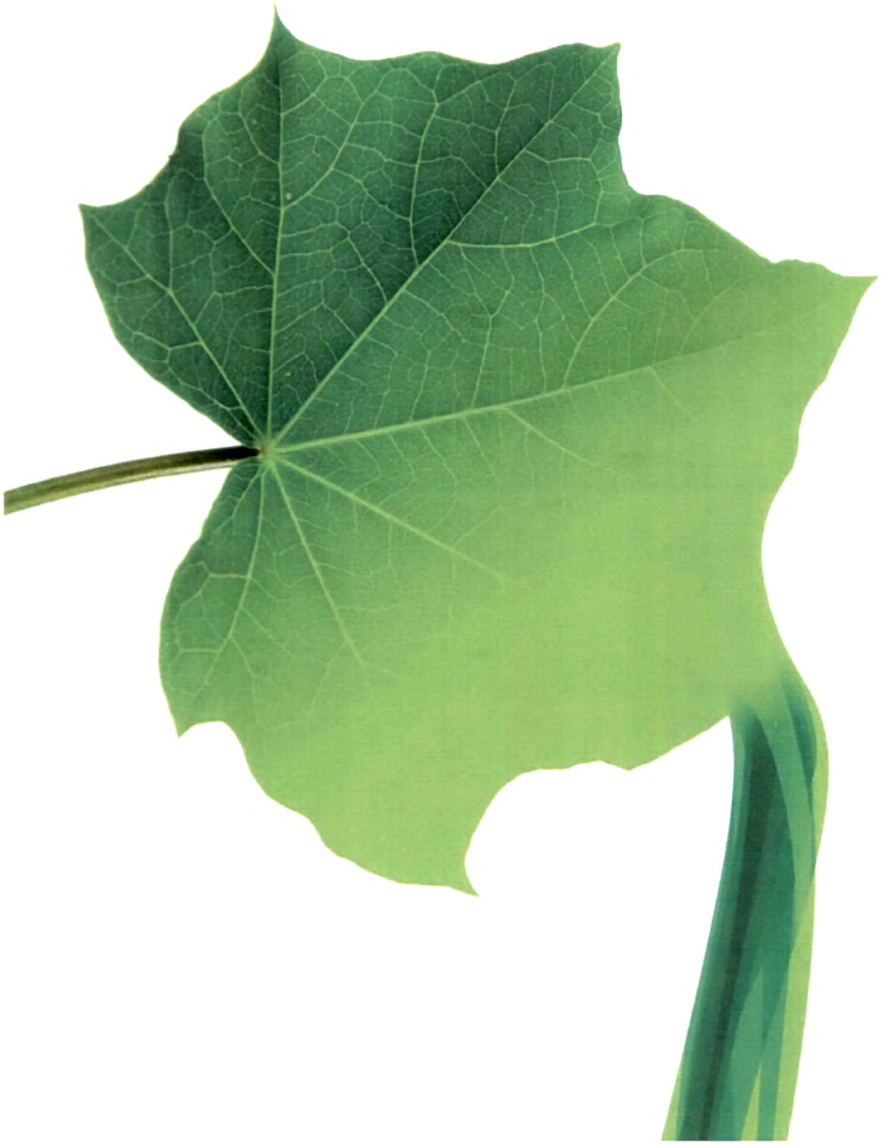
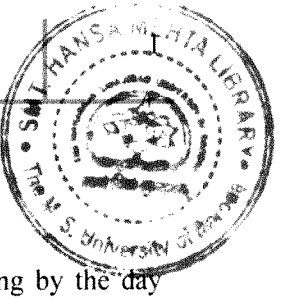
CHAPTER 1



Introduction



1.1 Present Energy scenario

The consumption and demand for petroleum products is increasing by the day due to increase in population, standard of living and urbanization. The total primary commercial energy consumption in India is around 380 MMT of which diesel consumption is around 42 MMT (Petroleum Conservation and Research Association, India). Our country's import bill is around 1,20,000 Cr INR. This has a serious impact on the economy of our country. In addition, consumption of diesel leads to serious problems of air pollution due to emission of polluting gases. Acid rain, global warming and health hazards are some of the ill effects of increased emission of polluting gases like SOx, CO and particulate matter in atmosphere due to use of diesel.

The increase in demand of diesel leads to depletion of reserves of fossil fuel and increase in import bill. This is an important trigger for urgent initiative to search for an alternate source of energy, which can supplement or replace fossil fuels. In recent years, research has been directed towards exploring plant-based fuels. Due to their renewable nature, plant oils and fats as fuel source have bright future. The most common fuel that is being developed and used at present is biodiesel, which is fatty acid methyl ester (FAMEs) of seed oils and which has been found suitable for use as fuel in diesel engine. Biodiesel is the only alternative fuel that can be used directly in any existing diesel engine without engine modification. Because it has similar properties to diesel fuel, biodiesel can also be blend with diesel fuel. Hence, biodiesel a renewable and domestically produced liquid fuel can help to reduce the countries dependence on foreign oil imports.

1.1.1 Alternative energy

Alternative energy refers to a source of energy intended to replace fossil fuel sources. Alternate energy source should replace and hence retrofit current technologies with alternatives that have comparable or better performance but do not emit carbon dioxide. Alternative energy sources are renewable and are called "free" energy sources. They all have lower carbon emissions, compared to conventional energy sources. The

known sources of alternate energy include Biomass Energy, Wind Energy, Solar Energy, Geothermal Energy and Hydroelectric Energy.

1.1.2 Alternative energy sources

Algae fuel

During photosynthesis, algae and other photosynthetic organisms capture carbon dioxide and sunlight and convert it into oxygen and biomass. This biomass can then be converted to biodiesel. The benefits of algal biofuel are that it can be produced industrially, thereby obviating the use of arable land and food crops (such as soy, palm, and canola), and that it has a very high oil yield as compared to all other sources of biofuel.

Biomass briquettes

Biomass briquettes are being developed in the developing world as an alternative to charcoal. The technique involves the conversion of almost any plant matter into compressed briquettes that typically have about 70% calorific value of charcoal.

Biogas digestion

Biogas digestion deals with harnessing the methane gas that is released when waste breaks down. This gas can be retrieved from garbage or sewage systems. Biogas digesters are used to process methane gas by having bacteria break down biomass in an anaerobic environment. The methane gas that is collected and refined can be used as an energy source for various products.

Biological Hydrogen Production

Hydrogen gas is a completely clean burning fuel; its only by-product is water. It also contains relatively high amount of energy compared with other fuels due to its chemical structure. To generate it, requires high-energy inputs, making commercial use of hydrogen fuel very inefficient (Ghirardi et al., 1997). Use of a biological vector as a means to split water, and therefore produce hydrogen gas, would allow for the only energy input to be solar radiation. Biological vectors can include bacteria or more commonly algae. This process is known as biological hydrogen production (Radmer et

al., 1977). It requires the use of single celled organisms to create hydrogen gas through fermentation. If it could be implemented on a large scale, then we could use sunlight, nutrients and water, to create hydrogen gas to be used as a dense source of energy (Gaffron et al., 1942). Large-scale production has not yet been successful.

1.1.3 Biodiesel -The Next Generation Sustainable Fuel

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 the now most quoted statement that *"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 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.

1.1.4 Biodiesel Scenario in India

As India is a developing country and not self sufficient with its edible oil requirement, non-edible oil is the preferred choice for producing biodiesel. According to the policies laid down by the Indian government, some development has been made and efforts are on to popularise the production and use of transesterfied non-edible oil as biodiesel. Institute such as Indian Institute of Science, Bangalore, Tamilnadu Agriculture University, Coimbatore and Kumaraguru College of Technology have initiated use of biodiesel. Generally, a Blend of 5% to 20% of biodiesel with conventional diesel is used in India and these are termed B5 and B20 respectively. Indian Oil Corporation has taken up Research and Development work to establish the parameters for the production of transesterified Vegetable oil and use of bio diesel in its

R&D center at Faridabad. Research is carried out in Kumaraguru College of Technology for marginally altering the engine parameters to suit the Indian biodiesel and to minimize the cost of transesterification.

1.2 Biodiesel

The accepted definition of biodiesel is Alkyl esters of long chain fatty acids derived from triacylglycerols from biological sources. It can be derived from natural, renewable biological sources such as vegetable oils like Sunflower, Canola, Jatropha etc. Its name indicates, use of this fuel in diesel engine as an alternate to diesel fuel. 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.

1.2.1 Advantages of biodiesel

- Produced from sustainable / renewable biological sources
- Ecofriendly and oxygenated fuel
- Decrease emission of Sulphur, CO, HC, particulate matter and aromatic compounds
- Income to rural community
- Fuel properties similar to conventional fuel
- Used in existing diesel engines without modifications
- Reduce expenditure on oil imports
- Non toxic, biodegradable and safe to handle
- Decreases Global Warming

Biofuels will help to create new markets for agricultural products and stimulate rural development because they are generated from crops. They hold enormous potential for farmers. In the near future, especially for the people in the developing world who derive their incomes from agriculture, biodiesel would be a boon. Hence, biofuels have

enormous potential to change the economic situation for the rural farmer. At the community level, farmers who produce dedicated energy crops can increase their income and have their own supply of affordable and reliable energy. At the national level, producing more biofuels will generate 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. 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.

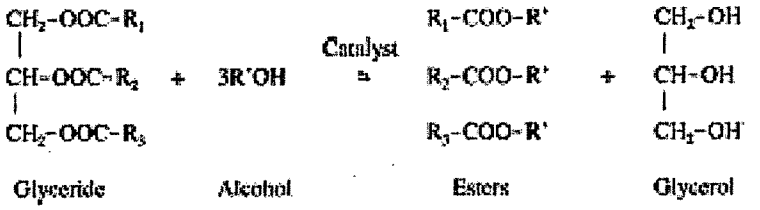
1.2.2 Disadvantages of biodiesel

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.
- **High Cost:** To refine biodiesel make it an efficient energy source and to build the necessary manufacturing plants to increase biofuel quantities will require a high initial investment.
- **Food Prices:** As demand for land to grow crops for biodiesel production increases, 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:** Biofuels are not 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.

1.2.3 Chemistry of biodiesel production

Biodiesel is produced by transesterification of fatty acids of triglycerides in to respective 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 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.

1.2.4 Putative plants for bio-diesel

Various plants have been identified as putative source for biodiesel production. Western countries use edible sources like Corn, Soybean, Canola etc. India being a developing country cannot afford to use edible oil for non edible use. In India the following plants have been identified as putative biodiesel sources.

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

- *Azadirachta indica* (neem)

All the above mentioned plants have been cultivated or found in the wild with various medicinal and other economic 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.

Why *Jatropha curcas* was selected for this study?

- Its seed oil content is high (35-40%) in comparison to other putative plants.
- It can easily grow in arid region (20 cm rainfall) and this potentiates its role as a preferred crop for the saline wastelands. It is adaptable 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 is highly pest, disease and drought resistance, which leads to optimum crop harvesting.
- It has many medicinal properties and different parts of the plant have been utilized in traditional medicines.
- Short gestation period: 2 years
- Long productive life: 50 years
- Close physico-chemical properties to petro-diesel

1.2.5 Scientific classification:

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

1.3 *Jatropha curcas*- An Overview

Jatropha curcas (Linnaeus) is a multipurpose bush/small tree belonging to the family Euphorbiaceae. It is a plant with many attributes, multiple uses and considerable potential. The plant can be used to prevent and/or control erosion, grown as a live fence, especially to contain or exclude farm animals and be planted as a commercial crop. It is a native of tropical America, but now thrives in many parts of the tropics and sub-tropics. It has few pests and diseases and grows under a wide range of rainfall regimes from 200 to over 1500 mm per annum. In low rainfall areas and in prolonged rainless periods, the plant sheds its leaves to counter drought.

Linnaeus was the first to name the commonly called physic nut, *Jatropha curcas* L. The genus name Jatropha derives from the Greek word jatros (doctor) and trophe (food), which implies its medicinal uses (Linnaeus, 1753). The plant is a shrub/small tree with height generally ranging from 3-5 m. The productivity of the plant was found to vary from 1.5 to 6 tons/ha/year. The Jatropha seeds could be harvested from a 5-month-old plantation, but productivity is stable after the first year.

The natural habitat of *Jatropha curcas* is quite diverse. It grows well in semi-arid, arid and tropical humid conditions (Jones and Miller, 1992; Makke et al., 1997; Openshaw, 2000). Its rooting patterns are significantly influenced by propagation method. Plant originating from seed and directly sown into the soil normally develops a rooting system with thick primary taproot and four lateral roots with abundant straight secondary roots (Heller, 1996), whereas plants propagated by cuttings only develop secondary roots. Growth containers in nurseries may hamper the initial growth of *Jatropha curcas* seedlings if container volume is insufficient. This is caused by reduced space for root expansion and not by lower availability of nutrients in the soil. It was reported that adult leaves are adapted to high radiation intensities. It is easy to establish, grows relatively quickly and is hardy, being drought tolerant. It is not browsed, as its leaves and stems are toxic to animals, but after treatment, the seeds or seed cake could be used as an animal feed. Various parts of the plant are of medicinal value, its bark contains tannin, the flowers attract bees and thus the plant has a honey production

potential; its wood and fruit can be used for numerous purposes including fuel. Of particular importance, the fruit of Jatropha contain viscous oil that can be used for soap making, in the cosmetics industry and as a diesel/kerosene substitute or extender. However, for several reasons, both technical and economic, the full potential of Jatropha is far from being realized. The growing and management is poorly documented and there is little experience in marketing its products. Thus, frequently, growers do not achieve the optimum output of products, such as the fruit that would bring the greatest rewards. Neither do they have much information about other lucrative potential. Therefore, potential growers may be reluctant to invest time and money in a crop that only has promise rather than concrete rewards. Some of the current strategies used to promote Jatropha may be sub optimal and could act as a deterrent instead of a stimulus in promoting rural development (Openshaw,, 2000). Thus, it is high time to examine problems encountered in the growing and use of Jatropha and the achievements made to date. Reevaluation of the present strategy in promoting this potentially useful and versatile plant is the need of the hour.

1.3.1 Other uses of *Jatropha curcas* (Gubitz et al., 1999)

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.

1.3.1.1 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 villages, it is used as an illuminant as it burns like castor oil. It is used for wool spinning in England. It is feasible to use Jatropha oil as hydraulic oil.

1.3.1.2 Potential as medicinal plant

The latex of *Jatropha curcas* contains an alkaloid known as "Jatrophine" which is believed to be having anti-cancerous properties. *Jatropha curcas* oil possesses purgative properties. It differs from castor oil in that it has a low viscosity. It is applied externally for skin diseases and rheumatism, it is reported to be abortificient and efficacious in dropsy, sciatica and paralysis. In some places, the tender twigs of the plant are used for cleaning teeth. The juice is reported to relieve toothache and strengthen gums. The leaf juice is used as an external application for piles. It is also applied for inflammations of the tongue in babies. The twig sap is used for dressing wounds and ulcers. An emulsion of the sap with benzyl benzoate is said to be effective against scabies, wet eczema and dermatitis. A decoction of leaves and roots is given for diarrhoea. The root is reported to contain yellow oil with strong antihelmintic action. The root bark is used as external application for sores. A decoction of the bark is given for rheumatism and leprosy. Similarly, roots are also reported to be used as antidote for snakebite.

1.3.1.3 Potential as raw material for dye

The bark of *Jatropha curcas* yields a dark blue dye that is reported to be used in Philippines for dying cloth. The dye may be extracted from leaves and tender stems and concentrated to yellowish syrup or dried to blackish brown lumpy mass. The dye imparts to cotton different shades of tan and brown that is fast.

1.3.1.4 Potential for enrichment of soil

Jatropha oil cake is rich in nitrogen, phosphorous and potassium and can be used as organic manure. Tender branches and leaves are used as manure for coconut trees. Jatropha oil cakes could replace synthetic fertilizers by undertaking plantations of *Jatropha curcas* on wastelands. *Jatropha curcas* leaves provide good organic matter and increases the microbial activity including earthworms, which is an indication of ecological improvement of site.

1.3.1.5 Potential as a feedstock

Jatropha leaves are used as feed for the tusser silk worm. The oil cake is rich in protein but contains some toxic principle and as such, it is considered unfit for use as cattle feed. However, it is reported that the poisonous principle appears to exist in the alcohol soluble fraction of the oil. With suitable research, it could be possible to convert the nonedible oil cake into protein rich cattle and poultry feed on a massive scale.

1.3.1.6 Potential as insecticide/pesticide

The seeds are considered anthelemintic in Brazil. They are ground with palm oil and used as rat poison in Gabon. Aqueous extract of leaves is reported to have insecticidal properties. In Ghana, the leaves are used for fumigating houses against bed bugs. The ether extract shows antibiotic activity against *Staphylococcus aureus* and *Escherichia coli.*

1.4 Soil requirement

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 come up on stony, gravelly or fallow and even calcareous soils. It can be conveniently propagated from seeds as well as branch cuttings. It can also be grown as a profitable non-edible oil crop on irrigated or on partially irrigated lands as a perennial crop (Radich, 2004).

The land should be ploughed once or twice depending on the nature of soil. In the case of heavy soils, deep ploughing is required whereas in light soils shallow ploughing is enough. The seed/cutting should be planted in the main field with the onset of monsoon. Two seeds are dibbled at each spot at spacing of 30 x 30 x 30 cm. When the seedlings are 4 weeks old, weaker seedlings are removed to retain one healthy seedling on each spot and the seedlings so removed could be used for gap filling (Gubitz et al., 1999).

1.5 Plantation of *Jatropha curcas* (Radich, 2004; Gubitz et al., 1999)

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 also 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 greater 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. During its first two years, it needs to be watered during dry season. The use of pesticides is not necessary, due to the pesticide and fungicidal properties of the plant. 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).

1.5.1 Climate conditions

Jatropha can be grown over a wide range of climatic conditions like arid or semi-arid climate conditions. For the emergence of seeds, hot and humid climate is preferred. Therefore, warm summers with rains are beneficial for proper germination of seeds. Jatropha can be cultivated with success in areas with scanty or low rainfall.

1.5.2 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 1 meter or 1.5 x 1.5 meter accommodating 5000 or 4444 plants per hectares were shown to be productive.

1.6 Nature of pollination

Breeding plays a critical role in deciding the route of plant evolution (Grant, 1981). There are three kinds of mechanisms for plant breeding; geitonogamy, xenogamy and apomixsis (Les, 1988). In spite of the common existence of geitonogamy, the tendency to promote xenogamy is still universally significant way of evolution in angiosperms (Stebbins, 1970; Faegri et al., 1979). Plants have various adaptive ways to guarantee xenogamy, such as dioecism (separation between male and female sexual plants), dichogamy (timing separation of male and female sexual functions), opposite styles, styles of different lengths and self-incompatibility (Wyatt, 1983; Fang, 1996). High fruit setting under open pollination reveals 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). The fruit sets of artificial self-pollination, artificial cross-pollination and natural cross-pollination were observed to be 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 et al., 2002). 50% of female flowers set fruit with 53% fecundity rate, 32% apomixis rate and 2:3 seed-ovule ratio (Bhattacharya et al., 2005; Chang-wei et al., 2007 and Abdelgadir et al., 2008) this suggests that fruit production can be increased by manipulating biological processes of pollination and growth.

1.7 Flowering in *Jatropha curcas*

Jatropha curcas, by definition, is a small perennial tree or large shrub, which can reach a height of three to five meters, and can attain a height of 8 or 10 meters under favorable conditions. The plant shows articulated growth (Kumar et al., 2008) straight trunk, thick branchlets with a soft wood and a life expectancy of up to 50 years (Achten et al., 2008). Flowering occurs during the wet season (Raju et al., 2002) often with two flowering peaks, in summer and autumn. In the permanently humid regions, flowering occurs throughout the year (Heller, 1996). The inflorescence is axillary, paniculate, polychasial cymes formed terminally on branches and are complex, possessing main and

co-florescences with paracladia. Flowers are unisexual, monoeceious, greenish yellow colored in terminal long, peduncled paniculate cymes. Male flowers open for a period of 8 - 10 days whereas female flowers open for 2 -4 days.

Characteristics of male and female flowers are:

Male flowers: Calyx segments 5, nearly equal, elliptic or obviate; corolla is campanulate, lobes 5, connate, hairy inside, exceeding the calyx, each lobe bear inside a gland at the base, stamens 10 in two series, outer five filaments free, inner five filaments connate, anthers dithecous erect, opening by longitudinal slit.

Female flowers: Sepals up to 18 mm long, persistent; calyx as in male, corolla 4 scarcely exceeding the calyx lobes united, villous inside; ovary 3-locular, ellipsoid, 1.5–2 mm in diameter, style bifid, ovules solitary in each cell.

The inflorescences form a bunch of green trilocular ellipsoidal fruits yielding approximately 10 or more ovoid fruits (Tewari, 2007) (Figure 1.1). The exocarp remains fleshy until the seeds are mature. Wiehr (Wiehr et al., 1930; Droit et al., 1932).

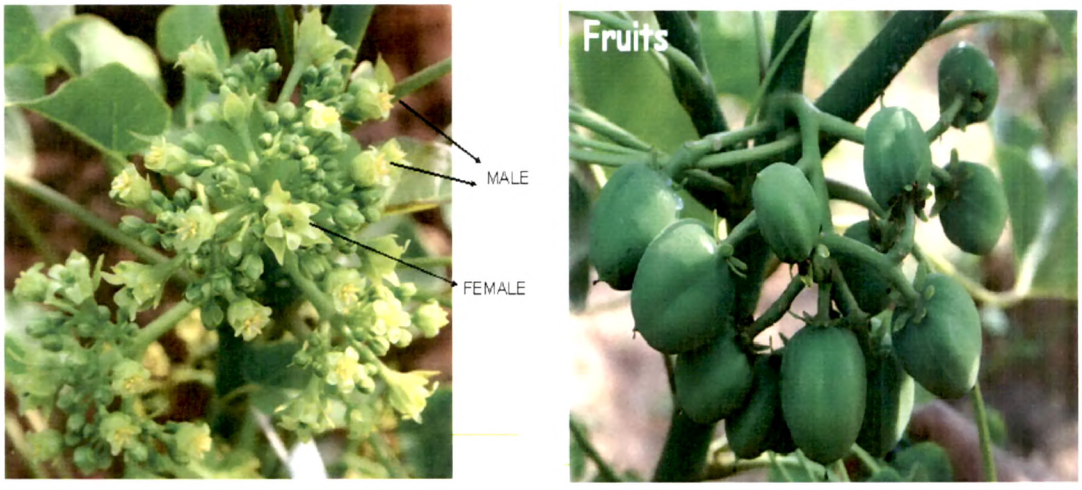


Figure 1.1: *Jatropha curcas* Inflorescence and bloom of fruits

1.7.1 Floral architecture (receptivity of male and female gametes)

Jatropha curcas is monoecious, and produces male and female flowers in the same inflorescence (Figure 1.1). Microsporogenesis and male gametogenesis of *Jatropha curcas* was studied by Liu et al., 2007. Male flowers of *Jatropha curcas* have ten

stamens, each of which bears four microsporangia. The development of the anther wall is of the dicotyledonous type, and is composed of an epidermis, endothecium, middle layer(s) and glandular tapetum. Chang-wei et al., 2007 studied the pollen viability, stigma receptivity and reproductive features and found that, the life span of the male flower is about two days. Its pollen viability is relatively high – 9 h after blooming and gets low 33 h later – and pollen hardly has any viability after 48 h. The life span of the female flower is about 5–12 days. The stigma receptivity is strong during the first four days, begins to decline by 5th day, and completely loses its receptivity on 9th day. There is no obvious secretion on the stigma. The higher proportion of green stigma indicates stronger receptivity. Normally, *Jatropha curcas* shows protandry, where the female flowers open later, with 60% of them opening from 3rd to 5th day. Many unopened male flowers remain even after all female flowers are opened. This provides time for receptive stigma to get pollens from male flowers, and enhances the opportunity of reproductive success. In a few racemes, the female flowers open first, and showed a tendency to promote xenogamy (cross-pollination) and minimize geitonogamy (self-pollination) (Chang-wei et al., 2007). Dhillon et al., 2006, observed flowering at the terminal end of branches after rainy season in India. However, some plants flower even in spring season (March– April). Average male to female flower ratio is 27: 1 and flowering normally starts after a dry and dormant period and continues until soil and water is available (i.e. September to December) (Ezradanam et al., 2002). The arrangement of the flowers is such that a central female flower is surrounded by a group of male flowers (1- 5 female flowers and 25 - 93 male flowers are produced per inflorescence, Ezradanam et al., 2002). The ratio changes drastically (108:1) due to fall in temperature. Usual peak period of flowering varies from 3 to 20 days. Bhattacharya et al., 2005 examined a forenoon pattern of anthesis with subsequent pollen release with each male flower producing 1617 ± 100 pollen with Pollen: Ovule ratio as 539:1. The stigmas become receptive 2 hours after anthesis, coinciding with nectar secretion and pollen presentation schedule. Each female flower (4.54 ± 0.82 µL) produces higher amount of nectar than male flower (1.92 ± 0.44 µL). Qing et al., 2007 recorded 17 species of floral visitors among which 11 species were pollinators (Apis dorsata, A. florea, A. mellifera, Eumenes conica, Vespa sp.) with two floral visiting peaks at 10.00–12.00 and 16.00–17.00 h every day. Among the different insect visitors Apis spp. were the most frequent. Sucrose level influences

flower-visit duration, pollen removal and deposition on stigmas by honeybees (Bhattacharya et al., 2005). Period of fruit development and maturity ranged from 55 to 61 days from date of first fruit initiation (Dhillon et al., 2006).

1.7.2 Potential of *Jatropha curcas* seeds as oil source

The analysis of *Jatropha curcas* seeds shows 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 35 to 40% in the seeds and 50 to 60% in the kernel. The oil contains 21% saturated fatty acids and 79% unsaturated fatty acids (Table 1.1).

Table 1.1: Seed mass, oil content, and protein content of *Jatropha curcas* seeds (King et al., 2009)

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

The seeds of *Jatropha curcas* form within seedpods. Each seedpod typically contains three seeds (Figure 1.2). The typical mass and composition of the seeds is detailed in Table 1.1.

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 analysed, 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 the 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 could potentially be converted to 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. The seeds of *Jatropha curcas* contain a range of toxins and antinutrients resulting in the toxicity of *Jatropha curcas* seeds. The toxicity has been attributed to the presence of a protein (curcin) and phorbol-esters (diterpenoids).

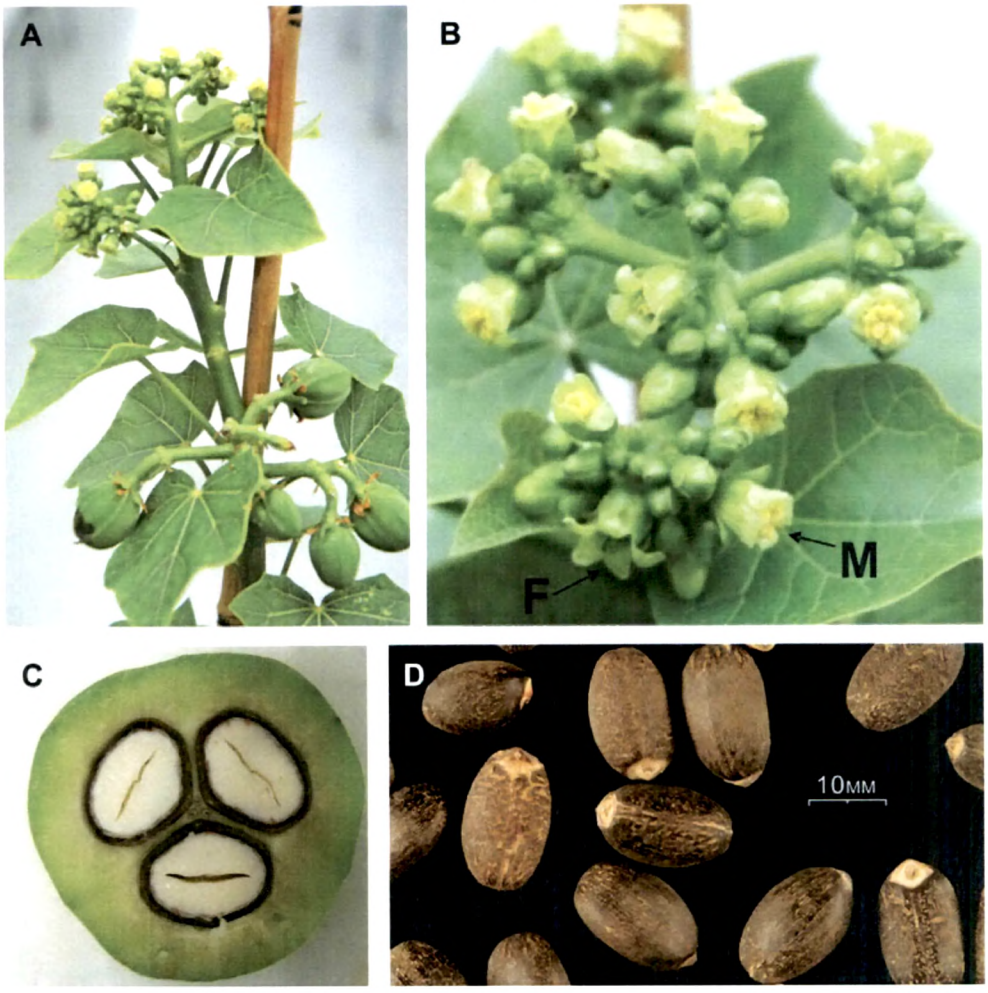


Figure 1.2: Images of *Jatropha curcas* (A) Young *Jatropha curcas* plant with both flowers and developing seedpods. (B) *Jatropha curcas* inflorescence containing both male staminate flowers (M) and female pistillate flowers (F). (C) Cross-section of a *Jatropha curcas* seedpod containing three developing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

1.7.3 *Jatropha curcas* Oil as an energy source

Jatropha curcas oil gives esters of 16-18 carbon chain length compared to diesel that has hydrocarbon of 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-diesel with properties similar to mineral diesel. This reduces their viscosity and increases their cetane number (Table 1.2). However, this requires considerable investment and currently it is not cost effective. As, Jatropha oil is not as cost effective as diesel, except in exceptional circumstances and also as identifying an alternate source of energy is an urgent need, a systematic study of the plant as fuel source and options to improve its inherent property is necessary.

1.7.3.1 Fuel properties

The important chemical and physical properties of *Jatropha curcas* oil have been determined and compared with diesel (Table 1.2). 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.

Table 1.2: Physical and Chemical properties of diesel and *Jatropha curcas* oil

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 number	40–55	38.00
Flash point °C	45–60	210.00
Calorific value, MJ/kg	42–46	38.20
Saponification value	–	198.00
Iodine No.	–	94.00

1.7.3.2 Jatropha oil blend with diesel

Dilution or blending of vegetable oil with other fuels like alcohol or diesel fuel brings the viscosity close to the specification range (Agarwal et al., 1998; Sinha et al., 1997). Jatropha oil has been blend with diesel oil in varying proportions with intention of reducing its viscosity and bringing it close to that of the diesel fuel (Table 1.3). 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.

Table 1.3: Properties of *Jatropha curcas* oil–diesel blends

% 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	0.900	23.447	55.56	Stable mixture
60	40	0.890	19.222	62.13	Stable mixture
50	50	0.853	17.481	66.86	Stable mixture
40	60	0.880	13.953	73.55	Stable mixture
30	70	0.871	9.848	81.00	Stable mixture
20	80	0.862	6.931	86.86	Stable mixture

1.7.3.3 Lipid and Fatty acid composition of seed oil and triacylglycerols

Triacylglycerol (TG), the dominant lipid present in *Jatropha curcas* seed and its constituent fatty acid would determine the properties of biodiesel from Jatropha. The percentage of different lipid classes found in Jatropha oil is given in Table 1.4.

Tabel 1.4: 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	2.0

The fatty acid compostion in *Jatropha curcas* seed oil is well characterized. Fatty acid composition is presented in Table 1.5. 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.

Table 1.5: Fatty Acid composition of seed oil of *Jatropha curcas* (Larson &Graham, 2001)

Fatty acid		Percentage (%)
Myristic acid	14:0	0 - 0.1
Palmitic acid	16:0	14.1-15.3
Stearic acid	18:0	3.7- 9.8
Arachidic acid	20:0	0- 0.3
Behenic acid	22:0	0- 0.2
Palmitoleic acid	16:1	0- 1.3
Oleic acid	18:1	**34.3- 45.8**
Linoleic acid	18:2	**29.0- 44.2**

Though Jatropha oil has high oil content, a beneficial fatty acid composition 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 (Biswas et al., 2007).
- Low seed yield due to a Female: Male flower ratio of 1:25-1:30 in the wild.
- Great variability in TG content in seeds (35% - 45%).

1.7.3.4 Biosynthesis of Triacyglycerol

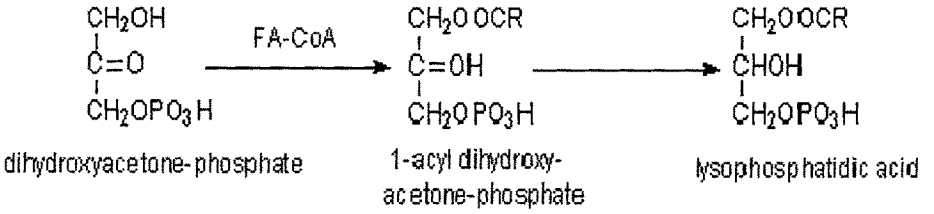
All eukaryotic organisms and even a few prokaryotes have the ability to synthesize triacylglycerols, and the process has been studied extensively in plants. Many cell types and organs have the ability to synthesize triacylglycerols. Within all cell types, triacylglycerols are stored as 'lipid droplets' (also termed 'fat globules', 'oil bodies', 'lipid particles', 'adiposomes', etc) enclosed by a monolayer of phospholipids and hydrophobic proteins, such as oleosins in seeds.

Two main biosynthetic pathways are known in animals, the *Sn*-glycerol-3-phosphate pathway and a monoacylglycerol pathway. In maturing plant seeds and some animal tissues, a third pathway has been recognized in which a Diacylglycerol acyl transferase enzyme (DGAT) is involved.

In the *sn*-glycerol-3-phosphate or α-glycerophosphate pathway, the main source of the glycerol backbone is *sn*-glycerol-3-phosphate produced by the catabolism of glucose or to a lesser extent by the action of the enzyme glycerol kinase on free glycerol. Subsequent reactions occur in the endoplasmic reticulum. First, the precursor *sn*-glycerol-3-phosphate is esterified by a fatty acid coenzyme A ester in a reaction catalyzed by a glycerol-3-phosphate acyltransferase at position *sn*-1 to form lysophosphatidic acid, and this is in turn acylated by an acylglycerophosphate acyltransferase in position *sn*-2 to form phosphatidic acid. The phosphate group is removed by the enzyme phosphatidic phosphohydrolase, and the resultant 1, 2-diacyl-*sn*-glycerol is acylated by a diacylglycerol acyltransferase to form the triacyl-*sn*-glycerol.

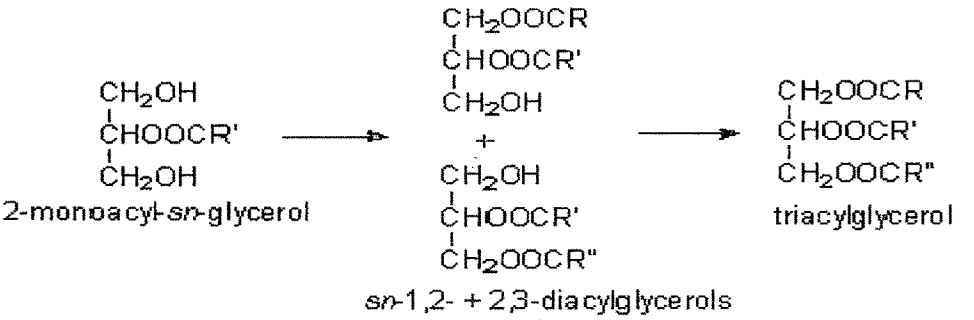
As the glycerol-3-phosphate acyltransferase has the lowest specific activity of these enzymes, this step is considered rate-limiting one.

Lysophosphatidic acid and phosphatidic acid can also be synthesized in mitochondria, but must then be transported to the endoplasmic reticulum before they enter the pathway for triacylglycerol production. In addition, dihydroxyacetone-phosphate in peroxisomes or endoplasmic reticulum can be acylated by a specific acyl-transferase to form 1-acyl dihydroxyacetone-phosphate, which is reduced by dihydroxyacetone-phosphate oxido-reductase to lysophosphatidic acid (Part of the biosynthetic route to plasmalogens), which can then enter the pathway to triacylglycerols.



In the glycerol-3-phosphate pathway, each of the enzymes catalyzing the various steps in the process is distinctive and can have preferences for particular fatty acids (as their coenzyme A esters) and for particular fatty acid combinations in the partially acylated intermediates. Natural triacylglycerols exist in enantiomeric forms with each position of the *sn*-glycerol moiety esterified by different fatty acids.

In plants, many questions remain unanswered such as regarding the nature and compartmentalization of the process. In particular, little is known of how the membrane and storage lipids acquire their very different fatty acid compositions and positional distributions.



In the third biosynthetic pathway, which is less well known, triacylglycerols are synthesized by an acyl-CoA independent transacylation between two racemic Diacylglycerols. The reaction was first detected in intestinal microvillus cells and is catalyzed by a diacylglycerol transacylase. Both diacylglycerol enantiomers participate in the reaction with equal facility to transfer a fatty acyl group with formation of triacylglycerols and a 2- monoacyl-***sn***-glycerol. A similar reaction has been observed in seeds.

1.7.3.5 Disadvantages of Jatropha Oil

Jatropha Oil (bio-oil) and its biodiesel (transesterified form of bio-oil) have some performance disadvantages due to its fatty acid composition. The performance of biodiesel in cold conditions is not as good as that of petroleum diesel. At low temperatures, diesel fuel forms wax crystals, which can clog fuel lines and filters in a vehicle's fuel system. The "cloud point" is the temperature at which a sample of the fuel starts to appear cloudy, indicating that wax crystals have begun to form. At even lower temperatures, diesel fuel becomes a gel that cannot be pumped. The "pour point" is the temperature below which the fuel will not flow. The cloud and pour points for biodiesel are higher than those for petroleum diesel (Radich, 2004). It was reported that biodiesel might be incompatible with the seals used in the fuel systems of older vehicles and machinery, necessitating the replacement of those parts if biodiesel blends are used. The initial use of B20 (proportion of Bio-diesel 20% and petro-diesel 80%) or B100 (Biodiesel 100%) in any vehicle or machine requires care. Petroleum diesel forms deposits in vehicular fuel systems, and because biodiesel can loosen those deposits, they

can migrate and clog fuel lines and filters (Radich, 2004). Another disadvantage being reported is that it tends to reduce fuel economy of the vehicle. Energy efficiency is the percentage of the fuel's thermal energy that is delivered as engine output, and biodiesel has shown no significant effect on the energy efficiency of any test engine. Volumetric efficiency, a measure that is more familiar to most vehicle users, usually is expressed as miles travelled per gallon of fuel (or kilometers per liter of fuel). The energy content per gallon of biodiesel is approximately 11 percent lower than that of petroleum diesel. Vehicles running on B20 are therefore expected to achieve 2.2 percent (20 percent x 11 percent) fewer miles per gallon of fuel (Radich, 2004). To mitigate these problems associated with the use of Jatropha oil as biodiesel biotechnological based approaches are necessary.

1.8 Approaches to improve oil content in *Jatropha curcas*

Jatropha curcas is a diploid species with a 2n chromosome number of 22 (Dehgan, 1984). It is an attractive candidate for genome sequencing with genome size (1C) to be 416 Mbp (Carvalho et al., 2008). *Jatropha curcas* is an introduced plant to many countries of Asia, Africa and Latin America and there have not been many systematic efforts for improvement of this crop. Improved varieties with desirable traits for specific growing conditions are not available, which makes growing Jatropha a risky business (Jongschaap et al., 2007). The development of high-yielding crop varieties through plant breeding has significantly increased agricultural productivity (Evenson and Gollin, 2003). There are a number of traits, which could be targeted for improvement of *Jatropha curcas* as a source of biodiesel, which are increasing seed yield, oil content, and eliminating seed toxicity (phorbolester content). Improvements in seed yield could be achieved in a number of ways. *Jatropha curcas* is monoecious, and has a male: female flower ratio of around 29:1. The plant is insect pollinated, although self-pollination is possible via geitonogamy (Raju and Ezradanam, 2002). Figure 1.2 shows distinct presence of male and female flowers. Increasing the ratio of female flowers may lead to increase in the seed yield. One recent report highlighted a correlation between male: female flower ratio and yield, and noted that this trait was highly heritable (Rao et al., 2008). Yield increases in a number of plant species have also been obtained through

the modification of plant architecture (Sakamoto and Matsuoka, 2004). Increasing the number of branches by application of BA (Benzyl adenine) may lead to an increase in number of inflorescences, and, ultimately, the number of seeds produced per plant (Bang-Zhen Pan et al., 2010). Increasing the oil content in seeds can be achieved by altering the expression levels of enzymes in the triacylglycerol biosynthetic pathway. Overexpression of diacylglycerol acyltransferases has been shown to increase oil content in Arabidopsis (Jako et al., 2001) and soybean (Lardizabal et al., 2008). The regulation of seed development and triacylglycerol biosynthesis in seeds has been studied in some depth (Santos-Mendoza et al., 2008). A number of studies have indicated that it is possible to increase the oil content of seeds via manipulation of the expression levels of key regulators of seed oil accumulation.

Other target traits for improvement of *Jatropha curcas* include synchronous flowering, increase in femaleness by sex modification through **phytohormone**. The main objectives for genetic upgradation of the crop should aim at more number of female flowers or pistillate plants, high seed yield with high oil content, early maturity, resistance to pests and diseases, drought tolerance/resistance, reduced plant height and high natural ramification of branches. Iodine number is under strong genetic control, using genotype having low iodine number will be prelude to efficient bio-diesel yielders with high cetane number (Divakara et al., 2009). *J. curcas* can be improved through assessment of variation in wild source and selection of superior/elite genotypes and application of mutation, alien gene transfer through inter-specific hybridization and biotechnological interventions to bring the change in the desired traits. Enhancement of productivity can be achieved through development of pistillate plants and/or to identify divergent parents, which can later be exploited by heterosis. Development of pistillate plants through mutation and inter-specific hybridization techniques is time consuming. In contrast to this physiological manipulation of sexuality by phytohormones could give a desirable M:F ratio.

The typical seed and oil yield per hectare plantation is given in Table 1.6. If, male to female flower ratio of 27:1 is considered then total number of M:F flowers in one plant would be around 32,400:1200.

Table 1.6: Total flowering, Seed and Oil yield of *Jatropha curcas*

Total Numbers of plant/Hectare	2500
Total seed yield per plant	3-5 Kgs
Numbers of seed per kg.	1200-1500
Male: Female raio	27:1
Numbers of female flowers in inflorescence	1-5
Approx numbers of Male: Female flowers in plant	32,400:1200
Oil yield per plant	900-1500 gms
Total oil yield per hectare plantation	2250 Kgs

An appreciable increase in seed and oil yield by increasing flowering and femaleness through sex alteration can be achieved. There are many reports on other plant system such as Cucumber, Bitter melon etc. in which femaleness has been increased by sex alteration through phytohormone application (Yamasaki et al., 2005). In the same way, if a small increase of 1 to 5 % in female: male flower ratio is achieved by exogenous phytohormone spray on foliar bud then the hypothetical yield would be appreaciable. This is shown in Table 1.7.

Table 1.7: Increase yield in Seed and Oil from Jatropha plantation by phytohormone treatment

If an increase in female flowers is achieved	**By 1%**	**By 5%**
Total seed yield per plant	3.8 - 6.4 Kgs	7.2 – 9 Kgs
Number of seeds per Kg	1200 – 1500	1200 – 1500
Male: Female raio	26.7 : 1.3 (21:1)	23 : 4 (16:1)
Numbers of female flowers in inflorescence	1.3 – 6.4	2.4 – 12
Approx numbers of Male: Female flowers in plant	32064 : 1536	30720 : 2880
Oil yield per plant (gms)	1152 – 1752	2160 – 2660
Total oil yield per hectare plantation	2880 Kgs	5400 KGs
Percentage increase over control	28 %	140 %

1.9 Phytohormones

Plant hormones are small endogenous molecules that regulate plant growth. They act as signal molecules and occur in very low concentration. Hormones regulate cellular processes in targeted cells locally or when moved to other location. Phytohormones are involved in regulating every aspect of plant growth and development like shape of the plants, seed growth, time of flowering, sex of flowers, sex alteration, senescence of leaves and fruits, growth direction i.e. upward or downward, leaf formation, stem growth, fruit development and ripening, plant longevity and even plant death. Phytohormones can be used to increase sex ratio to favour more female flowers. There are five main classes of phytohormones, which are:

- Cytokinin
- Abscisic Acid
- Auxin
- Ethylene
- Gibbrellic acid (GA)

1.9.1 Cytokinin

The cytokinins were discovered in the 1950s during the "golden age" of tissue culture. It has been widely used to stimulate plant cell to divide (i.e. undergo cytokinesis). Cytokinins have been shown to have effects on many physiological and developmental processes such as cell expansion in leaves, stem and roots, nutrient mobilization, apical dominance, the formation and activity of shoot apical meristems, floral development, the breaking of bud dormancy and seed germination. Cytokinin also appears to mediate many aspects of light regulated development, including chloroplast differentiation, the development of autotrophic metabolism, and leaf and cotyledon expansion. Although cytokinins regulate many cellular processes, its control of cell division is the most well studied. It is well known that cytokinin controls branching of shoots in most plant species. The most common form of naturally occurring cytokinin in plants today is called zeatin, which was isolated from corn (Zea mays).

A list of some of the known physiological effects caused by cytokinins is given below. The response will vary depending on the type of cytokinin and plant species (Davies, 1995).

- Stimulates cell division
- Stimulates morphogenesis (shoot initiation/bud formation) in tissue culture
- Stimulates the growth of lateral buds-release of apical dominance
- Stimulates leaf expansion resulting from cell enlargement
- Promotes the conversion of etioplasts into chloroplasts via stimulation of chlorophyll synthesis

1.9.2 Abscisic Acid (ABA)

Abscisic acid was called "abscisin II" originally because it was thought to play a major role in abscission of fruits. ABA promotes desiccation tolerance in the embryo and promotes the accumulation of seed storage protein during embryogenesis. The dormancy response caused by ABA in plants may result from suppression of RNA and protein synthesis. ABA leads to promotion of flowering in short-day plants. Though ABA generally is thought to play mostly inhibitory roles, it has many growth promoting functions as well (Arteca, 1996; Mauseth, 1991; Raven, 1992; Salisbury and Ross, 1992).

The following are some of the physiological responses known to be associated with abscisic acid (Salisbury and Ross, 1992).

- It promotes seed and bud dormancy
- It accelerates leaf abscission
- Stimulates the closure of stomata (water stress brings about an increase in ABA synthesis)
- Inhibits shoot growth but will not have as much affect on roots or may even promote growth of roots
- Inhibits the affect of gibberellins on stimulating de novo synthesis of a-amylase
- Induces gene transcription especially for proteinase inhibitors in response to wounding which may explain an apparent role in pathogen defence

1.9.3 Auxin

The word auxin is derived from the Greek word *auxem*, meaning "to increase" or "to grow". It was the first plant hormone to be discovered and believed to be the "master" plant hormone. Among auxins, Indole-3-acetic, acid (IAA) was the first to be isolated. Auxin is mainly involved in stem elongation. Auxin is also involved in regulation of apical dominance and formation of lateral and adventitious roots. Auxin delays onset of leaf abscission. Auxin promotes fruit development and induces vascular differentiation. Auxin transport regulates floral bud development. Auxin also affects phototropism (growth with respect to light) and gravitropism (growth in response to gravity). Synthetic auxins have a variety of commercial uses. In some plant species, seedless fruit formation is induced by the treatment of unpollinated flowers with auxin. Rooting can be enhanced by dipping excised leaf or stem cutting in an auxin solution. This is the basis of commercial rooting compound, which consists of synthetic auxin mixed with talcum powder. Auxins are widely used as herbicides. 2, 4-D and dicamba are most widely used synthetic auxins. Synthetic auxins are very effective because the plants do not metabolize them as quickly as IAA. Because maize and other monocotyledons can rapidly inactivate synthetic auxins by conjugation, these auxins are used by farmers for the control of dicot weeds in commercial cereal fields (Arteca, 1996; Mauseth, 1991; Raven, 1992; Salisbury and Ross, 1992).

The following are some of the responses that auxin is known to cause (Yunde Zhao, 2010).

- It controls the cell elongation
- It prevents leaf and fruit drop by inhibits zone of abscission
- Stimulates cell division in the cambium and, in combination with cytokinin in tissue culture
- Stimulates differentiation of phloem and xylem
- Stimulates root initiation on stem cuttings and lateral root development in tissue culture
- Involved in assimilate movement toward auxin possibly by an effect on phloem transport

- Delays fruit ripening
- Promotes flowering in Bromeliads
- Stimulates growth of flower parts
- Promotes (via ethylene production) femaleness in dioecious flowers
- Stimulates the production of ethylene at high concentrations

1.9.4 Gibberellin

Gibberellins were first discovered when Japanese researchers noticed a chemical produced by a fungus called *Gibberella fujikuroi* that produced abnormal growth in rice plants. Gibberellins stimulate stem growth in dwarf and rosette plants. They control various aspects of seed germination, including loss of dormancy and mobilization of endosperm reserves. In reproductive development, gibberellin can affect the transition from the juvenile to the mature stage, as well as floral initiation, sex determination and fruit set. The gibberellins are named GA_1....GA_n in order of discovery. Gibberellic acid, which was the first gibberellins to be structurally characterised, is GA_3. There are currently 136 GA identified from plants, fungi and bacteria and every plant has at least 10-12 different GAs.

Active gibberellins show many physiological effects, each depending on the type of gibberellins present as well as the species of plant. Some of the physiological processes stimulated by gibberellins are outlined below (Mauseth, 1991; Raven, 1992; Shinjiro Yamaguchi, 2008).

- Stimulate stem elongation by stimulating cell division in sub apical meristems and cell elongation
- Stimulates bolting/flowering in response to long days
- It leads to stem, leaf and fruit development and expansion
- Stimulates enzyme production (a-amylase) in germinating cereal grains for mobilization of seed reserves
- Induces maleness in dioecious flowers (sex expression)
- It also promotes dormancy and senescence
- It enhances the process of seed germination

1.9.5 Ethylene

Ethylene was identified as the active component of coal gas during nineteenth century. Ethylene is produced in all plants and plays a critical role in ripening of some fruits. It induces lateral cell expansion. It breaks seed and bud dormancy in some species. It promotes the elongation growth of submerged aquatic species. Ethylene induces formation of roots and root hairs and also enhances rate of leaf senescence. Ethylene induces flowering in pineapple family (Arteca, 1996; Mauseth, 1991; Raven, 1992; Salisbury and Ross, 1992).

Ethylene is known to affect the following plant processes (Davies, 1995; Mauseth, 1991; Raven, 1992; Salisbury and Ross, 1992).

- Stimulates the release of dormancy
- Stimulates shoot and root growth and differentiation (triple response)
- May have a role in adventitious root formation
- Stimulates leaf and fruit abscission
- Stimulates flower induction and femaleness
- Stimulates flower and leaf senescence
- Stimulates fruit ripening

1.9.6 Silverthiosulfate ($Ag_2S_2O_3$)

Apart from phytohormones, molecules which alter their levels have also been used. Inhibition of ethylene synthesis by $Ag_2S_2O_3$, has been used to regulate sex determination in plant (Theresa et al., 2002). $Ag_2S_2O_3$ is used as an inhibitor of ethylene synthesis. It plays a role in induction of femaleness in some plants. It has been established that silverthiosulphate enhanced stamen development in female white campion (*Silene latifolia*). In wild type females, stamen development is arrested before the microspore mother cells are formed. In contrast, stamens of $Ag_2S_2O_3$ treated females completed meiosis and produced microspores. Stamen development for these females was incomplete. $Ag_2S_2O_3$ stimulated stamen development to the same extent in asexual white campion that retained a Y chromosome but had lost Y linked genes needed for

early stages of stamen development. It is important to note that $Ag_2S_2O_3$ had no effect on the development of male flowers. $Ag_2S_2O_3$ has a strong masculinising effect on female white campion (Beyere, 1976).

The above discussion has highlighted the role of phytohormones in regulating plant growth and development. More than absolute levels of phytohormones, changes in hormone concentration and tissue sensitivity mediates the range of developmental processes. Many of these involve interactions with environmental factors and each other. In plants, more than in mammals, most of the physiological response, can be attributed to a cross talk between the phytohormones.

1.10 Cross-talk of phytohormones

In understanding the role of phytohormones in regulating flower sex expression, no specific phytohormones have been identified. This may suggest that interactions with other hormones may play a major role in this physiological action. This necessitates the existence of efficient and sensitive cross talk mechanisms among the corresponding signaling pathways (Figure 1.3). Recently, several studies have focused on the molecular machinery behind the interactions between various phytohormones uncovering a complex network. Here the focus is limited to crosstalk between phytohormones during flowering. For the successful development of flower and subsequent fruit set, differentiation of cells has to occur. In all multicellular organisms including plants, the organized destruction of cells is important for differentiation leading to specific organ shapes and for removing unwanted, damaged or infected cells. Thus, the outcome of crosstalk between phytohormones on sex expression could also be via Programmed Cell Death (PCD).

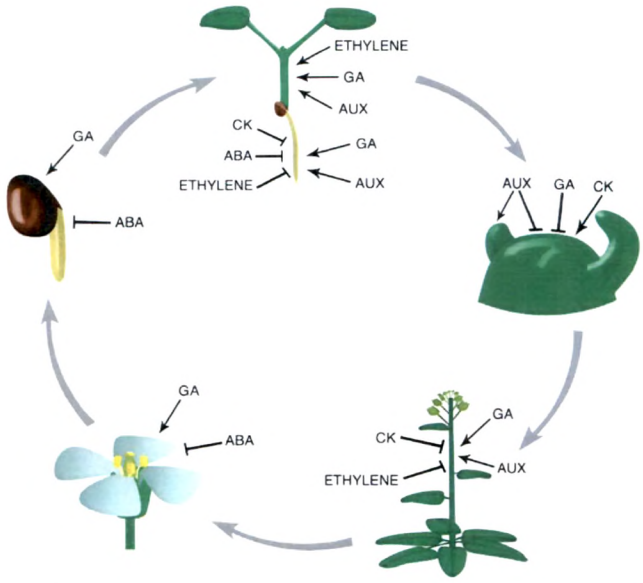


Figure 1.3: Interaction of various plant hormones throughout the plant's life cycle

1.10.1 Phytohormone crosstalk in sex expression in flowers

Endogenous hormones regulate sex expression in plants and this could be mimicked by the exogenous application of hormones or its inhibitors (Grant et al., 2002). No hormone has a consistently masculine or feminine effect on all species. Application of cytokinins increases female flowers in mercury (Durand et al., 1991) and spinach, but have no effect on cucumber and maize (Chailakhyan, 1979). Sex-determination mechanisms involve different hormones in different plant systems (Taiz and zeiger, 2nd edition). It was known that sexual differentiation of higher plants is controlled by sex chromosomes, genetic factors, plant hormones, and/or environmental factors (Dellaporta and Calderon Urrea, 1993; Tanurdzic and Banks, 2004). Higher plants have common plant hormones and the processes involved in their biosynthesis and actions are also reasonably conserved. In spite of this commonality, plant hormones have specific affect on sex expression in each plant. For example, Maize and cucumber have developed specific and different mechanisms for the hormonal regulation of sex expression. In Arabidopsis and tomato, GA deficiency leads to male sterility because of abnormal anther development. GA has been used to increase fruit yield in most studies however, Almeida et al., 2004 have reported decreased fruit yield in response to GA in oranges.

Ethylene-releasing compounds such as Ethrel, have been used to cause male sterility in wheat (Rowell and Miller, 1971; Hughes et al., 1974). Kazmierczak (2004) showed participation of ethylene in GA induced male sex expression in ferns. Ethylene is known to play a critical role in sex determination of cucurbit species. Ethrel induces change in sex expression in bitter melon (Thomas, 2008). Thus, there is a strong possibility that the induction of femaleness by ethylene in cucumber plants is related to regulatory affects of ethylene on expression of specific floral organ identity genes. Also, higher levels of ethylene were detected in gynoecious cucumber plants than in monoecious ones, where GA levels were lower in gynoecious cucumber plants than in monoecious ones (Atsmon et al., 1968; Rudich et al., 1972). In maize plants, GA acts to arrest the stamen primordial in the primary and secondary florets of the ears, and low levels of gibberellins do not cause the arrest of pistil primordial in the primary florets of the ears. On the contrary, in cucumber plants, ethylene acts on both the development of pistil primordia and the arrest of stamen primordia, which results in the induction of femaleness. Therefore, ethylene has opposing effects on the development of sexual organs, stamens and pistils. With regard to the hormonal regulation of sex expression, it is interesting that both plant hormones, gibberellins and ethylene, cause the arrest of stamen primordial in maize and cucumber, respectively. This indicates that ethylene and gibberellin signalling pathways mediate the arrest (by PCD or senescence) of stamen primordial in maize and cucumber. To understand the hormonal regulation of sex expression in plants, therefore, the relationships between plant hormone signaling pathways and PCD needs to be understood.

1.10.2 GA interaction with Auxin

The activities of GA and Auxin overlap with respect to the regulation of cell expansion and tissue differentiation. Auxin affects GA signaling as well as GA biosynthesis (Figure 1.4). In Arabidopsis, GA stimulation of root elongation has been shown to require auxin. GA-induced root elongation was inhibited by the removal of the shoot apex that is a major auxin source, and this effect was reversed by auxin application (Ross et al., 2000; Wolbang and Ross, 2001). Auxin promotes GA responses by destabilizing DELLA, a protein important for repression of GA action, and by promoting

the expression of GA biosynthetic genes. Auxin also affects GA production in the stem by positively regulating the expression of GA biosynthetic genes (Nemhauser et al., 2006), GA20ox in tobacco and Arabidopsis, whereas in pea, the hormone induced the expression of GA3ox and suppressed the expression of GA2ox, which is involved in GA deactivation (O'Neill and Ross, 2002; Frigerio et al., 2006). Loss of function of DELLA genes had no effect on the induction of GA20ox by auxin. Thus, auxin induces GA biosynthesis through a DELLA-independent pathway or via other DELLA like proteins. The effect of auxin on GA biosynthesis was shown to be via the degradation of auxin signaling suppressors Aux/IAA proteins (Teale et al., 2006) and the resulting activation of the transcription factor *AUXIN RESPONSE FACTOR7 (ARF7).* Therefore, auxin positively interacts with GA either at the biosynthesis level or by promoting DELLA degradation.

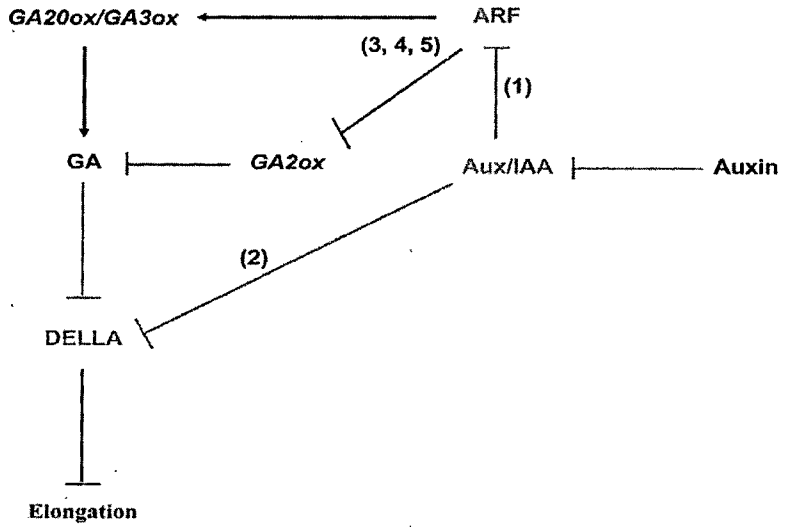


Figure 1.4: Network of interactions between GA and Auxin (Weiss and Ori, 2007). Numbers in parentheses indicate the respective reference as follows: 1, Frigerio et al., 2006; 2, Fu and Harberd, 2003; 3, O'Neill and Ross, 2002; 4, Ross et al., 2000; 5, Wolbang and Ross, 2001. Interactions mediated by changes in protein activity or stability are in gray and those mediated by gene expression are in black.

1.10.3 Interaction between GA and Ethylene

The interaction between GA and Ethylene is rather complex, as both negative and positive reciprocal effects have been seen (Figure 1.5). Ethylene inhibits growth in a GA-antagonistic manner. Achard et al., (2003) have shown that ethylene inhibits the process of growth by its interaction with GA that is mediated by the DELLA proteins. GA promotes seedling root elongation in Arabidopsis, and this effect is inhibited by ethylene. In agreement with this, ethylene inhibited RGA degradation in root-cell nuclei in response to GA. The effect of ethylene on RGA stability was mimicked by the loss of its signaling suppressor *CONSTITUTIVE TRIPLE RESPONSE1 (CTR1)*, suggesting that ethylene's RGA stabilizing signal is transduced via a *CTR1*-dependent pathway (Guo and Ecker, 2004). Furthermore, ethylene delayed the transition to flowering in Arabidopsis under short day condition, and this effect was suppressed by GA treatment (Achard et al., 2007). While in seedlings, ethylene was shown to affect DELLA stability directly, during the transition to flowering, it affected GA biosynthesis. The resulting reduction in the level of biologically active GAs repressed the expression of two central flowering genes, *LEAFY (LFY)* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1)* and the consequent transition to flowering. This effect of ethylene on the accumulation of active GA is transduced via *CTR1* and the downstream transcriptional regulator *ETHYLENE INSENSITIVE3*. This study suggests that the antagonistic interaction between Ethylene and GA mediates the timing of the decision to flower in response to changing environmental conditions. Ethylene represses GA biosynthesis or suppresses GA responses via DELLA stabilization.

GA is a major factor controlling cell elongation in light-grown hypocotyls and ethylene enhances this effect. Whether the DELLA proteins mediate this positive interaction, i.e. ethylene reduces DELLA stability, is not yet known. These studies indicate that GA and ethylene stimulate each other's activities reciprocally under specific circumstances. Ethylene plays a central role in regulating the plant's developmental reaction to stress. Submergence promotes ethylene responses in dark and light grown seedling (apical hook formation in the dark and hypocotyls elongation in the light). Hence, depending on the developmental process and environmental conditions, ethylene

interacts both positively and negatively with GA. Furthermore, the interaction between these two hormones operates at both the biosynthesis and signal transduction levels, exhibits reciprocal effects of these hormones on one another, and involves both additive and synergistic effects.

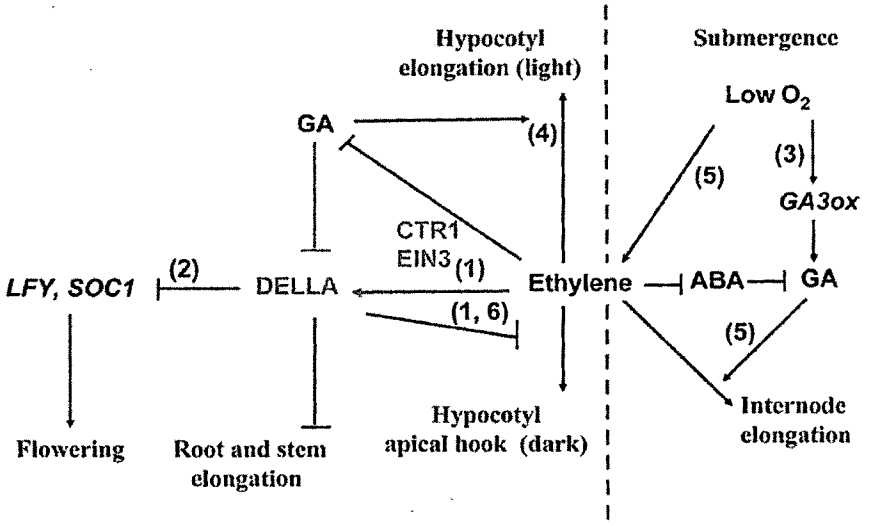


Figure 1.5: Network of interactions between GA and Ethylene (Weiss and Ori, 2007). Numbers parentheses indicate the respective reference as follows: 1, Achard et al., 2003; 2, Achard et al., 2007; 3, Benschop et al., 2006; 4, Sabio et al., 2003; 5, Sauter et al., 1995; 6, Vriezen et al., 2004. The interaction between phytohormones and the changes mediated in protein activity or stability (shown in gray) and those mediated by gene expression (shown in black).

1.10.4 Interaction between GA and Cytokinin

GA and Cytokinin exert antagonistic effects on numerous developmental processes, including shoot and root elongation, cell differentiation, shoot regeneration in culture, and meristem activity (Greenboim-Wainberg et al., 2005; Jasinski et al., 2005). Several recent studies have shown development-dependent reciprocal interactions between the two hormones, where cytokinin inhibits the production of GA and promotes its deactivation and GA inhibits cytokinin responses (Figure 1.6). High cytokinin and low GA signals are required for normal shoot apical meristem (SAM) function

(Sakamoto et al., 2001; Jasinski et al., 2005; Yanai et al., 2005). *KNOXI* (KNOTTED1-like homeobox proteins) controls the balance between the GA and cytokinin hormones in SAM by inducing cytokinin production, directly inhibiting GA synthesis, and indirectly promoting GA deactivation. *SPY* (Spindly) regulates the balance between the response pathways of these two hormones via suppression of GA signal and promotion of cytokinin responses. Interestingly, GA and *SPY* suppress phenotypes caused by *KNOXI* overexpression (Hay et al., 2002). Hence, cytokinin and GA act mostly in an antagonistic manner. The reciprocal interaction is regulated at both the biosynthesis and signal transduction levels.

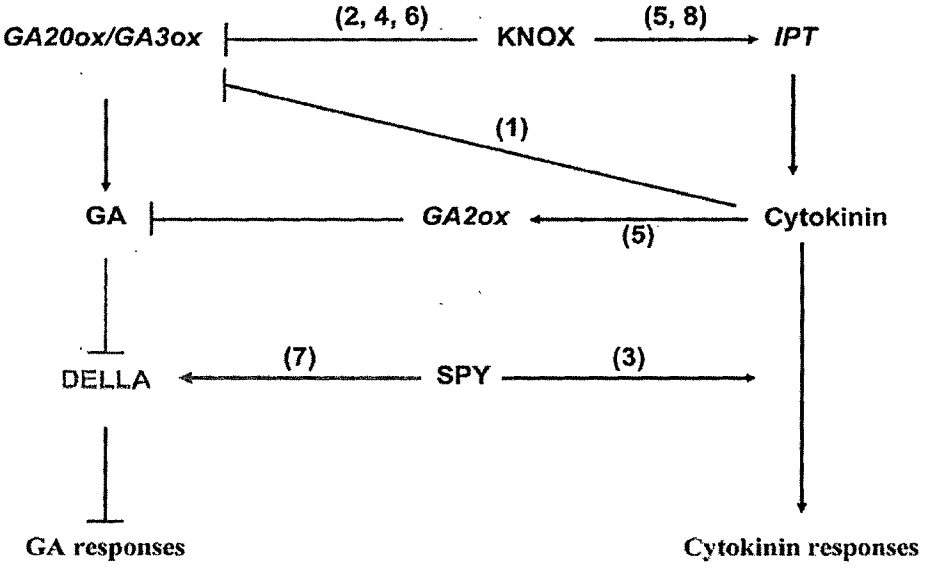


Figure 1.6: Network of interactions between GA and Cytokinin (Weiss and Ori, 2007). Numbers in parentheses indicate the respective reference as follows: 1, Brenner et al., 2005; 2, Chen et al., 2004; 3, Greenboim-Wainberg et al., 2005; 4, Hay et al., 2002; 5, Jasinski et al., 2005; 6, Sakamoto et al., 2001; 7, Silverstone et al., 2007; 8, Yanai et al., 2005. The interaction between phytohormones and the changes mediated in protein activity or stability (shown in gray) and those mediated by gene expression (shown in black).

1.10.5 Ethylene and Auxin interactions

Early observations showed that ethylene and auxin could each regulate the activities and levels of the other. For example, auxin can reduce the ability of ethylene to accelerate ageing dependent processes such as ripening and abscission. Auxin [IAA, 2, 4-D, and Naphthalene acetic acid (NAA)] also increases the rate of ethylene production, for example in Etiolated mung-beans (Grierson et al., 1982). The function is achieved, at least in part, by its interactions with other hormone signalling pathways. ACC synthase (ACS) catalyzes the step from S-adenosyl methionine (SAM) to 1-aminocyclopropane-1-carboxylate (ACC), following which ACC oxidase catalyzes the final step in ethylene formation. It was known that ACC synthase genes expressions are regulated by Auxin. The highest amount of IAA-induced ethylene occurred in the root or inflorescence tip, with regions below this producing less, suggesting that ethylene and auxin synergistically regulate these developmental processes (Figure 1.7). The enhancement of feminization by Auxin possibly occurs through the induction of ethylene biosynthesis (Takahashi et al., 1984). Ethylene biosynthesis in the abscission zone is regulated by Auxin (Taiz and Zeiger 2nd edition). Auxin can induce flowering in pineapple through ethylene biosynthesis (Burg and Burg, 1966).

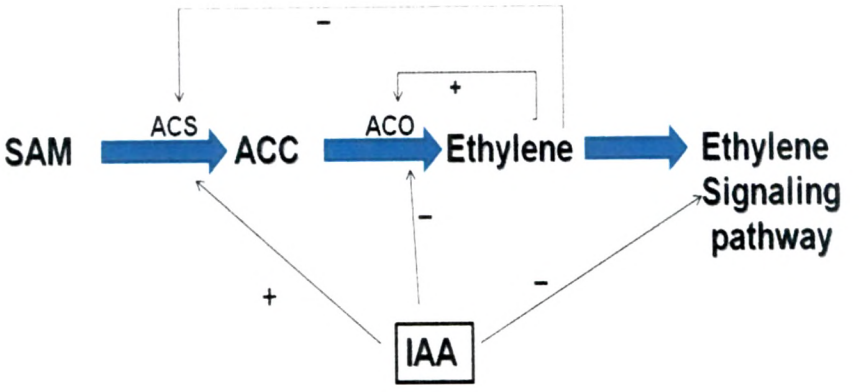


Figure 1.7: Regulation of Ethylene biosynthesis by IAA (Malgorzata et al., 2002)

1.10.6 Crosstalk between phytohormones in Abscission due to Cell death

For plants to develop properly and survive, PCD is an important response strategy, which controls cell death leading to plant development and survival (Lam E, 2004). If cells are no more needed they die by activating intracellular death program termed Programmed cell death (PCD). PCD is a key process for normal cell and organ development, integrity and homeostasis, in all multicellular organisms. Its involvement in development serves many functions including: 1) sculpting structures, 2) deleting unwanted structures, 3) controlling cell numbers, 4) removing abnormal, misplaced, non-functional or harmful cells, 5) producing differentiated cells without organelles and 6) generating functional structures.

During development, plants often shed leaves, fruit and flowers independently through a complex and highly regulated process known as abscission. Abscission is preceded by abscission zone (AZ) formation. Hydrogen peroxide is the chief mediator of AZ, which eventually leads to PCD and hence abscission. Ethylene and Abscissic acid have been implicated as main regulatory phytohormones responsible for Abscission and cell death. PCD occurs in many processes in plants, including: reproductive development (*e.g.* embryogenesis; flower petal senescence) and vegetative development (*e.g.* xylogenesis; lysigenous aerenchyma formation). PCD is also seen as a response to stress or environmental conditions, *e.g.* salt-stress or pathogen attacks. In plants, PCD is classified into three different types based on morphological features. **Apoptosis-like cell-death**, which occurs in some stress-related and developmental processes, involves rapid degradation of the nucleus and loss of cell organization. DNA laddering, nuclear shrinkage and chromatin condensation are the main characteristics of Apoptosis. It is often induced by signalling through Mitochondria and Reactive oxygen species. The second type is **cell death occurring during senescence and abscission processes.** This very slow process is associated with high recovery of cell contents and optimal reallocation of nutrients. The third type of cell death, illustrated by **tracheary element differentiation**, is PCD induced by vacuolar degradation (Figure 1.8). It involves the central action of vacuole-localised proteases, which once released into the cytosol degrade the cell contents then finally, under the influence of the lytic enzymes, the

nuclear DNA breaks down. The disruption of both the nucleus and the vacuole occurs at the very end of the cell death process, after complete degradation of the plastids.

PCD during treachery element differentiation is initiated and regulated by Auxin and Cytokinin and utilizes a defined signal transduction pathway that includes a calcium flux shown to be necessary and sufficient to initiate death. An extracellular signal triggers this calcium influx, which leads toward the disruption of the vacuole. Ethylene is important for inducing cortical root cells to die and autolyse during lysigenous and aerenchyma formation. Ethylene also evokes a signaling pathway that involves a calcium influx during aerenchyma formation that is necessary for this type of death. Calcium increases prior to gibberellin-induced death of aleurone cells. It is clear that calcium plays a common central role in death execution.

Cells integrate various combinations of survival and death signals to decide whether to die and subsequently how the corpse will be managed. This acquired program of death begins well before cells die. How the cell corpse is managed is a function of vacuole hydrolases (and toxins) that are loaded into the vacuole and these are regulated by several signals. Death is triggered and two events are shared among most plant cell deaths which are calcium flux and vacuole collapse. Collapse of the vacuole marks the beginning of corpse management. The different profiles of hydrolases loaded into the vacuole determine the manifestation of death. For tracheary elements, the protoplasm but not secondary cell walls are autolyzed. During the formation of lysigenous aerenchyma, the entire corpse is removed, whereas the corpse from the hypersensitive response is left to be crushed by expanding tissues. Death and corpse management for senescing cells is similar. However, many obvious signs of cell disassembly occur before vacuole disruption in senescent cells. This skeletal model is intended to serve as a unifying theory of many, but not all, PCDs in plants and represents those features of PCD that are in common. This indicates the fact that many of the cytoplasmic changes that are occurring during the "preparation to die" differ between programs and that these changes are an integral part of the manifestation of death.

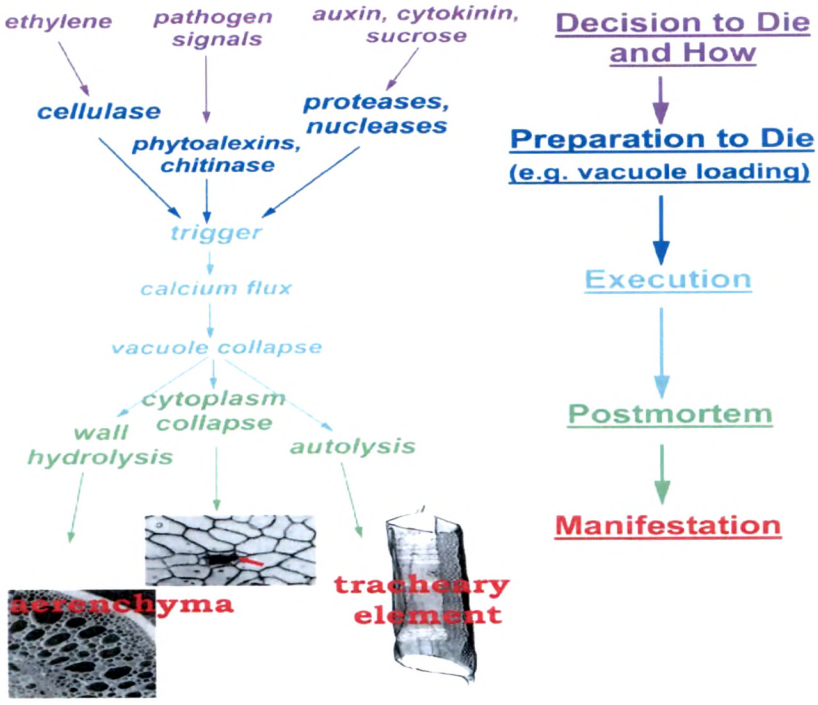


Figure 1.8: A model for general mechanism of PCD in plant

In Barley aleurone protoplasts, GA has been known to synchronize and accelerate the procsess of cell death (Kuo et al., 1996). The Mesocarp tissue of winter squash (Cucurbita maxima) fruit produces a large amount of ethylene in response to wounding (Hyodo et al., 1983). Ethylene is involved in H_2O_2 release during PCD in tomato suspension cells. Ethylene is indispensable for PCD in tomato suspension cells. Ethylene is powerful potentiator of H_2O_2 accumulation, which subsequently leads to PCD. Ethylene is involved in the regulation of cell death in pea carpel senescence (Orzaez and Granell 1997). Ethylene is also involved in induction of cell death in ozone-exposed plants (Overmyer et al., 2000). In Arabidopsis, ethylene was shown to be essential for normal floral organ abscission (Butenko et al., 2003; Patterson and Bleeker, 2004). Ehylene is the major co-ordinator of senescence in many flowers. The deterioration of the corolla in such species is hastened by exogenous ethylene and senescence is accompanied by increased endogenous ethylene biosynthesis (Nicolas, 1977). In Maize root, Aerenchyma formation (A key characteristic of PCD) is specifically initiated by ethylene produced endogenously or applied exogenously (Drew et al., 1979).