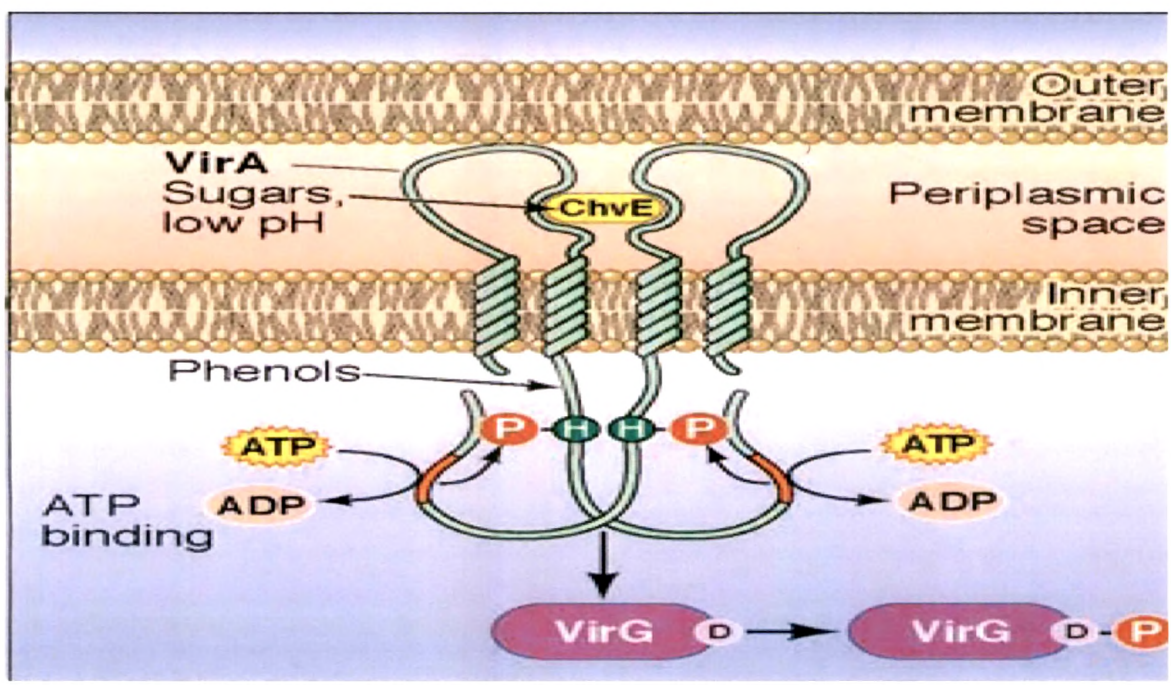


Figure 1.10: The ChvE/VirA/VirG signal transducing system by Mc Cullen and Binns, 2006

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

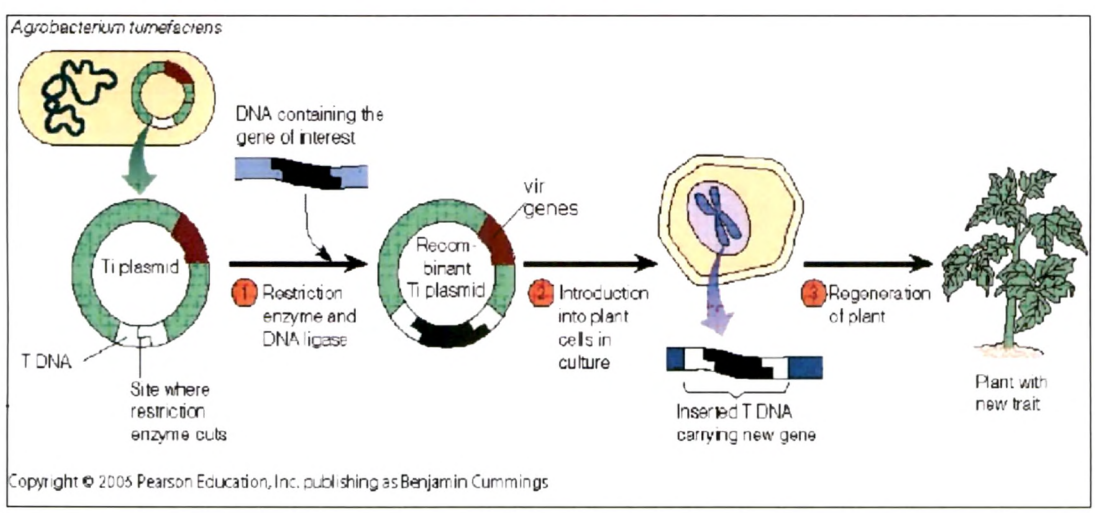


Figure 1.11: Cloning of gene of interest into Ti plasmid and its use in plant transformation

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.

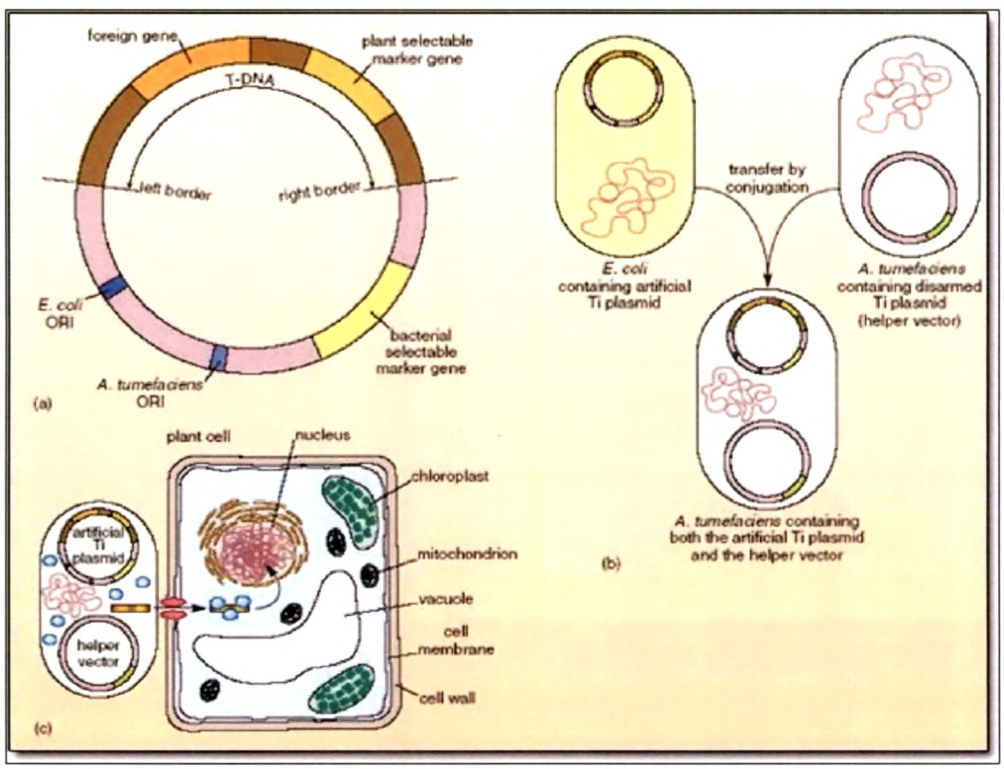


Figure 1.12: Plant transformation using binary vectors

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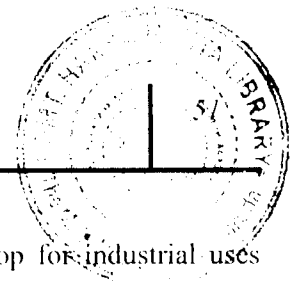

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