DIVERSITY AND PROPAGATION STUDIES OF *JATROPHA CURCAS* L.

THESIS SUBMITTED TO UNIVERSITY OF PUNE
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IN BOTANY

BY

Mr. GOPALE KALURAM DNYANESHWAR

UNDER THE GUIDANCE OF

DR. RAJENDRA S. ZUNJARRAO

HEAD,
DEPARTMENT OF BOTANY
AND
PRINCIPAL,
P. E. SOCIETY'S MODERN COLLEGE OF ARTS SCIENCE AND COMMERCE,
SHIVAJINAGAR, PUNE 411005

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Declaration by the Candidate

I declare that the thesis entitled '**Diversity and Propagation Studies of *Jatropha curcas* L.',** submitted by me for the degree of Doctor of Philosophy in botany is the record of work carried out by me during the period from 20th October, 2007 to 20th October 2011, under the guidance of Dr. Rajendra S. Zunjarrao and has not formed the basis for the award of any degree, diploma, associateship, fellowship, titles in this or any other University or other institutions of Higher learning.

I further declare that the material obtained from other sources has been duly acknowledged in the thesis.

Place : Pune

Date :

Mr. Gopale Kaluram Dnyaneshwar

(Research Scholar)

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Abstract

'Diversity and Propagation Studies of *Jatropha curcas* L.'

The present study provided most recent and useful insights on the implications for investments on *Jatropha* in Maharashtra. It also provides the most recent and realistic information to researchers, farmers and organizations who intend to set up large scale or small scale plantation sites in Maharashtra. Much information has been collected during the research period (2007-2011) about *Jatropha*, towards learning to distinguish between 'reality' and 'promise'. Data have been presented on Morphological diversity, Biochemical diversity, Molecular diversity and Propagation systems. A reflection is also given about long-term sustainability.

Morphological diversity

Weight of the whole seeds and its components showed maximum weight in Marathwada sources while it was significantly low for Kokan sources. The coefficient of variance (CV) for whole seeds, seed coat and kernel were recorded 12.67, 7.05 and 10.25% respectively. Seed size, i.e. length, width and thickness, has also shown a similar trend. Maximum seed size was recorded for Marathwada source and minimum for Western Maharashtra source. The CV of these traits was found to be 1.76, 1.00 and 2.67% respectively. Variation in seed viability, germination percentage, germination energy was found significant among different seed sources with CV of 8.49, 12.54, and 10.31 % respectively. Maximum values for these parameters were recorded for Marathwada seed sources while minimum for Kokan sources. The highest phenotypic variance was found for germination energy, while lowest for below ground biomass. The 'genetic variance' varied from 116.66 to 0.039 for germination energy and below ground biomass. In 'environmental variance', the highest value of germination was 16.52 for PCV whereas it was 16.24 for GCV.

The patterns of variation exhibited for various characters were substantially different. The presence of such difference among populations has probably produced by different intensities of natural selection acting upon these traits in their natural habitat. Some of the variation found associated with the discrete populations from which seeds were collected. The Kokan seed sources in the present study were ranked low, in comparison to the growth producing sources, i.e. Marathwada and Vidarbha. It is

indicative that there is a better choice of using selected promising genotypes and population from the range of distribution. The seed sources in most of the cases were significantly different growth variables. It showed a considerable amount of genetic variability within the distribution range indicating a good scope of genetic gain through selection.

The present study showed that there exists considerable amount of genetic variability in this species in Maharashtra with respect to growth performance, which offers scope to breeder for selection and breeding. It is advisable that these seed sources should be used for collection of bulk quantity of seeds to achieve better productivity. It is quite clear that Marathwada source is good in growth performance, particularly in the prevailing conditions in Maharashtra. The Western Maharashtra and Vidarbha sources also performed satisfactory in respect of growth parameters. Perhaps, this is a first attempt and report, which surveys and assesses morphological diversity of *J. curcas* population from four agro-climatic regions of Maharashtra.

Biochemical diversity

The *Jatropha* oil contains Palmitic, Stearic, Oleic and Linolenic acids in good proportion. Although there is a growing demand for quality planting material of *Jatropha*, efforts are lacking for the selection of elite planting material based on their biochemical composition. Seed protein, oil and phorbol ester contents revealed variation with accessions from said four regions. Presence of the toxic phorbol esters is a major concern and analysis of twenty accessions resulted in identification of high and low phorbol ester contents. The overall oil percentage in the said agro-climatic regions varied from 26 to 37%. Marathwada, Vidarbha and W. Maharashtra regions showed 34.37, 34.78 and 34.25% respectively, while Kokan region was showed 27.22%. The oil samples were further analyzed by GLC to study the variability of fatty acid composition. Significant variability of fatty acid was also observed. The Palmitic acid ranged from 14 to 36 %, Stearic acid from 4 to 15%, Oleic acid from 38 to 58% and Linolenic acid from 11 to 43%. Rich diversity among the ecotype of Maharashtra, in terms of oil percentage, Phorbol ester content, and fatty acids profile, indicates the need for exploitation of germplasm in breeding programme.

There was no significant variation at inter population level of Phorbol ester in four agro-climatic regions of Maharashtra. While, at intra population level significant variations were found, which vary from 0.66 to 4.40mg/g. It indicates the synthesis of Phorbol ester was under the strong influence of genetic control. In the present study, it was also observed that, there is linear relationship between protein and oil content. Significant variability in fatty acids was observed.

Molecular diversity

Random amplified polymorphic DNA (RAPD) markers were used to evaluate the genetic diversity in representative population of *Jatropha curcas L*. from four agro-climatic regions of Maharashtra. Each region was uniformly represented by five accessions. Ten selected markers have been used for amplification. A total of 125 DNA bands were obtained, of which 94 (74.62%) were polymorphic. The polymorphism was scored and used in band sharing analysis to identify genetic relationship. Cluster analysis based on Jaccard's similarity coefficient using UPGMA grouped all the 20 genotype in to two major groups at similarity coefficient of 0.54. Similarity indices from the 20 genotypes selected ranged from 0.14 to 0.98 with average of 0.63, indicating moderate to high genetic variability among the genotypes. The highest similarity coefficient was detected from Kokan region accessions and lowest to moderate from W. Maharashtra, Marathwada and Vidarbha. In addition, the *Jatropha* populations from diverse agro-climatic regions were more dispersed on the principle co-ordinate plot, revealing a wide genetic base. The results of molecular diversity study revealed that *J. curcas* germplasm within Maharashtra constitutes a broad genetic base. From the clustering pattern and genetic relationship obtained from different clusters can be employed in their future breeding programme.

Propagation studies

A cutting treated with 2000mg/l IBA showed 78% survival during Spring (summer) season while, in Monsoon and Winter it was 62% and 54%, respectively. Sand: Soil: Vermicompost (1:2:1), was found to be better substrate with 78% rooting and survival. Similarly, Sand: Soil: Cocopeat (1:2:1) and Sand: Soil: FYM (1:2:1) showed satisfactory survival percentage, which was 70% and 72%, respectively, in spring season. Stem cuttings with 20 cm length were suitable for quicker regeneration. It

showed 76% of survival and rooting in Spring season. Spring season is more suitable for quicker vegetative propagation of *Jatropha curcas*. For the large-scale production of genetically pure and improved planting material of *J. curcas*, vegetative reproduction through 20cm long stem cutting treated 2000mg/l IBA for 5min. (IBA) is the most effective and economic method in Spring season. A trend of relatively better survival and rooting of stem cuttings were obtained with Sand: Soil: Vermicompost (1:2:1) as compared to other substrate with 20cm length of stem cuttings. The observed results could be due to better aeration and high water holding capacity of substrate. The further study should evaluate the field performance of such plant produce by stem cuttings.

Hence, this study implies that the stem cuttings with above mentioned growth hormone treatment, size of stem cuttings and type of substrate help to develop uniform plant stock in nursery during Spring season.

In-vitro study revealed that leaf disc was the best explants for micro propagation through direct induction of adventitious shoots when cultured on Murashige and Skoog's (MS) medium supplemented with thidiazuron (TDZ) (2.27 µM), 6-benzylaminopurine (BA) (2.22 µM) and indole-3-butyric acid (IBA) (0.49 µM). The presence of TDZ in the induction medium has greater influence on the induction of adventitious shoot buds, whereas BA in the absence of TDZ promoted callus induction rather than shoot buds. Induced shoot buds were multiplied and elongated into shoots following transfer to the MS medium supplemented with BA (4.44 µM), kinetin (Kn) (2.33 µM), indole-3-acetic acid (IAA) (1.43 µM) and gibberellic acid (GA_3) (0.72 µM). Well-developed shoots were rooted on half strength MS medium supplemented with IBA (0.5 µM) after 30 days. Regenerated plants after two months of acclimatization were successfully transferred to the field without visible morphological variation. This protocol might find use in mass production of true-to-type plants and in production of transgenic plants through Agrobacterium/ biolistic-mediated transformation.

Embryogenic callus were also obtained from leaf explants on MS basal medium supplemented with only 9.3 µM Kn. Induction of globular somatic embryos from 48% of the cultures was achieved on MS medium with different concentrations of 2.3 to 4.6 µM Kn and 0.5 to 4.9 µM IBA; 2.3 µM Kn and 1.0 µM IBA proved to be the

most effective combination for somatic embryo induction in *Jatropha curcas*. Addition of 13.6 µM adenine sulphate stimulated the process of development of somatic embryos. This protocol of somatic embryogenesis in *Jatropha curcas* may be an ideal system for future transgenic research. However, asynchronous development of somatic embryo is major limitation to use this protocol for mass multiplication of this plant, further research in this direction is required.

Significant outcome of the study

- Morphological, Biochemical and Molecular diversity showed that the considerable amount of genetic variability within the four agro-climatic regions of Maharashtra. Which offer ample scope to breeders for the selection and breeding.

- Marathwada seed sources showed better performance, thus this region is ideal for the plantation of *J. curcas* as compared to the rest of the regions.

- Present study showed that highest oil percentage (34.78%) was recorded for Marathwada region. There is no significant variation in phorbol ester (toxic substance) within said agro-climatic regions. It indicates the synthesis of phorbol ester was under the strong influence of genetic control.

- Mass multiplication of *J. curcas* can be carried out by using stem cutting method in Spring season. Where 20cm long stem treated with 2000mg/l IBA and substrate was san:soil:vermicompost (1:2:1).

- *In vitro* mass multiplication could be done by using leaf disc as explant on MS medium supplemented with TDZ (2.27 µM), BAP (2.22 µM) and IBA (0.49 µM).

- Somatic embryogenesis could be induced in development of somatic embryo, however asynchronous development of somatic embryo is major limitation to use this protocol for further multiplication.

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Abbreviations

TTC	2, 3, 5-Triphenyl Tetrazolium Chloride
Vp	Phenotypic variance
Vg	Genotypic variance
Ve	Environment variance
PCV	Phenotypic Coefficient of Variation
GCV	Genotypic Coefficient of Variation
ECV	Environment Coefficient of Variation
IS	Indian Standard
GLC	Gas Liquid Chromatography
HPLC	High Performance Liquid Chromatography
PCR	Polymerase Chain Reaction
RAPD	Randomnly Amplified Polymorphic DNA
RCBD	Random Complete Block Design
FYM	Farm Yard Mannure
LSD	Least Significant Difference
CV	Critical Variance
SE	Standard Error
PGR's	Plant Growth Regulators
SE's	Somatic Embryos
BAP	6-Benzylaminopurine
GA_3	Gibberlic acid
IAA	Indol-3-acetic acid
IBA	Indol-3-Butyric acid
Kn	Kinetin
MS	Murashige and Skoog medium
TDZ	Thidiazuron

Contents

1. Introduction

India is targeting economic growth rate of 8-9 % in coming years. It is likely to have a significant consumption of energy resources in future for meeting the targeted growth rate and fulfilling the energy needs of its increasing population. It lacks sufficient domestic energy resources to meet its growing energy requirements. In India, it has projected that there is need to increase the primary energy supply by at least 10-12 times from their 2003-04 levels by 2031-32 in order to maintain 8% growth rate. Maximum contribution of renewable energy in an optimistic scenario will be around 5-6% by 2031-32 with an import dependence on crude oil is expected to be in the range of 90% in next decade making energy security a concern (Planning Commission of India, 2006).

Crude oil is the largest consumed fossil fuel after coal in India. Known crude oil reserves are estimate to be depleted in less than 50 years, at the present rate of consumption (A report by US Department of Agriculture and Energy, 1998). Many countries lacking crude oil resources are facing foreign exchange crisis and high inflation rate mainly due to import of crude oil (Demirbas, 2005). It has been advocated to have large-scale usage of renewable energy to meet the energy challenges in India (Ravindranath and Balachandra, 2010). India's energy supply system has failed to meet the growing needs and at the same time contributing significantly to the environmental degradation.

It is believed that dependence on imported crude oil, environmental issues and employment in rural areas are reasons for replacement of fossil fuels by biofuels (Senthilkjumar and Gunasekaran, 2005). Self-sustainable energy sources are likely to hold the key to economic development of India in future. India should not look towards a certain group of countries to meet its ever growing needs but it is mandatory to seriously implement bioenergy development programs as a part of environmental sustainability in the form of clean development mechanism (CDM) (Kumar et al., 2010). The CDM, established by the Kyoto Protocol, promotes the industrialized nations to provide resources to developing countries in order to support their sustainable development, while at the same time reducing the global green house gasses (GHG) emissions.

Increasing population density and the additional decline in availability of productive land due to degradation would further, the large-scale migration of people into the already overpopulated metropolis in Asia and Africa in future, unless urgent corrective measures adopted to increase rural incomes and generate new perspectives in the weaker regions. Economic development in many developing countries has led to huge increases in the energy demand.

As most of the countries now enjoying rapid development (China and India e.g. - both countries already rank among the top five net CO_2 emitters in the world) are also large petroleum importers, their dependence on external energy sources from highly unstable regions would increase to uncomfortable levels. Energy security has thus become a key issue for many countries. India produces about 25% and imports 75% of its oil requirement. India is the least explored region for oil. India's import bill is about Rs.1500 billion per annum and consumption is about 2% of world's oil. Moreover, due to uncertain supplies and fluctuations in prices of fossil fuel in international market, the need to search renewable, safe and nonpolluting sources of energy assumes top priority (Ravindranath and Balchandra, 2010).

1.1 Global status of bio-diesel requirement

The global interest in biofuels was not unnoticed. The keen interest in biofuels is mainly inspired by climate change issues, aiming to reduce CO_2 emissions, as well as by geopolitical issues, aiming to reduce nation's dependence on fossil fuels (Verrastro and Ladislaw, 2007). Biomass as a source of renewable energy is fundamental for development and sustenance of civilization. In view of growing interest for renewable energy sources, liquid bioenergy production from vegetable oils is one of the possible options to reduce greenhouse gas (GHG) emissions. Bio-diesel production from vegetable oils during 2008–2009 estimated to be 4.36 million tones globally.

1.2 Bio-diesel plants

Bio-diesel is expanding very fast because of demand, necessary policy support and technological availability. India consumes approximately 40 million tones of diesel and ranked fifth in the world after the US, China, Russia and Japan in terms of fossil fuel consumption. Recently, Government of India launched ‘‘National Mission on Bio-diesel" with a view to find a cheap and renewable liquid fuel based on vegetable

oils (Shukla, 2005). However, shortage of raw material to produce bio-diesel is a major constraint (Wani et al., 2006). The total number of oil-bearing species range from 100 to 300 and of them 63 belonging to 30 plant families hold promise for bio-diesel production (Hegde, 2003). Many developed countries are using edible oil seed crops such as soybean, rapeseed, groundnut, sunflower for production of bio-diesel. However, developing countries like India, having scarcity of huge quantity of edible oil (6.31 million tonnes) for consumption, cannot afford to use edible oils for bio-diesel production and hence non-edible oil seeds such as *Pongamia (Pongamia pinnata* Vent.*),* Mahua (*Madhuca indica* L.) and *Jatropha (Jatropha curcas* L.*)* are important plants. *Jatropha cur*cas takes a special place in this debate, as it is claimed to produce biofuel and enhance socio economic development while, reclaiming marginal and degraded lands in semi-arid regions, without competing with food production or depleting natural carbon stocks and ecosystem services (Francis et al., 2005).

The seeds were crushed to extract raw oil, a process that also provides organic fertilizer from the husks. *Jatropha* oil is used for making soap, candles, varnish and lubricants, etc. The oil can be mixed up to 50% and used for tractors and oil engines. For initiating rational use of wild plants, knowledge of genetic variability of concerned species is necessary. Introduction and evaluation of provenance progenies is an essential aspect in agroforestry research (Kaushik, 2007). The variation could be a useful source of future genetic selection, provided the desired ideo types for agroforestry are clearly defined. Genetic variation in seed physiology and oil content of *Jatropha curcus* L. can be of great potential in tree improvement programmes, particularly selection of genotype.

1.3 *J. curcas* L. as a choice plant

The much required green energy revolution would provide India an opportunity to change its standing from a fuel importing nation to one that generates clean and affordable energy. Among various alternatives to diesel, Planning Commission of India has identified *Jatropha*, a non-edible oil bearing tree capable of producing oil that is easily convertable in to bio-diesel with properties almost similar to diesel (Planning Commission of India, 2003). *Jatropha curcas* plant is a drought resistant, perennial plant living up to 50 years and has the capability to grow on marginal soils.

It requires very little irrigation and grows in all types of soils, thus making *Jatropha* a more sustainable choice than other vegetable oils (Reubens et al., 2011). *Jatropha* bio-diesel can be used for decentralized microgrid electricity generation at the village/taluka level and as a replacement for diesel fuel in irrigation pump sets, diesel generators and as an alternative to kerosene. Thus, *Jatropha* bio-diesel has to be seen, not in isolation but as part of a total environment/energy management system.

J. curcas is native of tropical America, but is now found abundantly in many tropical and sub-tropical regions throughout Africa and Asia because of likely distribution by Portuguese ships via the Cape Verde islands and Guinea Bissau. *J. curcas* has spread beyond its original distribution because of its hardiness, easy propagation, drought endurance, high oil content, low seed cost, short gestation period, rapid growth, adoption to wide agro-climatic condition, bushy/shrubby nature and multiple uses of different plant parts. Added to this, qualitative sustainability assessment, focusing on environmental impacts and strengthened by some socioeconomic issues, is quite favorable as long as *Jatropha* is cultivated on wastelands/degraded lands. In view of these advantages, many investors, policy makers and clean development mechanism (CDM) project developers are interested to tackle the twin challenges of energy supply and GHG emission reduction.

The production of ethanol relied on sugar (when possible with cellulose), but the conversion process is energy intensive requiring distillation to separate ethanol from water. In contrast, if oil is extracted from oil bearing fruit, such as *Jatropha* conversion to bio-diesel is simpler based on catalytic transesterification of the oil. The process also generates modest quantities of glycerine and oil cake. The bio-diesel properties are similar to those of petro diesel (Ref. Table 1.1). Its cetane number is 48–60, comparable to ptro diesel. The sulphur content is less than 15 ppm. Experiments with bio-diesel have resulted in lower emissions of CO and particulate matter (Planning Commission, Report, 2003). Bio-diesel has higher viscosity and it has to warm before injection to avoid gum deposition, especially in colder climates. This is not a major issue in many parts of India. Engines can operate totally on bio-diesel or with varying blends of bio-diesel and conventional diesel. In either case, engine performance reported to be satisfactory. A number of European diesel vehicles

were certifie to operate over a wide range of blends and the emissions were reported to be lower (Report, National Bio-diesel Board, USA).

The Planning Commission set up a committee to examine various issues pertaining to large-scale production of bio-diesel. The committee's report (Planning Commission Report, 2003) argued that large-scale *Jatropha* cultivation on about 11.2 m ha of land could substitute for fossil diesel to the extent of 20% by 2031–32. Some workers have suggested that given an estimated 60 m ha of wastelands, there is sufficient scope to grow *Jatropha* on at least 30 m ha. land.

Table 1.1 Comparisons of fuel properties of *Jatropha* bio-diesel and Petrodiesel

Properties	**Unit**	***Jatropha* Bio-diesel**	**Petrodiesel**	***ASTM**
Density at 15°C	Kg/m^3	880	850	875-900
Viscocity at 40°C	mm^2/s	4.84	2.60	1.9-6.0
Flash point	°C	162	70	›130
Pour point	°C	-6	-20	-
Water content	%	NIL	0.02	‹0.03
Ash content	%	NIL	0.01	‹0.02
Carbon residue	%	0.025	0.17	-
Sulphur content	%	NIL	-	‹0.3
Acid value	mg KOH/gm	0.24	0.35	‹0.8
Iodine value	-	104	_	_
Saponification value	-	190	_	_
Calarific value	MJ/kg	37.2	42	_
Cetane Number		51.6	46	_

*ASTM- American Society for Testimg and Materials

The use of the tree on wastelands is of vital importance for the human population in developing countries. Bio-diesel has drawn attention because, it is environmentally safe and can be made from renewable sources and prepared locally. Since, India is deficient in edible oils, the non-edible oil as if *Jatropha* could be the desirable source for India for production of bio-diesel. This plant could be grown on wasteland, about 40 millions hectare of which is available in India. The crop grows in arid and semi-arid region and requires almost no post plantation management and care. Since, almost all the wasteland is available in rural and economically underdeveloped region, the large-scale bio-diesel production has an enormous potential for employment and development of these areas. *Jatropha* is a perennial tree living for 40 to 50 years or more depending upon local conditions. The *Jatropha* takes about 2 to 3 years to commence fruiting and another 2 years or more to come to the stage of full bearing. Thus, only after 4 or 5 years the grower will be in a position to reap the reward for his labours. If the original planting material used happens to be poor in quality, it will result in the establishment of a plantation giving poor yields and turn out to be a source of loss to the grower as long as the plantation lasts. He has no other option but to uproot the unproductive and uneconomic trees. These facts will underline the need to plant quality planting material that will ultimately give good yields.

1.4 Taxonomy and Botanical Description

Fig. 1.1 *Jatropha curcas* L.

Systematic Position	
Kingdom	Plantae
Division	Embryophyta
Class	Spermatopsida
Order	Malpighiales
Family	Euphorbiaceae
Genus	*Jatropha*
Species	*curcas*
Botanical name ***Jatropha curcas*** L.	

The genus *Jatropha* belongs to the family Euphorbiaceae 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. The physic nut, by definition, is a small tree or large shrub, which can reach a height of three to five meters, but under favorable conditions, it can attain a height of 8 or 10 m. The plant shows articulated growth, with a morphological discontinuity at each increment.

The branches contain latex. A taproot is not usually formed in vegetatively propagated plants. Leaves five to seven lobed, hypostomatic and stomata are of paracytic (Rubiaceous) type. The trees are deciduous, shedding the leaves in dry season. Flowering occurs during the wet season i.e. rainy season. The inflorescence is axillary paniculate polychasial cymes. The plant is monoecious and flowers are unisexual, occasionally hermaphrodite flowers occur (Dehgan and Webster, 1979). A flower is formed terminally, individually, with female flowers (tricarpellary, syncarpous with trilocular ovary) usually slightly larger and develop in the hot seasons. In conditions where continuous growth occurs, an unbalance of pistillate or staminate flower production results in a higher number of female flowers. Ten stamens are arranged in two distinct whorls of five each in a single column in the androecium and in close proximity to each other. In the gynoecium, the three slender styles are connate to about two-thirds of their length, dilating to massive bifurcate stigma (Dehgan and Webster, 1979). The rare hermaphrodite flowers can be self-pollinating. The flowers are pollinated by insects especially honey bees. Each inflorescence yields a bunch of approximately 10 or more ovoid fruits, in good rainfall conditions. Nursery plants may bear fruits after the first rainy season and directly sown plants after the second rainy season. Three, bivalved cocci is formed after the seeds mature and the fleshy exocarp dries. The seeds mature about 3–4 months after flowering. The seeds are black and the seed weight per 1000 seeds is about 727g. There is 1375 seeds/kg in the average. Singh (1970) has described the microscopical anatomy of fruits. Gupta (1985) investigated the anatomy of other plant parts (Ref. fig. 1.2). The physic nut is a diploid species with 2n = 22 chromosomes.

Dormancy is induced by fluctuations in rainfall, temperature and light. Normally, five roots are formed from seedlings, one central and four peripheral. Vegetatively propagated plants (Kobilke, 1989) Droit (1932) has described the microscopical anatomy of the seeds in detail. The brief description along with the sketches, as reported by Dehgan (1984) has been shown in fig.1.2.

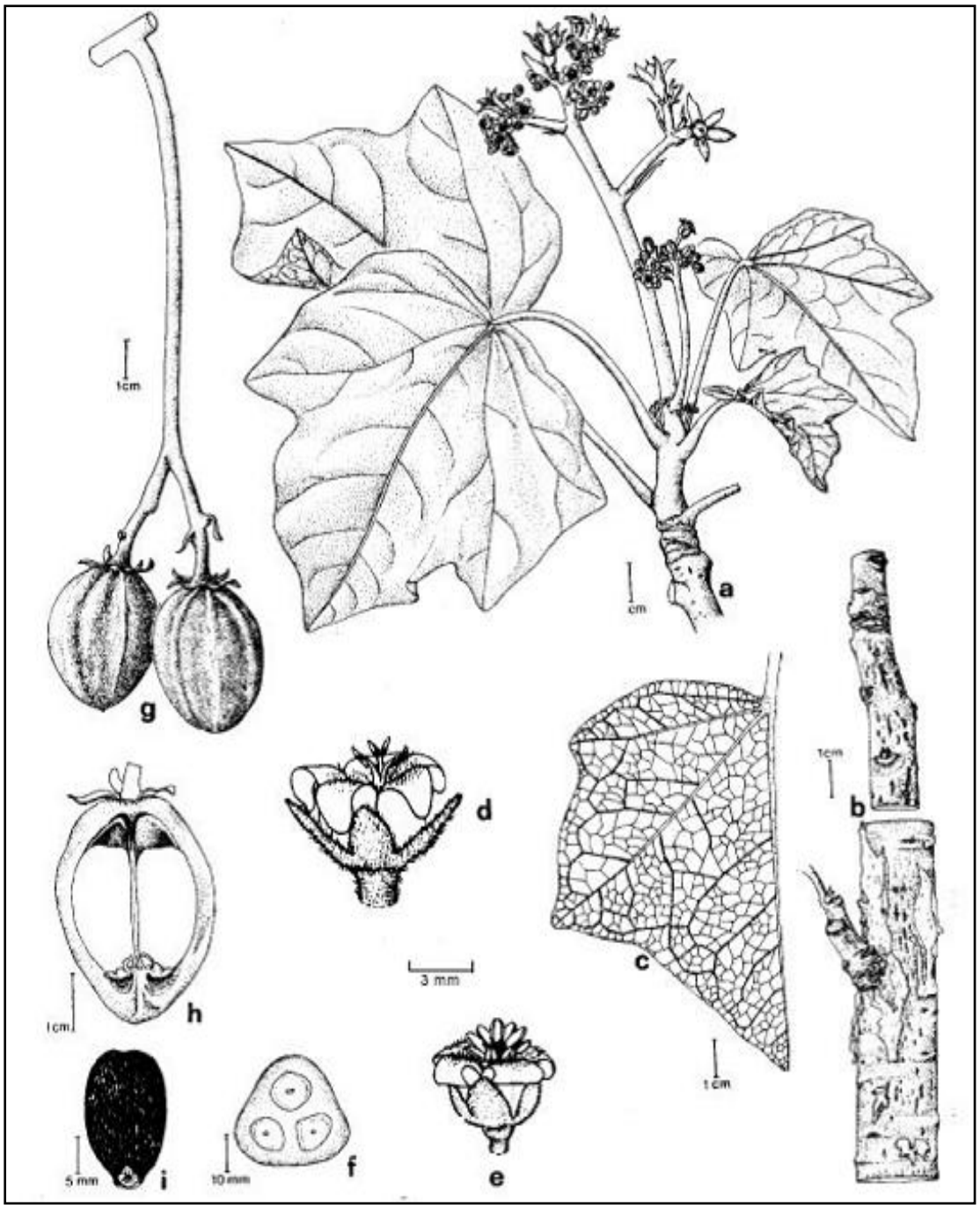

Fig. 1.2 Important parts of the physic nut: **a** flowering branch, **b** bark, **c** leaf veinature, **d** pistillate flower, **e** staminate flower, **f** cross section of immature fruit, **g** fruits, **h** longitudinal cut of fruits; **a-c** and **f-h** from Aponte 1978; **d** and **e** from Dehgan 1984.

1.5 Origin and centre of diversity

A number of scientists have attempted to define the origin of physic nut, but the source remains controversial. Martin and Mayeux (1984), identified the Ceara state in Brazil as a centre of origin but without giving any arguments. Dehgan and Webster (1979), have reported that *Jatropha* has north Central American origin before the arrival of Cortez and it most likely originated there. According to other sources, the physic nut seems to be native to Central America as well as to Mexico where it occurs naturally in the forests of coastal regions (Aponte, 1978). It is highly probable that the centre of origin of the physic nut is in Mexico and Central America. Since, it is not found in these forms of vegetation in Africa and Asia but only in cultivated form. The center of diversity and the current distribution of *Jatropha*, as suggested by Munch (1986), have been given in fig. 1.3.

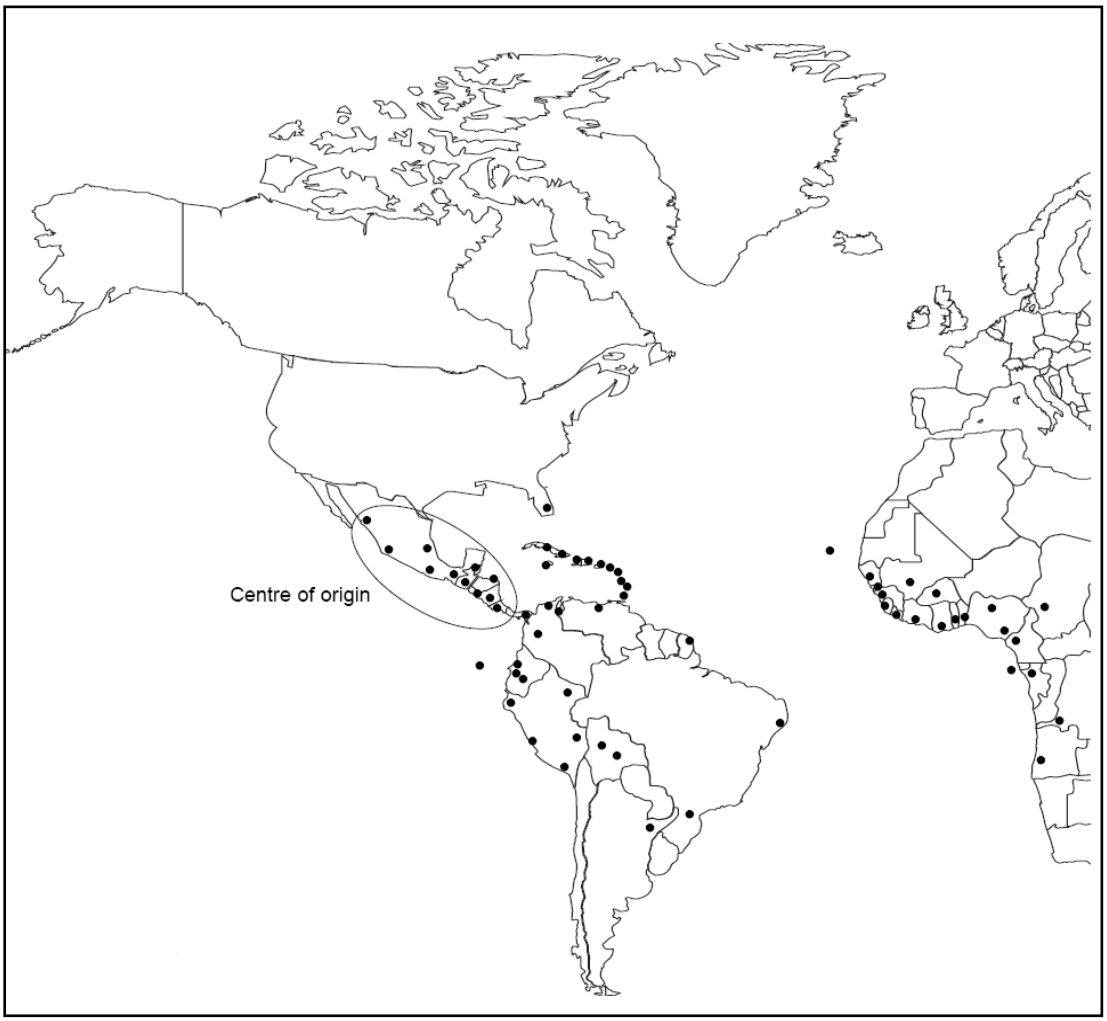

Fig.1.3 Current distribution of *Jatropha curcas* L. and proposed center of diversity (According to Munch, 1986)

Uses of *Jatropha* products

1.6.1 As hedge: *Jatropha* is an excellent hedging plant generally grown in most parts of India. It is usful as live fence for protection of agricultural fields. It is also as unpalatable to cattle and goats. Thus, in addition to seed yields, it serves the purpose of biofence with respect to cost effectiveness as compared to wire fence.

1.6.2 As green manure and fertilizers: Seed cake or press cake is a by-product of oil extraction. *Jatropha* seed cake contains curcin, a highly toxic protein similar to ricin in castor, making it unsuitable for animal feed. However, it does have potential as a fertilizer or biogas production (Gubitz et al., 1999). Kumar and Sharma (2008), reported that, it has nitrogen content ranging from 3.2 to 3.8 %, similar to that of neem oil cake and cow dung manure. A fertilizer trial with pearl millet where the effects of on pearl millet were compared (Henning, 2004). Pearl millet yields per ha were maximum in *Jatropha* oil cake treatment (5t/ha) compared to that with farm yard manure (5t/ha) and NP fertilizer (100 kg ammonium phosphate and 50 kg urea/ha) (Henning, 2004).), if available in large quantities; it can also be used as a fuel for steam turbines to generate electricity. The defatted meal has been found to contain a high amount of protein in the range of 50–62% and the level of essential amino acids except lysine is higher than the FAO reference protein (Makkar et al., 1998).

Being rich in nitrogen, the seed cake is an excellent source of plant nutrients. In a green manure trial with rice in Nepal, the application of 10 tonnes of fresh physic nut biomass resulted in increase yield of many crops (Sherchan et al., 1989). In preliminary experiment, *Jatropha* seed cake is utilized as feedstock for biogas production (Karve, 2005; Visser and Adriaans, 2007). Experiments on use of biogas slurry as a fertilizer are still in the early stages. Recently, experimentation on solid-state fermentation of *Jatropha* seed cake showed that, it could be a good source of low cost production of industrial enzymes (Mahanta et al., 2008).

1.6.3 As food: The physic nut seed is eaten in certain regions of Mexico once it has been boiled and roasted (Delgado and Parado, 1989). *Jatropha* can be toxic when consumed, however, a non-toxic variety of *Jatropha* is reported to exist in some provenances of Mexico and Central America, said not to contain toxic Phorbol esters

(Makkar et al., 1998). This variety is used for human consumption after roasting the seeds and the young leaves may be safely eaten, steamed or stewed (Duke, 1985a; Ochse, 1931).

1.6.4 Soap: The glycerin that is a by-product of bio-diesel can be used to make soap, and soap can be produced from *Jatropha* oil itself. In either case the process produces a soft, durable soap and is a simple one, well adapted to household or small scale industrial activity.

Table 1.2 An indicative list of potential attributes of *Jatropha curcas* L. for environment and economic development

Sr. No.	Multipule attributes of *Jatropha curcas*	Reference
1	Bio-diesel Production	Foidl et al. (1996); Openshaw (2000)
2	Soil carbon sequestration, soil quality improvement of degradable soil, wasteland reclamation and local sustainable development	Openshaw(2000); Ogunwole et al. (2008), Achten et al. (2008); Srivastava (2010)
3	Restoration of fly ash dykes and phytoremediarion of heavy metals from fly ash dykes	Jamil (2009); Jamil et al., (2009)
4	Phytoremediation of organochlorine pesticide (Lindane)	Abhilash (2009); Abhilash et al. (2009)
5	Phyotoremediation of heavy metals contaminated soils (As, Cr, Zn, Pb and Cd.)	Yadav et al. (2009) Mangkoedihardjo and Surahmaida (2008)
6	Waste product (Seed cake, seed hulls etc) as a possible source of Animal feed and fertilizer or feed stock for bio-diesel production	King et al. (2009); Gunaseelan (2009)
7	Rhizoremediation of soil contaminated with used lubricating oils	Agamuthu et al. (2010)

8	Medicinal and therapeutic properties of various plant parts (seed, fruit, bark, oil, latex, root powder etc.) like anti inflamatory, purgative, wound healing, anticoagulant etc.	Heller (1996); Samy et al. (1998); Osoniyi and Onajobi (2003);
9	Other industrial application (green synthesis from latex i. e., synthesis of silver nanoparticles using *Jatropha* latex as capping and reducing agent)	Bar et al. (2009)

1.6.5 Pesticide: The oil and aqueous extract from oil has potential as an insecticide. For instance, it has been used in the control of insect pests of cotton including cotton bollworm and on pests of pulses, potato and corn (Kaushik and Kumar, 2004). Methanol extracts of *Jatropha* seeds (which contain biodegradable toxins) are being tested in Germany for control of bilharzia-carrying water snails.

1.6.6 Medicinal uses: All parts of *Jatropha* (seeds, leaves and bark) have been used in traditional medicine and for veterinary purposes for a long time (Dalziel, 1955; Duke, 1985b). Some compounds (Curcacycline A) with antitumor activities were reportedly found in this plant (Van den Berg et al., 1995). Substances such as phorbol esters, which are toxic to animals and humans, have been isolated and their molluscicidal, insecticidal and fungicidal properties have been demonstrated in lab-scale experiments and field trials (Nwosu and Okafor, 1995; Solsoloy and Solsoloy, 1997). The seed oil can be applied to treat eczema and skin diseases and to soothe rheumatic pain (Heller, 1996). The 36% linoleic acid (C18:2) content in *Jatropha* kernel oil is of possible interest for skincare, furthermore, Goonasekera et al. (1995), showed that various solvent extracts of *Jatropha* have an abortive effect.

The oil has a strong purgative action and is widely used for skin diseases and to soothe pain such as that caused by rheumatism. The oil is used as a cathartic purgative (Jamalgota) and for the treatment of skin ailments (Duke, 1985). The latex itself has been found to be strong inhibitors to watermelon mosaic virus (Tewari and Shukla, 1982). The leaves and latex are used in healing of wounds, refractory ulcers and septic gums and as a styptic in cuts and bruises. A proteolytic enzyme (curcain) has been

reported to have wound healing activity in mice (Nath and Dutta, 1997; Villegas et al., 1997). Investigation of the coagulant activity of the latex of *Jatropha* showed that whole latex significantly reduced the clotting time of human blood. Diluted latex, however, prolonged the clotting time, at high dilutions, the blood did not clot at all (Osoniyi and Onajobi, 2003).

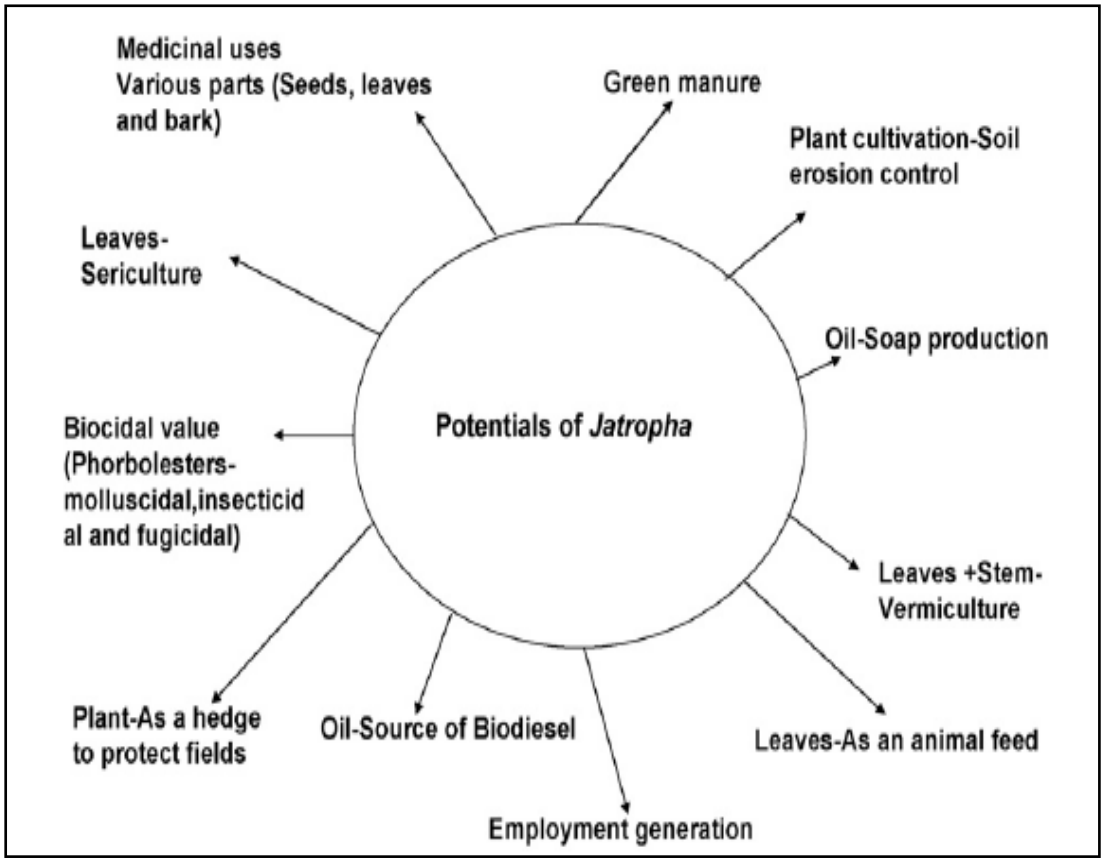

Fig. 1.4 Economic significance of *Jatropha curcas* L. (According to Divakar et al., 2008)

Topical application of *Jatropha* root powder in paste form is common ethnobotanical practices for the treatment of inflammation, which has followed by Bhil tribes from Rajasthan area in India, it confirmed in albino mice and the successive solvent extraction of these roots carried out by ether and methanol.

1.6.7 Bio-diesel: Bio-diesel is an environmental friendly replacement for petroleum based fuel. There are a number of plant species yielding oil and high molecular weight hydrocarbons, which can provide bio-diesel. A lot of work has been done on conversion of vegetable oils, however for sustainable production and utilization it is essential that the rich biodiversity existing in the country is optimally utilized.

Jatropha curcas, a plant of Latin America origin has shown promising potential to serve as a major raw material for bio-diesel production. Although, it is available widely across the country there has been no systematic study on the yield and oil content under different climatic conditions. There is a strong GxE interaction and it is therefore essential that agronomic trials are conducted for selection of superior material.

Bio-diesel is made from virgin or used vegetable oils (both edible and non-edible) and animal fats through transesterification. Bio-diesel is a substitute for a diesel and requires very little or no engine modifications up to 20% blend and minor modification for higher percentage blends. *Jatropha* oil can be used as fuel in diesel engines directly and by blending it with methanol (Gubitz et al., 1999). The seed oil of *Jatropha* was used as a diesel fuel substitute during the World War II. Engine tests with *Jatropha* oil were done in Thailand, showing satisfactory engine performance (Takeda, 1982). For African countries, the feasibility of the production of fatty acid ethyl esters from *Jatropha* oil was studied (Eisa, 1997).

The economic evaluation has shown that, the bio-diesel production from *Jatropha* is very profitable provided the by-products of the bio-diesel production can be sold as valuable products (Foidl and Eder, 1997). Tiwari et al. (2007), have developed a technique to produce bio-diesel from *Jatropha* with high free fatty acids contents (15% FFA), in which two stage transesterification processes was selected to improve methyl ester yield. The first stage involved the acid pretreatment process to reduce the FFA level of crude *Jatropha* seed oil to less than 1% and second was the alkali base catalyzed transesterification process gave 90% methyl ester yield. In order to reduce the cost of bio-diesel fuel production from *Jatropha*, the lipase producing whole cells of *Rhizopus oryzae* immobilized onto biomass support particles was used and found to be a promising biocatalyst for producing bio-diesel (Tamalampudi et al., 2007). More efficient expeller system can be used to extract a higher percentage of oil from the seeds, which in turn should produce higher profits in a *Jatropha* system, since oil sells for more than the residual seed cake.

The simple technology specially developed for this chemical process can also be performed in less industrialized countries (Mittelbach et al., 1983; Connemann 1994).

Use of methyl ester of *Jatropha* oil and dual fuel operation with methanol induction can give better performance and reduced smoke emissions than the blend. Dual fuel operation showed the lowest smoke and NO levels (Senthil et al., 2003). Tamalampudi et al. (2007), have examined the blends of *Jatropha* and Palm oil, bio-diesel for their physical chemical properties. It gets optimum mix of them to achieve better low temperature and improved oxidation stability needed for South Asian and South East Asian countries.

1.7 Merits of *Jatropha* for Bio-diesel Production

The Tree Borne Oil seeds (TBOs) namely *Jatropha (Jatropha curcas),* Karanj (*Pongamia pinnata*), Jojoba (*Simmondsia chinensis*), Neem (*Azadirachta indica)* etc. have potential to grow in categorized wasteland. These TBOs posses 20-60% oil content in seeds, which are non-edible and it can be utilized as source of bio-diesel production. *Jatropha* is one of the best alternatives of energy plantation due to its following characteristics:

- It can be grown in areas of low rainfall (600 mm per year) and in problematic soils. In high rainfall and irrigated areas too it can be grown with much higher yields.
- *Jatropha* is easy to establish, grows relatively quickly and is hardy. *Jatropha* lends itself to plantation with advantage on lands developed on watershed basis and on low fertility marginal, degraded, fallow, waste and other lands such as along the canals, roads, railway tracks, on borders of farmers' fields as a boundary fence or live hedge in the arid/semi-arid areas and even on alkaline soils. As such it can be used to reclaim waste lands in the forests and outside.
- Animals do not browse *Jatropha*.
- Being rich in nitrogen, the seed cake is an excellent source of organic manure.
- One hectare of *Jatropha* plantation will produce 3 to 4 MT seed.
- Gestation period is two years
- Various parts of the plant have medicinal value, its bark contains tannin, the flowers attract bees and thus the plant has honey production potential.
- Like all trees, *Jatropha* removes carbon from the atmosphere, stores it in the woody tissues and assists in the build up of soil carbon. It is thus environment friendly.

- *Jatropha* can be established from seed, seedlings and vegetatively from cuttings. Use of branch cutting for propagation is easy and results in rapid growth
- The bush can be expected to start bearing fruit within two years and in some cases after one year of planting.
- The plant is undemanding in soil type and does not require tillage.
- It can meet a number of objectives, such as meeting domestic needs of energy services including cooking and lighting. It has as an additional source of household income and employment through markets for fuel, fertilizer, animal feed, medicine and industrial raw material for soap, cosmetics, etc. in creating environmental benefits – protection of crops or pasturelands or as a hedge for erosion control, or as a windbreak and a source of organic manure.

Although ability to control land degradation and oil production are the most important environmental uses of *Jatropha*, its products provide numerous other benefits that would additionally improve the living conditions of the rural people and offer greater income opportunities through enhanced rural employment. For instance, the *Jatropha* oil can be used for soap production and cosmetics production in rural areas and all parts of the plant have traditional medicinal uses (both human and veterinary purposes) that are being scientifically investigated.

The oil is a strong purgative, widely used as an antiseptic for cough, skin diseases and as a pain reliever from rheumatism. *Jatropha* latex can heal wounds and also has anti-microbial properties. *Jatropha* oil has been used commercially as a raw material for soap manufacture for decades, both by large and small industrial producers. The oil cake cannot be directly used as animal feed because of the presence of toxic compounds, but it is valuable as organic manure having nitrogen content comparable to chicken manure and castor seed cake. *Jatropha* oil cake is rich in nitrogen (3.2%), phosphorous (1.4%) and potassium (1.2%).

1.8 Quality Planting Material

The Planning Commission (2006) Govt. of India has initiated an ambitious program of growing *Jatropha curcas* on wastelands for bio-diesel production, an initial demonstration of the potential of *Jatropha* oil as a bio-diesel is being established. The

entire cost economics is dependant on the quality and performance of the raw material. Yield and oil content are the two primary factors responsible and special emphasis is being laid on improved production and productivity of the planting material. Both of these characters can be further factored in to relatively simpler traits such as number of mature fruits per cyme/raceme, 100-seed weight, seed-kernelratio and kernel oil content (%). Other desirable traits are superior fatty acid composition, plant hardiness, short and compact canopy (for easy harvesting and high-density plantation) and synchronised maturity. All these characters need to be pyramided together through stepwise screening and selection.

There are as number of issues which need to be resolved to ensure cost effective large-scale commercial potential of this species as a petrocrop in this region.

I. Systematic germplasm collection and evaluation programme to identify superior material from the existing natural variations.
II. Establish clonal seed orchards and develop mass multiplication techniques to ensure easy supply of elite planting material to growers.
III. A series of multi-locational trials under different agro-climatic are required

1.9 DBT Micromission on Quality Planting Material

The Department of Biotechnology, Government of India has launched a micromission on production and demonstration of quality planting material. The main objectives of this micromission are

- Selection of superior material based on established criteria-Oil content 30-40%; yield 3-5/ha.
- Production of superior quality material- Micro and macropropagation
- Standardizing agrotechnology packages
- Nursery establishment and Demonstrations in identified areas at 10-12 agroclamatic locations.

Under this mission, material/germplasm from different parts of the country is being screened, complete characterization of oil/yield is also being done and based on these characteristics an effort is being made to identify the superior plant. This identified superior material is being mass multiplied at different locations across the country

through clonal propagation. Nurseries have been developed at 14 locations in 12 states. Demonstration plots of the superior high material are being established and these would serve as a mother stock/source material for further large-scale multiplication plantation of superior material.

1.10 Critical research needs

- The estimated potential area of *Jatroph*a plantation is 486 million hectares worldwide and the potential production is 613 million ton dry seed per year (Li et al., 2010). This kind of information can be used for policy makers and prospective countries should identify their land areas suitable for *Jatropha* plantations using GIS and other land suitability modeling.

- Although, it is widely reported that *Jatropha* can thrive on marginal or substantial soil conditions, the seed production and oil yield under marginal condition is not yet validated (Wiskerke et al., 2010). Since the yield of any crop is a direct result of cultivation practices, suitable agronomic practices are most important for the large-scale plantations (Achten et al., 2008). As an undomesticated plant, the yield of *Jatropha* varies widely (0.4–12 ton ha^{-1}) and there is no complete information about the genetics, common cultivation practices or breeding aspects of this plant (Achten et al., 2008). Therefore, most estimations of *Jatropha* yield are site specific and not accurate for large-scale exploitation (Achten et al., 2008). Therefore, there is an urgent need to optimize the agro-practices of this species like developing mass multiplication strategies, optimization of spacing pattern, standardization of pruning techniques, pruning time for good plant architecture and lateral branching. Application of growth regulators, integrated nutrient management, integrated water management, integrated pest management, etc. for different agro-climatic and the real yield of the *Jatropha* should be calculated based on edaphic and climatic conditions.

- Similarly, *Jatropha* accessions should be collected from different agro-climatic regions of the world for assessing the seed yield, oil content and disease resistance. The collected accessions should be raised in different parts

of the world and detailed morphological, genomic and molecular characterizations should be done. The better accessions could be used for the breeding and crop improvement programme. For this, a cordial research and knowledge exchange program should be facilitated at the international level.

- It is estimated that *Jatropha* biomass production would sequester 5.5 t CO_2 ha^{-1} $year^{-1}$ (Achten et al., 2008). However, the carbon sequestration of *Jatropha* plantations in different agro-climatic regions (under standard agro-practices) should be quantified and this information can be used for carbon crediting and clean development mechanisms especially for India, China, Brazil, Mexico, Columbia, and Peru. An effort in this regard is already started in India and the evaluation of carbon sequestration potential of *Jatropha* plantations growing under varying edaphic condition is going on (Srivastava, 2010).

- Despite, the presence of plant-borne toxic chemicals like curcain, the occurrence of phorbol esters in *Jatropha* seed is also a serious health concern. The phorbol esters are acutely toxic, skin irritants and purgatives (Gandhi et al., 1995). They can act as co-carcinogens or tumor promoters (Griner and Kazanietz, 2007). Therefore, measures should be taken to protect those, who have an occupational exposure to *Jatropha* seeds. At present, the varieties being used to establish plantations in Africa and Asia are inedible due to presence of toxic substance as phorbol ester (King et al., 2009). Therefore, the seed cake can not be used as a source of animal feed. However, it needs to be investigated, whether biotechnological and genetic interventions may reduce the plant-borne toxicity of phorbol esters.

- Many workers have suggested that, *Jatrop*ha seed cake can be used as an organic fertilizer. However, research into the fate of phorbol esters in the environment and their impact on soil ecology is required to study, before the large-scale field utilization (King et al., 2009).

- Molecular markers should be developed for delineating non-toxic varieties. Importantly, non-toxic (edible) varieties could be developed by altering the phorbol ester biosynthetic pathway so that seed can be converted as animal feed. Although, a range of methods have been established to detoxify defatted seed cake, including extraction with organic solvents and combined heat/$NaHCO_3$ treatment, it is not economically feasible for large-scale exploitation (King et al., 2009).

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1.11 Objectives

Throughly reviewing literarure it released that, growers are unable to achieve the optimum economic benefits from the plant, especially for its various uses. The markets of different products from this plant have not been properly explored or quantified. Consequently, the actual or potential growers including those in the subsistence sector do not have an adequate information base about the potential and economics of this plant to exploit it commercially. In Indian context, development of bio-diesel would not only serve to reduce import of petrodiesel but also in generation of employment opportunities, accelerated rural development and meeting the environmental obligations such as reduction of green house gases (India can tap the US $53 billion global market for carbon trading by promoting biofuels uses and production), carbon sequestration, etc. Further large wasteland could be utilized for the cultivation of non-edible oil producing trees for production of bio-diesel.

Jatropha as it is successful growing under varied climate, edaphic environmental and biogeographic zones, indicates that through natural process appropriate selection must have taken place for preparation of species to thrive under these diverse agro-climatic conditions. The wide distribution of *Jatropha curcas* in this varied climatic, environmental and edaphic condition implies genetic variability. It will offer scope for selection of required characters for the improvement of the species. In the above background, it is found worthwhile to examine the extent of variation existing in the population of *Jatropha curcas* plants available in the entire agro-climatic regions of Maharashtra.

Keeping all these aspects in view, a study was under taken with the following objectives.

A) Diversity studies: (w.r.t. Maharashtra)

1. Morphological: With references to seed size, seed weight and seed viability.
2. Biochemical: With respect to oil content, fatty acid profile and toxic substances phorbol ester (toxic substance).
3. Molecular: studied with appropriate RAPD based molecular markers.

B) Propagation studies:

Refining of protocols for multiplication of selected elite plants by

1. *In vivo* studies
2. *In vitro* studies

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2. Review of Literature

2.1 Introduction

India is emerging as a major force in the global economy. During the present phase of accelerated development, it is inevitable that energy consumption increases many folds as compared to the present per capita consumption. Country produces only 30% of its annual crude oil requirement of 111 MMT. It imports crude oil to the tune of Rs.1,02,500 crores for meeting the remaining requirement (Bhattacharya and Joshi, 2006). Constantly increasing global oil prices and depleting resources of fossil fuels have further become burden on the country's economy. Estimate reveals that by the year 2030, India would be dependent on nearly 95% imported oil. This alarming situation calls for emphasis on the use of non–conventional energy source. In this scenario, biofuel is a ray of hope for meeting our energy challenges. Even 5% replacement of fossil fuel by bio-diesel would help to save over Rs. 4000 crores annually in foreign exchange (Bhattacharya and Joshi, 2006). Bio-diesel is a renewable source of energy, which can help in lowering the dependence on fossil fuels and minimizing the greenhouse gases and other pollutants. Environmental degradation could be arrested through the production and use of bio-diesel. It will enable compliance of approval norms for April 2010 in the entire country as targeted in the national auto fuel policy (Katwal and Soni, 2003).

During the last 10 years, *Jatropha curcas* L. has gained considerable attention as a potential feedstock of bio-diesel and many *Jatropha* plantations have been established in tropical and subtropical regions worldwide (Li et al., 2010). *Jatropha* is regarded as an oil plant with multiple attributes, uses and considerable potential (Openshaw, 2000). Its peculiar features are drought tolerance, rapid growth, easy propagation (Heller, 1996). Higher oil content than other oil crops (Achten et al., 2008), small gestation period, adaptation to a wide range of environmental conditions and the optimum plant size and architecture (that make the seed collection more convenient) make, it as a special candidate for further consideration (Sujatha et al., 2008).

Many scientists started about one decade ago, by Gubitz et al. (1999), reviewed the utilization of various parts of *Jatropha*. In addition, some of the recent review articles provide status and perspectives of *Jatropha* bio-diesel program in various countries e.g., India (Biswas et al., 2010, Jain and Sharma, 2010), UK (Janaun and Ellis, 2010),

Malaysia (Lim and Teong 2010), Sweden (Arvidsson et al., 2010), China (Ye et al., 2009), Thailand (Siriwardhana et al., 2009). Zhou and Thomson (2009), has highlighted the current status of biofuel production including the national development targets, strategies, incentives, and policies in Asia's largest producing countries like Indonesia, Malaysia, Philippines, Thailand, China, and India. Although large-scale investment in *Jatropha* plantations have been made throughout tropical areas, particularly in the countries of sub-Saharan Africa, Latin America and South and South Eastern Asia (Li et al., 2010), many of the field promises offered by this shrub is yet to be finalized. Furthermore, still there are several economical, technological and toxicological issues related to the plant selection, cultivation, maintenance and management of *Jatropha* plantations, bio-diesel production existing across the world and these concerns should be addressed in detail. This review aimed to highlight all these issues and provides a very brief commentary on the progress in *Jatropha* research during the last 10 years. It will help identify the critical research needs for developing and implementing successful *Jatropha* bio-diesel program.

2.2 *Jatropha* bio-diesel fuel for Aviation Industry

Media reports say that biofuel produced from seeds of *Jatropha,* can be successfully used for fueling aircrafts, a report by Yale's School of Environmental Studies, funded by Boeing. According to Business Matchmaking, Inc., many airlines, including Japan Airlines, Air New Zealand, Continental, Brazil's TAM Airlines and most recently the Mexican carrier Interjet, in cooperation with European manufacturer Airbus, successfully tested the oil produced from *Jatropha* seeds as a potential substitute for traditional jet fuel.

James Garton, president of Mission New Energy, said on 31st March 2011, that "We are particularly pleased to learn of repeated testing of *Jatropha* in aviation with positive results". According to Reuters, report said an Interjet Airlines Airbus 320 passenger plane, using the biofuel landed at Angel Albino Corzo of Tuxtla Gutierrez airport in Chiapas on 1st April 2010. The flight, from Mexico City's International Airport, was the first in Mexico partially powered (27%) by biofuel. According to the Mexican airport authority Aeropuertosy Servicios Auxiliares (ASA) Mexico is one of the few countries pursuing the production of biofuel from *Jatropha* for a couple of years now. As the weed widely became known for producing oil that could be used to fuel jet planes, the Mexican government wanted farmers to grow entire fields of it to

turn into bio-diesel. The United Nation's International Civil Aviation Organization has established the goal of reducing aviation-related carbon-dioxide emissions and the use of renewable fuels. The Yale study projected greenhouse gas reductions of up to 60 percent from *Jatropha* based fuel compared to petroleum based jet fuel. According to Garton, with the unprecedented challenges facing the airlines because of the constant increase in the price of jet fuel and the global need to accept sustainability as a key to environmental responsibility, the *Jatropha* solution is timely and efficient.

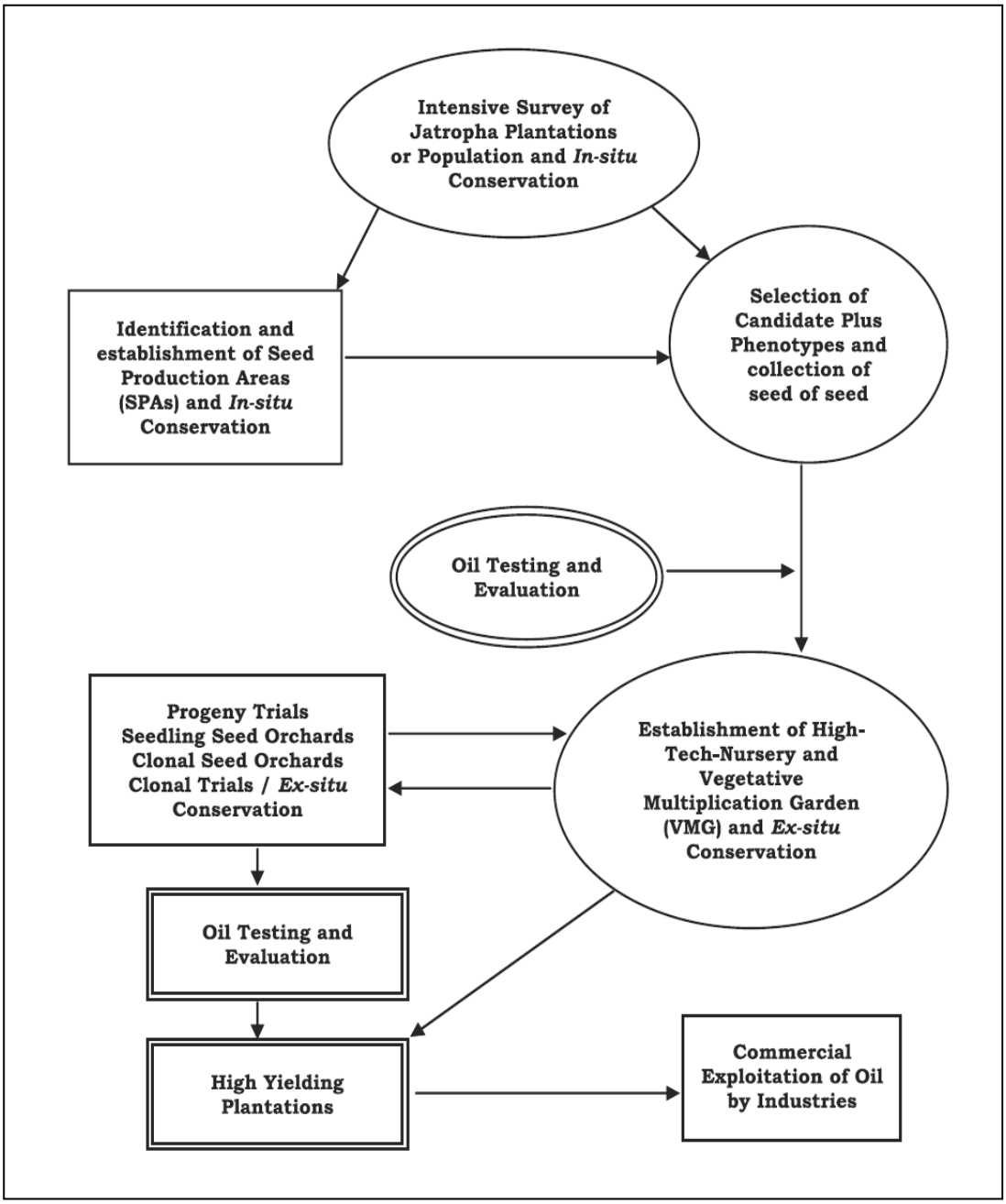

Fig. 2.1 Approach for genetic improvement of *Jatropha curcas* L (According to Kumar and Sharma, 2008)

2.3 Germplasm collection, characterization and availability

The key for success of any genetic improvement programme lies in the availability of genetic variability for desired traits (Heller, 1996). Genetic resource through global exploration, introduction, characterization and evaluation will provide strong base for development of elite varieties by various improvement methods. Comprehensive work on collection, characterization and evaluation of germplasm for growth, morphology, seed characteristics and yield traits is still in its infancy. The fact that *Jtropha* has adapted itself to a wide range of edaphic and ecological conditions suggested that, there exist considerable amount of genetic variability to be exploited for potential realization (Rao et al., 2008). Species and provenance trials contribute fundamental information for further breeding and genetic improvement (Burley et al., 1976). Systematic provenance trials have not yet carried out with to the necessary extent. The genetic background of the physic nut grown in Africa and Asia is unclear. Certain provenances may differ relatively from others if cultivated at different sites, which is due to G x E interaction (Zobel et al., 1988).

Priority should be give to assess intra and inter accessional variability in the available germplasm, selection of pure lines and then multiplication. Kumar and Sharma (2008), gave the approach for the genetic improvement programme (Ref. Fig 2.1). Existence of natural hybrid complexes was reported in the genus *Jatropha*, such as, *J. curcas-canascens* complex in Mexico (Dehgan, 1978) *J. integerrima–hastata* complex in Cuba and West Indian islands and *J. curcas–gossypifolia* (*J. tanjorensis*) in India (Prabhakaran et al., 1999). Hence, germplasm exhibiting gross morphological differences should be subjected for pollen studies and lines with pollen abnormality or poor seed set should be investigated in detail before drawing conclusions about the distinctness. Makkar et al. (1997), reported large variations in contents of crude protein, crude fat, neutral detergent fiber and ash on 18 different provenances of *Jatropha* from countries in West and East Africa, the Americas and Asia.

Wani et al. (2006), recorded variation in Indian accessions for oil content (27.8–38.4%) and 100 seed weight (44–77 g). Similarly Rao et al. (2008), found wide variation in 100 seed weight (57–79 g) and oil content (30–37%) for Andhra Pradesh, India, accessions.

2.4 Phenotypic diversity studies

Studies of genetic variability, divergence in seed trait and oil content can help to identify plants having better yield and more oil content. Best genotype selected will improve the poor sites of agro-forestry systems and energy plantation in wastelands. Little work has been done so far on germplasm collection and it's evaluatation for chemical composition (Oil content) of seeds of India's arid and semiarid species reported by Kaushiket et al. (2007). Kaushik et al. (2007), have assessed variability in seed traits and oil content of 24 accessions of *Jatropha curcas* collected from different agroc-limatic regions of Haryana state, India and observed there were significant differences ($P<0.05$) in seed size, 100-seed weight and oil content between accessions. In general phenotypic coefficient of variation was higher than the genotypic coefficient of variation indicating the predominant role of environment. Based on non-hierarchical Euclidian cluster analysis, six clusters were obtained and cluster analysis suggested that the crossing between accessions of cluster IV and VI would result in wide spectrum of variability in subsequent generations. Selection of seeds and providing of optimum conditions are essential for producing high quality seedlings. Kochhar and Kochhar (2005**),** have reported that the seeds are toxic due to the presences of cursive and curative ingredients, but after treatments, the seed cake can be used as animal feed. They have further stated that seeds posses a limited viability; they lose 50% viability within 15 months.

According to Kaushik and Sushil (2003), Selections of large seeds ensure production of quality seedlings in nursery. Gurunathan et al. (2006), have reported that bigger, heavier and dark black coloured seeds recorded the maximum seed and seedling quality characters on grading the seeds based on size, weight and colour. Kathiravan (2009), have also reported that bigger sized seeds produced elite seedling at nursery. According to Kaushik and Kumar (2004), sincere efforts are required to produce of quality planting materials so that the farmers grow *Jatropha curcus* without any hesitation. If the farmer's interests should taken care for, the entire supply chain of seed to bio-diesel will be stronger and longer.

Analysis of phenotypic diversity in germplasm collections can facilitate reliable classification of accessions and its identification with future utility for specific breeding purposes. There are very few studies on phenotypic diversity involving a

limited number of germplasm and their suggested use in hybridization. Sunil et al. (2008), have developed a methodology for identification of superior lines by assessing the phenotypic traits of plants recorded in situ. Further, they generated grid maps for the distribution pattern based on plant height, number of primary branches, collar length, number of fruits per cluster and oil content using Geographic Information System (GIS) to find the potential area for germplasm with high oil content based on rarefaction method of DIVA-GIS (Sunil et al., 2008). Heller (1996), have tested 13 provenances in multilocation field trials in 1987 and 1988 in two countries of the Sahel region: Senegal and Cape Verde. Significant differences in the vegetative development except leaf shape were detected among the various provenances at all locations. Gohil and Pandya (2008), have analysed diversity based on phenotypic traits of nine *Jatropha* genotypes and suggested that for varietal improvement, hybridization among the genotypes of divergent clusters (clusters – III, IV and V) may be done in order to obtain better results in terms of variability and diversity. Rao et al. (2008), observed four clusters with phylogeographic patterns of genetic diversity among 32 high yielding candidate plus trees of *J. curcas* for seed traits.

In seed sources evaluation trial of *Jatropha curcas* L. laid out in 1996 at Jabalpur (M.P), a semi arid region of India. Seeds collected from ten sources from central India representing the states Madhya Pradesh and Maharashtra viz., Gondia, Bichia, Balaghat, Niwas, Khandwa, Burhanpur, Nasik, Chindwara, Kundam and Jabalpur were evaluated for their growth performance from nursery stage (3 months) to field (two years). Variation in seed oil content of different sources was recorded. Significant differences between the seed sources of 27 months old were observed for height, collar diameter, number of branches leaf area and field survival. Seeds collected from different sources varied significantly in respect of seed and kernel weight and oil content in seed/kernel. The Chindwara (M.P.) source performed the best and yielded a maximum oil of 39.12% from whole seed and 58.12% from kernel. The oil content ranged from 33.02 to 39.12% in whole seeds and 47.08 to 58.12% in kernel, across the seed sources. The performance of Kundam (M.P.), Jabalpur (M.P.), Bichia (M.P.), Niwas (M.P.) and Nasik (Maharashtra) sources were found satisfactory for oil yield. Results indicate that genetic differences exist between the seed sources of *J. curcas.* The growth traits showed significant positive correlation with each other.

Fair differences between phenotypic and genotypic coefficient of variability were observed.

According to Ginwal et al. (2004), heritability, (broad sense) values were good with regard to leaf area, height and collar diameter in comparison to survival percent. The relative performance of these sources was consistent throughout the observation period. A significant seed source variation was observed in seed morphology (colour, size and weight), seed germination (viability, germination percent, germination energy, germination value) and seedling growth parameters (survival percentage, seedling height, collar diameter, leave/plant, and seedling biomass). The phenotypic and genotypic variance, their coefficient of variability and broad sense heritability also showed a sizeable variability. Further, high percentage of heritability coupled with moderate intensity of genetic gain, was observed for seed germination traits, which signifies that germination is under strong genetic control and good amount of heritable additive genetic component can be exploited for improvement of this species reported by Ginwal et al. (2005).

Kumar et al. (2005), reported that the phenotypic variability and the correlation between the species *Jatropha gossypifolia* showed wide morphological variation with respect to vegetative growth, reproductive and seed parameters at different location. Altitudes ranging from 400 to 1000 m elevation might have a relation with the temperature. It had a significant positive effect on various oil yield components, including the number of branches per tree (+2), number of fruits per branch (+12), number of fruits per tree (+100) and number of seeds per tree (+300). However, a significant reduction was observed in kernel oil content (43.10 at low vs. 30.66% at higher elevations) reported by Pant et al. (2006). According to Patil et al. (2006), the number of branches and collar diameter of *Jatropha* were significantly higher in 1m. pruning as compared to other pruning levels and also at 80:80:80 N:P:K g/plant than 40:40:40 N:P:K g/plant.

Dhillon et al. (2006), their study revealed that the bud began to appear as small protruding structure intermingled with the initiation of new leaves at the terminal end of branches. Majority of plants flower after rainy season i.e. September–October, however, some plants flower even in spring season (March–April). Flowers are

unisexual, male and female flowers produced in the same inflorescence. The average male to female flower ratio was 20:1, which changed drastically (108:1) with the fall in temperature. The male floral buds develop earlier than female. The flowering pattern was asynchronous. The peak period of flowering varied from 3-20 days. Period of fruit development and maturity ranged from 55 to 61 days. The results of breeding system indicated 32.9 per cent fruit setting under selfing and 89.7 per cent under natural pollination. The high fruit setting under open pollination revealed that the plant is capable of producing fruits through selfing (geitonogamy) and open pollination (xenogamy), such a breeding system represents facultative cross pollination. Luo Chang-wei et al. (2007), studied the reproduction characteristics of *Jatropha curcas* in Yuanjiang County and concluded that the plant produces flowers in dichasial inflorescences. Normally, the flowers are unisexual and male and female flowers were produced in the same inflorescence. By the treatments of emasculation, bagging and artificial pollination in this experiment showed that *Jatropha curcas* could produce fruit through apomixis but not wind pollination. When the inflorescence unbagged, unemasculated and free pollination showed that *Jatropha curcas* shows outcrossing is self-compatible and demanding for pollinators. However, a large number of female flowers open from the third to the fifth day, with some female flowers opening first in a few racemes. This shows a tendency to promote xenogamy and minimize geitonogamy.

According to Francis et al. (2005), seed yield can also be highly variable within plantation strands, vary from 0.2 to more than 2 kg per tree. This variability in yield is in contrast with the genetic variability, which is rather small in Indian germplasm, as reported by Basha and Sujatha (2007). Selected strains of *Bacillus spp.*, either supplemented with or without chitin were tested for their ability to promote growth of *Jatropha* seedlings in pot culture studies, as reported by Desai et al. (2007). The strains supported the growth of *Jatropha* seedlings up to 42 days after sowing. Among all strains, *Bacillus pumilus* (IM-3) supplemented with chitin showed over all plant growth promotion effect resulting in enhanced shoot length (113%), dry shoot mass (360%), dry root mass (467%), total dry plant mass (346%), leaf area (256%), and chlorophyll content (74%) over control. Treating seeds with strain IM-3 without chitin resulted in enhanced dry shoot mass (473%), total dry plant mass (407%) and chlorophyll content (82%). However, Bacillus polymyxa (KRU-22) with chitin

supported maximum root length (143%). Either strain IM-3 alone or in combination with other promising strains could be promoted further for enhanced initial seedling growth of *Jatropha*.

Provenance trials with local *J. curcas* accessions are reported in India (Ginwal et al., 2004; Basha and Sujatha, 2007; Kaushuk et al., 2007) and for various accessions, mainly from Africa (Makkar et al., 1997). They revealed that the genetic base of *J. curcas* provenance from India is quite small (Basha and Sujatha, 2007) significant difference in plant morphological aspects (plant height, leaf area index, stem girth, number of primary secondary and tertiary branches) and yield contributing factors (fruits per plant, seeds per plant, seed weight and oil%) could be distinguished between provenances. Significant trait differences were observed in all the seed characters viz., seed morphology and oil content as were observed in growth characters viz., plant height and female to male flower ratio and seed yield in the progeny trial. Broad sense heritability was high in general and exceeded 80% for all the seed traits studied. Female to male flower ratio showed near to 100% heritability followed by yield (83.61) and plant height (87.73). The path analysis revealed that female to male flower ratio had highest positive direct relationship with seed yield (0.789), followed by number of branches (0.612) and number of days from fruiting to maturity (0.431). Negative indirect effects were seen in number of days from flowering to fruiting, which indirectly and negatively influenced yield through plant height and number of branches. Hierarchical clustering by Ward's minimum variance cluster analysis showed phylo-geographic patterns of genetic diversity. Clustering revealed that trees from different geographic regions were grouped together in a cluster and as trees were from the same geographical area placed in different clusters suggesting that geographical diversity did not go hand in hand with genetic diversity. In addition clustering identified promising accession with favourable traits for future establishment of elite seedling seed orchard and clonal seed orchard for varietal and hybridization programmes reported by Rao et al. (2008).

2.5 Genetic studies of traits

Although selection based on seed characters would simplify the task, there still exist several limitations and doubts in these selected plants because of season and time bound expression of genes of interest. Knowledge of genetic variation in branching

pattern, M/F flower ratio, pest resistance, drought resistance and yield attributes in wild *Jatropha* germplasm can be of great potential in improvement programs. This is particularly important in selection of genotypes with more oil content and yield. Hence, a large-scale collection of germplasm from selected plus trees, their conservation and the evaluation program of various *Jatropha* accessions is essential to understand patterns of variability. Estimation of correlation among characters, broad sense heritability and genetic advance will be useful to determine the extent to which improvement is possible through selection. Kaushik et al. (2007), recorded coefficients of variance between 24 Haryana provenances, India, which envisaged that environment has comparatively low influence on the seed traits and oil content. High broad sense heritability and high genetic advance for oil content in *Jatropha* indicates potentiality of test material for further improvement through selection. Positive significant association was observed between seed weight and oil content reported by Kaushik et al. (2007). Rao et al. (2008), have evaluated genetic association and variability in seed and growth characters and found that M/F ratio had the highest positive direct relationship with seed yield (0.789), followed by number of branches (0.612) and number of days from fruiting to maturity (0.431) with high heritability for these traits. In another study using ten sources from central India, seed oil content was significantly correlated with seed weight (0.792), stem diameter (0.836) and total leaf area (0.883) reported by Ginwal et al. (2004). Observation on influence of regional climate (Kun et al., 2007) and altitudinal ranges (Pant et al., 2006) on seed oil content variations was recorded.

2.6 Edapho-climatic impact on biochemical content of *J. curcas* L.

According to Renu Swarup (2007), biotechnological interventions would play a major role for improvement of *Jatropha curcas* L. concerted research and development, efforts in this direction are essential, seed yield and oil content are the two most desirable traits in a biofuel species like *Jatropha*. Other desirable traits are dwarfness, fatty acid composition, early flowering and synchronous maturity. All of these characters vary with the geographical origin of the plant material. An effort towards pyramiding the economically important traits is needed. Fuel property and storage quality of vegetable oil is associated with its fatty acid composition. Therefore, it is necessary to have the desirable fatty acid profile to successfully utilize it as a diesel substitute. It is essential to screen *Jatropha* genotypes with high oil content and to

associate oil characters with molecular markers and estimation of genetic diversity of the available germplasm. In addition, screening for desirable fatty acid profile for fuel purpose is also essential. Swamy et al. (2002), have reported that lot of variability exists in natural populations of *Jatropha curcas* L. with respect to seed yield (0.2 – 2 kg/tree) and oil content (30-48%), since it is widely distributed in different edapho-climatic conditions in the country. According to them selection of good seed source is important and the primary need of developing high quality planting material of *Jatropha curcas* L.

2.7 Molecular markers

Traditionally, genetic variation of tree species has been understood through an assessment of survival and growth performance parameters. However, such exercise can be time consuming, labour-intensive and costly. The past 20 years have seen rapid advances in technologies available for assessing genetic diversity at the molecular level. Recent developments in molecular biology and biotechnology offer quick detection of genetic variation and characterization of genotypes using molecular markers.

2.7.1 Molecular markers and their use in characterization

The accurate identification of genotypes is of paramount importance in tree breeding and deployment operations, without which errors can be committed resulting in the wrong genotypes being used. If unidentified, these errors can cause lower than expected productivity. Using the right genotype is especially important where the breeding or deployment strategy uses clonal propagation, as the cost per clone is very high. With the ratification of UPOV convention, 1991, by several countries, the importance of Intellectual Property Rights of Plant Breeders (IPRPB) has recognized worldwide. The identity of individual tree clones will thus gain importance in future with commercial applications in clonal forestry.

2.7.2 Molecular markers in genetic diversity analysis and selection

The development and use of molecular markers for the detection and exploitation of DNA polymorphism is one of the significant achievements in the field of molecular genetics, which accelerate breeding by establishment of molecular fingerprints for distinct and most divergent accessions using diversity analysis reported by (Caetano,

1997). DNA based markers such as Random Amplified Polymorphism DNA (RAPD), Enhanced Random Amplified Polymorphism DNA (ERAPD), Simple Sequence Repeat Simple Sequence Repeat (Simple Sequence Repeat), Sequence Characterized Amplified Region (SCAR), Amplified Fragment Length Polymorphism (AFLP) and Inter-Simple Sequence Repeat (ISSR) can used to assist breeding through Marker Assisted Selection (MAS) to select prospective varieties from seedling stage. Microsatellite markers are available for a few Euphorbiaceae members and are being developed for castor (Okogbenin, 2006). As the developmental costs for microsatellite, markers are high, cross-taxa utility of molecular markers from could assessed as done in other plant species (Rossette, 2001).

Genetic variation in seed morphology and oil content of *Jatropha* is of great potential in tree improvement programs, unfortunately no much work has been done on germplasm conservation. However, Ginwal et al. (2005), reported seed source variability in central India and Kaushik et al. (2007), studied the variation in seed trait and oil content in 24 accessions collected from Haryana state, India. Use of molecular markers are of great significance in applying genetic technologies to crop improvement such as DNA fingerprinting of plant germplasm, introduction of new strain, marker assisted selection and targeted map based cloning etc. The first advancement came with the introduction of RFLP markers. It helps in assessing the molecular diversity of *Jatropha* germplasm and can be used in breeding program reported by (Mohan et al., 1997; Kumar, 1999). Recently a new full-length cDNA of stearoyl-acyl carrier protein desaturase was obtained by RTPCR and RACE techniques from developing seeds of *Jatropha* and the gene was functionally expressed in E. coli (Tong et al., 2006). It is an important enzyme for fatty acid biosynthesis in higher plants and also plays an important role in determining the ratio of saturated fatty acid to unsaturated fatty acids in plants reported by (Lindqvist et al., 1996).

Majority of the studies are confined to characterization of accessions available in India, except (Basha and Sujatha, 2007) having one non-toxic accession from Mexico, Montes et al. (2008), having accessions from 30 countries and Sun et al. (2008), having accessions from south China. Regardless of the number of accessions used, the robustness of the primer and number of marker data points, all accessions from India

clustered together. In general, diversity analysis with local germplasm revealed a narrow genetic base in India (Ganeshram et al., 2007) and south China (Sun et al., 2008) indicating the need for widening the genetic base of *J. curcas* through introduction of accessions with broader geographical background and creation of variations through mutation and hybridization techniques. In contrary to the above studies, AFLP based molecular characterization of *J. curcas* accessions from Andhra Pradesh were found diverse as these were scattered in different groups, showed occurrence of higher number of unique/rare fragments and had greater variation in percentage oil content reported by Leela et al. (2009). However, careful understanding of the phylogeny and use of adequate number of molecular markers are essential prerequisites for drawing valid inferences about the genetic affinities (Sujatha et al., 2008). However, Basha and Sujatha (2007), characterized *Jatropha* species occurring in India using nuclear and organelle specific primers revealed high inter-specific genetic variation (98.5% polymorphism). Further, characterization of both natural and artificially produced hybrids using chloroplast specific markers revealed maternal inheritance of the markers reported by (Basha et al., 2009). In support, genetic variation studies using RAPD, AFLP and combinatorial tubulin based polymorphism (cTBP) indicating higher possibilities of improving *J. curcas* by inter-specific breeding. Hence, molecular diversity estimates combined with the datasets on other agronomic traits will be very useful for selecting the appropriate accessions.

2.7.3 Molecular diversity studies on *Jatropha curcas* L.

According to Sathaiah and Tummal (1985), the seed protein profiles, obtained through polyacrylamide gel electrophoresis, on 56 castor accessions, comprising 3 height groups, viz, dwarfs, medium talls, and talls, were highly constant and uniform. The castor protein profile displayed 17 bands. Dwarfs exhibited a large number of bands when compared to medium talls and talls, and two bands, at Rf 0.22 and 0.42, were specific to them. Each of the *Jatropha* species, *J. curcas, J. heterophylla, J. gossypifolia,* and *J. panduraefolia,* has its own distinct protein profile. Eleven bands of *Jatropha* had their homologs in castor. Similarity indexes indicated *J. gossypifolia* as the closest one to *Ricinus.* Apparently, *Ricinus* and *Jatropha*, despite their morphological resemblances, are distinct genera, although perhaps with a common ancestor now extinct.

Although 42 Indian accessions of *J. curcas* showed moderate level of genetic diversity with 400 RAPD primers (Random Amplification of Polymorphic DNA; genetic fingerprinting technique), 42% molecular polymorphism and 100 ISSR primers (Inter-simple Sequence Repeat: genetic finger printing technique) 33.5% molecular polymorphism reported by Basha and Sujatha (2007). In other tests with 23 provenances from 300 selected provenances in central India, Reddy et al. (2007) was found that 8-10% (AFLP; Amplified Fragment Length polymorphism; genetic fingerprinting technique) and 14-16% (RAPD) polymorphism.

Table 2.1 Characterization of accessions of *Jatropha* by using molecular markers

Species/accessions	Primers	Number	Reference
142	AFLP	-	DBT India, 2007
5	RAPD	18	Ganeshram et al., 2007
22	RAPD DAMD	7 4	Ranade et al., 2008
13	RAPD ISSR	20 14	Gupta et al., 2008
7	RAPD AFLP	52 27	Sudheer et al., 2008
20	RAPD AFLP	- -	Reddy et al., 2007
43	RAPD ISSR	400 100	Basha and Sujatha, 2007
225	AFLP	-	Montes et al., 2008
58	SSR AFLP	30 7	Sun et al., 2008

Ganeshram et al. (2007), carried out an investigation to assess the genetic diversity of 12 *Jatropha* species based on random amplified polymorphic DNA markers. From 26 random primers used, 18 primers gave reproducible amplification banding patterns of 112 polymorphic bands out of 134 bands scored accounting for 80.2% polymorphism across the genotypes. Three primers viz., OPA 4, OPF 11, and OPD 14 generated 100% polymorphic patterns. The polymorphic information content was highest for the primer OPD 14 (0.50) followed by the primers OPF 11 and OPAD 11 (0.48). Jaccard's coefficient of similarity varied from 0.00 to 0.85, indicative of high level of genetic variation among the genotypes studied. UPGMA cluster analysis indicated three distinct clusters, one comprising all accessions of *J. curcas* L., while second included six species viz., *J. ramanadensis* Ramam., *J. gossypiifolia* L., *J. podagrica* Hook., *J. tanjorensis, J. villosa* Wight and *J. integerrima* Jacq. *J. glandulifera* Roxb. remained distinct and formed third cluster indicating its higher genetic distinctness from other species. The overall grouping pattern of clustering corresponds well with principal component analysis confirming patterns of genetic diversity observed among the species. These results provide valid guidelines for collection, conservation and characterization of *Jatropha* genetic resources.

Samanta and Maiti (2007), analysed 12 *Jatropha curcas* genotypes at the molecular level and found very poor genetic diversity. Among 24 RAPD primers, only 4 primers produced clear, reproducible bands. The primer RA1 produced maximum polymorphism, having maximum PIC content (0.44), RA2 has shown minimum PIC content (0.16). The percentage of loci that are polymorphic among the species studied was found to be 97.74% by RAPD and 97.25% by AFLP. The mean percentage of polymorphism (PP) was found to be 68.48 by RAPD and 71.33 by AFLP. The phylogram generated with RAPD and AFLP data showed maximum similarity. With the generated data, maximum relatedness was found between *J. curcas* and *J. integerrima* this may be the reason for the success of inter hybrid crosses between these two species. Neither RAPD nor AFLP data generated in this study supports the view of *J. tanjorensis,* a natural *interspecific hybrid* between *J. curcas* and *J. gossypifolia*.

The markers generated by RAPD and AFLP can be employed efficiently for interspecific hybrids identification, marker assisted selection and genetic resource

management reported by (Sudheer et al., 2008). The work on *Jatropha curcas* using molecular marker have been summarized in table 2.1.

2.9 Propagation methods and crop improvement

Jatropha curcas can also be successfully propagated by means of stem cuttings. It is the cheapest and economical methods of propagation. It will help in rapid multiplication of superior phenotypes/genotypes that contains highest seed and oil yield. The uniform and identical true to type planting stock can be developed through vegetative techniques. Clonal propagation techniques will be useful in capturing maximum genetic gains in quickest time (Swamy et al., 2002). The rooting potential of *Jatropha* mainly depends on season, age and size of the cuttings. The studies conducted on rooting of stem cuttings revealed that season is the most important parameter that strongly influences the rooting.

The tissue culture protocols serve as a prelude for genetic improvement through biotechnological tools. Various authors, (Sujatha and Mukta, 1996; Tiwari et al., 2006; Rajore and Batra, 2005; Datta et al., 2007) using different tissues explants from the field grown plants, have reported micropropagation of *J. curcas.* Regardless of the species and explant used, shoot regeneration occurred on medium supplemented with benzyladenine and indolebutyric acid but the concentration of growth regulators varied with the genotype and explant. Also in all of the cases, the multiplication rate was low for application. These studies revealed that the scope for improvement of *J. curcas* through somatic hybridization (Jha et al., 2007) in inter-specific crosses limited by crossability barriers. Somatic embryogenesis not only helps to obtain a large number of plants year round, but also can act as a powerful tool for genetic improvement of any plant species because of its single cell origin (Bhansali, 1990).

Jatropha grows readily from seeds or cuttings; however, trees propagated by cuttings show a lower longevity. It possess a lower drought and disease resistance than those propagated by seeds reported by (Heller, 1996) this might have been due to trees produced from cuttings do not produce true taproots (hence less drought tolerant). Rather they produce pseudo taproots that may penetrate only 1/2 to 2/3rd the depth of the soil as taproots produced on trees grown seed.

There are various methods to cultivate *Jatropha,* which vary from region to region and on climatic conditions. These are direct seeding, pre-cultivation of seedlings (nursery raising), transplanting of spontaneous wild plants and direct planting of cuttings. Wider spacing (3m×3m) is reported to give larger yields of fruit, at least in early years (Heller, 1996). In different countries and regions, the seed yields of *Jatropha* may range from 0.1 to 15 tonnes/ha/year (Ouwens et al., 2007). Kobilke (1989), in Cape Verde, conducted comparative research on the influence of different propagation methods on survival and vegetative development, in better rainfall or good moisture condition the plantation could also be established by direct seeding. The survival rate depended not only on sowing time and depth of sowing, but also on the trial year. When establishing a physic nut crop, the survival rate can be influenced by the choice of cultivation method. Two factors are generally responsible for sprouting, first one is the age of the plant from which cuttings are taken and second one is the position of the cutting within the plant, it might have been due to declines of rooting ability of many woody plants with age, when the source is a seedling derived mother plant. Distal portion of the stock plants are first to exhibit this reduced rooting potential, while cuttings from the lower or juvenile regions of the plants generally maintain a higher rooting capacity than those from the upper regions (Hartmann and Kester, 1983). The application of biofertilizers containing beneficial microbes showed a promoting effect on the growth of *Jatropha.* In most of the cases the biomass yield were found to be slightly higher with vermi-compost than farmyard manure but the reverse was observed in some cases wherein improvement of stem length was noticed with farmyardmanure reported by Kumar and Sharma (2005).

Rooting patterns were significantly influenced by propagation method. Plants originating from seeds and directly sown into the soil normally develop a rooting system with a thick primary taproot and four lateral roots, and with abundant and 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 was caused by reduced space for root expansion and not by lower availability of nutrients in the substrate (de Lourdes Silva de Lima et al., 2007). The seed yield per plant could be enhanced by employing biotechnological tools like marker-assisted selection of quality planting material, discussed earlier in this section. Tissue cultures have been

developed for the propagation and storage of selected genotypes of tropical plants (Engelmann, 1991). These techniques provide higher multiplication rate than the conventional breeding procedure. It will helpful to minimize the risk of infections by microbes and insect pests, reduced genetic erosion, space requirements and expenses in labor costs. Promising results from aseptic culture of various genotypes of *Jatropha* from both India and Nicaragua were obtained, which formed a base for future genetic improvement of this species (Sujatha and Dingra, 1993; Sujatha and Mukta, 1996; Machado et al., 1997; Sujatha and Prabakaran, 2003; Wei et al., 2004).

Swamy et al. (2002), have suggested propagation by stem cuttings for rapid and mass multiplication of superior phenotypes and genotypes of *J. curcas*. Preferably, cuttings should be taken from young plants during spring (February) and rainy (August) seasons. The rooting potential of stem cuttings can be improved by using phyto-environmental facilities *viz.* shade house, polyhouse and mist chambers. However, the cost of production of planting stock needs to be carefully assessed before adopting such improved technologies. They have strongly suggested use of quality planting stock to ensure sustainable yields of *Jatropha* for improving biofuel production. Introduction of non-toxic or less toxic variety of *J. curcas* will enhance the value of this crop in human and animal nutrition. The variety can be propagated through tissue culture. Crossing studies have clearly indicated that accidental out crossing with toxic *J. curcas* will not affect the phorbol ester content of seeds borne on the nontoxic variety. Mass propagation through shoot proliferation from axillary and apical bud cultures need to be optimized as suggested by Sujatha et al. (2006).

Sardana et al. (2000), have developed an efficient 2-stage method for plant regeneration from leaf explants derived embryogenic callus of *Jatropha curcus* L. They also studied influence of different culture media, hormonal regimes, light and sucrose on somatic embryogenesis. They have reported that somatic embryos develop into normal plantlets on full strength MS medium. Sujatha and Mukta, (1996) have observed callus induction from hypocotyls, petiole and leaf explants within weeks of incubation. Cytokinins like zeatin used singly (9.12 µM) induced shoots along with callus from hypocotyl explants. When auxins were used singly roots were formed, at cut ends of hypocotyl explants on IBA (9.8 and 24.7 µM) or NAA (5.37 µM) supplemented media. However, the response was very slow and overall frequency of

regeneration was very low. WEI Qin, (2004) obtained adventitious buds from epicotyl explants inoculated on MS medium supplemented with BA and IBA. However, according to him further research is needed to improve the protocol. Rajore and Batra (2005) reported shoot tips proliferation on MS medium with BAP (2.0 mg/l) and IAA (0.5 mg/l) along with adenine sulphate, glutamine and activated charcoal. *In vitro* produced shoots were induced to rooting on IBA (3.0 mg/l). Sujatha et al., (2005) reported that maximum shoot proliferation rate (10 – 12.3) with nodal culture, initially on medium with 2.3 – 4.5 µM TDZ. Nodal explants transferred from medium supplemented with BA and Kinetin produced shoots from axillary buds, while those from TDZ supplemented media differentiated shoots directly without any callus intervention. Nodes subcultured from TDZ supplemented media continued to expand and produced new shoots up to third subculture indicating it's carry over effect.

Mukherjee et al. (2007), have reported embryogenic callus formation from leaf explants on MS basal medium supplemented with only 9.3 µM Kinetin. They also mentioned induction of globular somatic embryos in *Jatropha curcus.* L. in addition with 13.6 µM adenine sulphate. Mature somatic embryos were converted to plantlets on half strength MS basal medium with 30% survival rate under field conditions. They also mention tissue culture studies were under taken in different species of *Jatropha.* Morphogenesis from endosperm tissues have been reported, in *J. panduraefolia.* High frequency regeneration from various explants of *Jatropha integrerrima,* has been reported. Using different explants, plant regeneration protocols have also been developed in *Jatropha curcus.* L. but multiplication rate was low for field applications. Economic importance and critical analysis of the earlier protocols have necessitated formulating a well standardized, simple and reproducible *in vitro* micro propagation protocol in this species. The literature survey on *Jatropha curcus* L. has been conducted for last 15 years. The work done on plant tissue culture has been summarized in table 2.2.

Table 2.2 Summary of tissue culture work reported on *Jatropha curcas* L.

References	Ex- plant	Medium Composition	Response
Sujatha and Dhingra, 1993	Hypocotyls	MS + 4.4 μM BA + 4.9 μM IBA	Shoot formation
Sujatha and Mukta, 1996	Hypocotyls	MS + 2.22 μM BAP + 4.9 μM IBA	Shoot bud induction on hypocotyls segment
	Hypocotyls	MS + 22.8 μM Zeatin + 4.9 μM IBA	Adventitious shoot formation
	Third leaves (Leaf disc)	MS+2.22 μM BA + 4.9 μM IBA	50% Leaf discs Producing Adventitious Shoots
	Leaf callus	MS + 4.44 μM BA + 2.46 μM IBA	4-5 shoots per callus
	Leaf callus	MS + 2.22 μM BA + 2.46 μM IBA	10-11 Shoots per Callus
Prabhakaran and Sujatha, 1999	Leaves	MS + 44.38 μM BA+ 4.9 μM IBA	Callus formation
WEI Qin and LU Wei-Da, 2004	Epicotyls	MS + 0.5 mg/l BA + 1.0 mg/l IBA	Adventitious buds formation
	Shoot tips	MS + 2.0 mg/l BAP + 0.5 mg/l IAA	Multiple shoots
Rajore and Batra, 2005	Rooting	MS + 3.0 mg/l IBA	Successful rooting
	Shoot tip	MS + 16^{-6} M BAP (Scrose 2%)	8-9 Shoots

	Shoot tip	MS + 16^{-6} M BAP +16^{-6} M Kin	15-18 Shoots
Tiwari et al., 2006	Nodal explant	MS + 5.0mg/l BAP + 3.0mg/l IAA	5-8 Multiple shoot
	Rooting	MS + 0.5mg/l IAA	Plantlet rooted
Sujatha et al., 2006	Auxiliary bud	MS + 2.3- 4.5 μM TDZ	2-3 Multiple shoot formation
	Subculture	MS + 8.9 Mm BA + 4.9 μM IBA	Highest frequency of regeneration
	Leaves	MS + 2.22 μM BA + 4.9 μM IBA	3-11 Adventitious shoots are formed
	Leaves	MS + 8.9 μM + 4.9 μM IBA	Highest frequency of regeneration
	Rooting	MS Basal	80% rooting
Rajore and Batra, (2007)	Leaf	MS + 5.0 mg/l BAP + 1.0 mg/l NAA	Callus containing meristemoids
	Subculture	MS + 1.5 mg/l BAP + 0.5 mg/l IBA	Shoot bud differentiation
	Rooting	MS + 3.0 mg/l IBA	Rooting (55%)
Datta et al., 2007	Nodal explant	MS + 22.2 μM BAP + 55.6 μM Adenine sulphate +	6.2 shoots per explant
	Subculture	MS + 2.3 μM Kinetin + 0.5 μM IBA + 27.8 μM Adenine sulfate	Shoot multiplication (30.8 shoots)

	Rooting	MS + 1.0 μM IBA	Rooting
Mukharjee et al., 2007	Leaf	MS + 9.3 μM Kinetin	Embryogenic callus
	Ebryogenic calli	MS + 2.3 μM Kinetin + 1.0 μM IBA + 13.6 μM Adenine Sulfate	58% Globular somatic embryos
	Somatic Embryos	½ MS Basal medium	Mature somatic embryos converted into plantlets
	Nodal explants	MS + 55.6 μM Adenine Sulphate + 22.2 μM BA	5 – 6 Shoot per explant.
Kalimuthu et al., 2007.	Nodal explants	MS + 1.5mg/l BAP + 0.5mg/l Kinetin + 0.1mg/l IAA	30 Shoots per explant
	Initiated Shoot	MS + 1mg/l IAA	Successfully roots are formed.
	Cotyledon leaves	MS + 2mg/l BAP	Somatic embryo formation
Deore A. and Johnson T. S. 2008	Cotyledon leaves/ leaves	MS + 2.27 Mm TDZ + 2.22 Mm BAP + 0.49 Mm IBA	Adventitious bud initiations
	Subcluture	MS + 4.44 Mm BAP + 2.33 Mm kinetin + 1.43 Mm IBA + 0.72 Mm GA_3	Multiplication and elongation
	Rooting	MS + 0.5 Mm IBA	Plantlet rooted

Meiru et al., 2008	Cotylydonary leaves	MS + 1.5 mg/l BAP + 0.05mg/l IBA + 1.0mg/l Phosphinothricin +500 mg/l cefataxime	Callus formation
	Subculture	MS + 1.5 mg/l BAP + 0.05 mg/l IBA + 1.0 mg/l Phosphinothricin +250 mg/l cefataxime	Shoot multiplication
	Subculture	1/2MS + 0.3mg/l IBA	78% rooting
Srivastava and Banarjee, 2008	Axillary node	MS + 3.0mg/l + 1.0mg/l + IBA + 25mg/l Adenine sulphate + 50mg/l Glutamine + 25mg/l Citric acid	Multiple shoot formation
	Subculturing	1/2MS + 3.0mg/l IAA	Rooting of shoot
Kumar S. et al., 2010	Leaf disc	MS+2 μM TDZ+2 μM BAP+1μM IBA	Adventitious bud initiations
	Subculture	MS+3 μM BAP	Multiplication (9.7/explants)
	Rooting	MS+1.5 μM IBA+200 μM Phloroglucinol	Rooting (76.7%)
Khurana et al., 2010	Leaf	MS+0.90 μM TDZ+0.98μM IBA+5 μM $CuSO_4$	Adventitious bud initiations
	Subculture	MS+2.22 μM BAP+1.44 μM GA_3+5 μM $CuSO_4$	Multiplication
	Rooting	1/2MS+2.46 μM IBA	Rooting (60%)

Lin Cai, Lin Fu and Lianghui Ji, 2011	Immature zygotic embryos	MS + 5-10mg/l 2, 4-D	Embryogenic callus
	Subculture	MS + Glutamine + Aspargine	Somatic embryogenesis

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3. Morphological diversity

3.1 Introduction

Jatropha curcas L. is considered to have originated in Latin America and is presently grown throughout the arid and semi-arid tropical and subtropical regions of the world. Success in the establishment and productivity of tree plantations is determined largely by species used and the source of seed within species (Lacaze, 1978). Growth and management of *J. curcas*, be it on private, public or community lands, have been poorly documented, with little field experience being shared amongst researchers and farmers (Saika et al., 2009), for reducing the dependence on crude oil and to achieve energy independence by the year 2030. *Jatropha* has been promoted under the National Bio-diesel Mission in India. Forson (2004), reported that the '*Jatropha* seed oil' can be easily processed to partially or fully replace petroleumbased diesel fuel. Therefore, the use of this plant for large-scale bio-diesel production is of great importance in order to solve the energy shortage, mitigating atmospheric CO_2 and enhancing the income of farmers (Banerji et al., 1985; Gubitz et al., 1999 and Openshaw, 2000). Systematic collection and investigation of the genetic distinctness in the regions where *Jatropha* has been introduced is identified as research gap (Hellar, 1996).

Most tree species, with a few exceptions, have a high degree of fecundity and their wild pollinated out crossing mating system, ensures large amount of heterozygosity and considerable genetic variability (Libby, 1987). Studies on provenance and seed sources have been made for many tree species indicating the usefulness of better quality, genetically improved seeds for plantations (Burley and Nikles, 1973; Lacaze, 1977; Wells and Wakeley, 1970). The planting stock raised from seeds is not genetically uniform due to cross-pollinated nature of plant. However, such studies on *Jatropha curcas* are lacking. Seeds collected from wild plants are not genetically uniform. Lot of variability exists in natural population of *Jatropha* with respect to seed yield (0.2 to 6 Kg/Tree) and oil content (30 to 48%) (Hellar, 1996). Therefore, selection of seed sources is important for raising high quality planting material of *Jatropha curcas*. Due to semi-wild distribution of *J. curcas* in Maharashtra, it would be expecte to have considerable genetic variations. According to Roy et al. (2004), environmental factors in combination with genetic and physiological factors play

important role in determination of plant potential for seed quality and it appears to be under strong genetic control. Morphology of seeds and its genetic traits vary according to geographical and environmental factors viz. latitude, soil type, temperature and degree of habitat disturbance. Research conducted across the world showed that *Jatropha* oil is an important emerging, low-cost and smokeless alternative of petrodiesel. *Jatropha* is viewed with a mixture of optimism and prudence. The cultivation of *Jatropha* has begun world over for bio-diesel production, but unfortunately, there is a lack of availability of quality planting material. In order to translate effective breeding programs, it is necessary to know each aspect of the plant and for this purpose study of provenance variation and initial growth performance of seedlings are considered to be important. So far, only few records were available of provenance trials of the *J. curcas* from Maharashtra. Hence, during present study explorations were undertaken in four agro-climatic regions of Maharashtra state of India, during 2007-2011. The present investigation has been aimed to develop a method of identifying superior lines using morphological traits.

3.1.1 Characteristic of study area: The study area comprises four agro-climatic regions of Maharashtra. As the study area is endowed with a variety of soils and varied climatic conditions, description of physiography, soils, climate and rainfall are given according to region (Ref. fig. 3.1 and table 3.1)

3.1.1 a. Kokan

The survey in Kokan region was carried out at Sindudurga, Ratnagiri, Dapoli, Alibag and Kalyan, ranging from 180 64'- 18^0 64'N to 72^0 15'- 73^0 38'E (Ref. fig 3.2). The important soil type is deep red loamy. The explored area is dry, semi evergreen tropical in nature. The annual rainfall varies from 2030 to 3114mm. The average minimum and maximum temperature vary between 20^0C and 41^0C respectively.

3.1.1 b. Western Maharashtra

The germplasm was collected from Pune, Nashik, Kolhapur, Solapur and Dhule in this region, ranging from 16^0 42'- 21^0 20' N to 73^0 51'-75^0 54'E (Ref. fig. 3.3). The important soil types encountered are basalt soil, deep red loamy, deep black cotton soils. The annual rainfalls vary from 549 to 1726mm. The average minimum and maximum temperature vary between 18^0 C to 42^0 C.

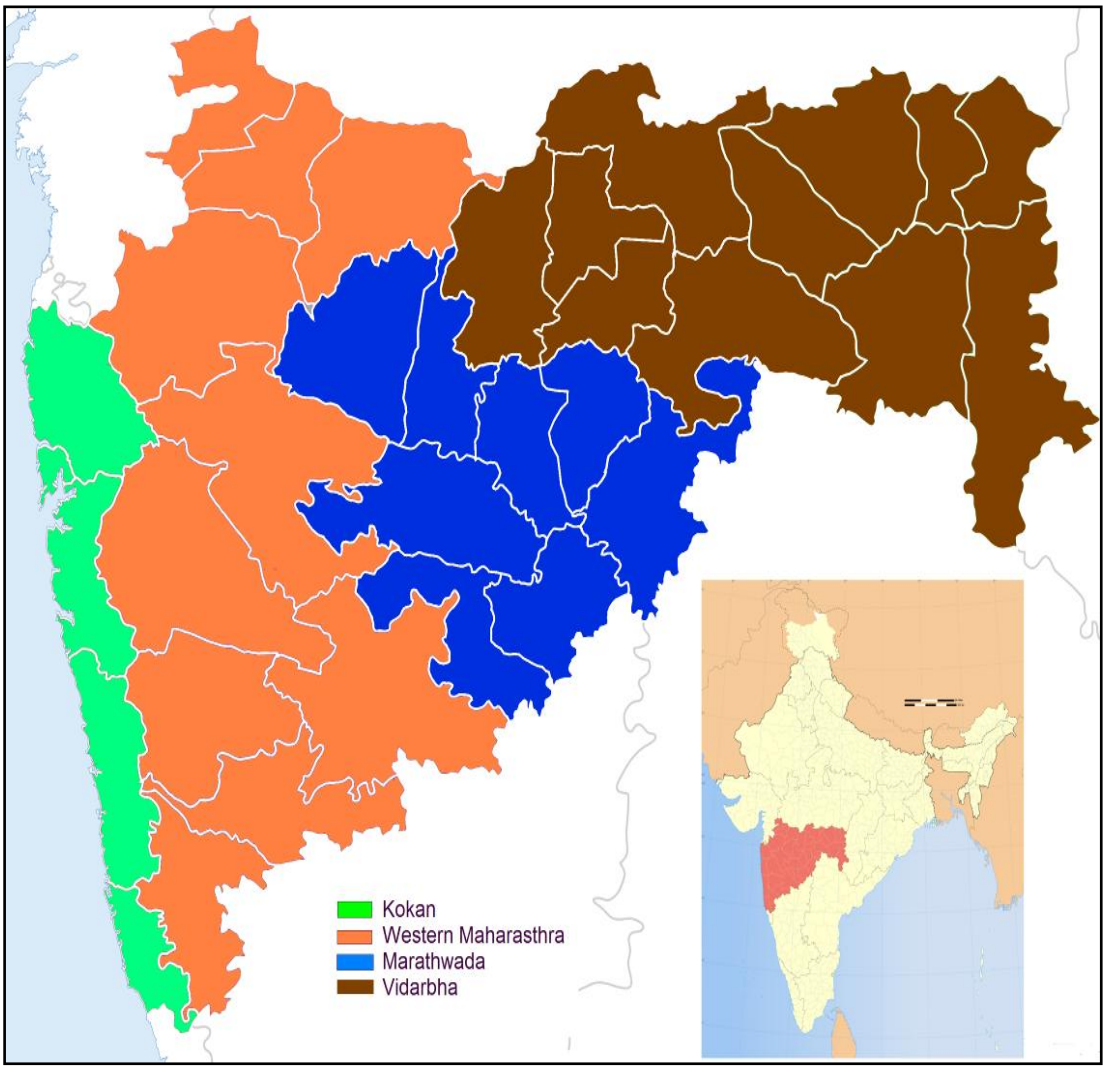


Fig. 3.1 Four Agro-climatic regions of Maharashtra

Fig. 3.2 Collection sites of Kokan region

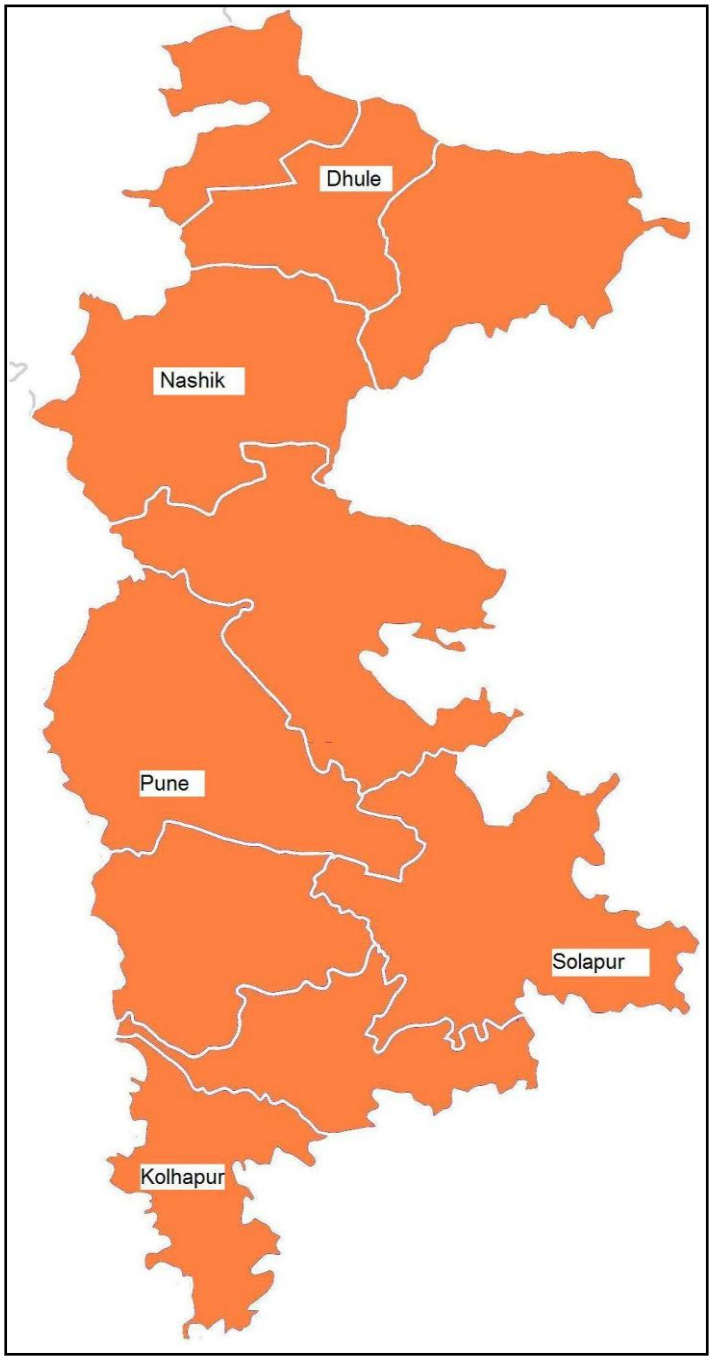


Fig. 3.3 Collection sites of Western Maharashtra region

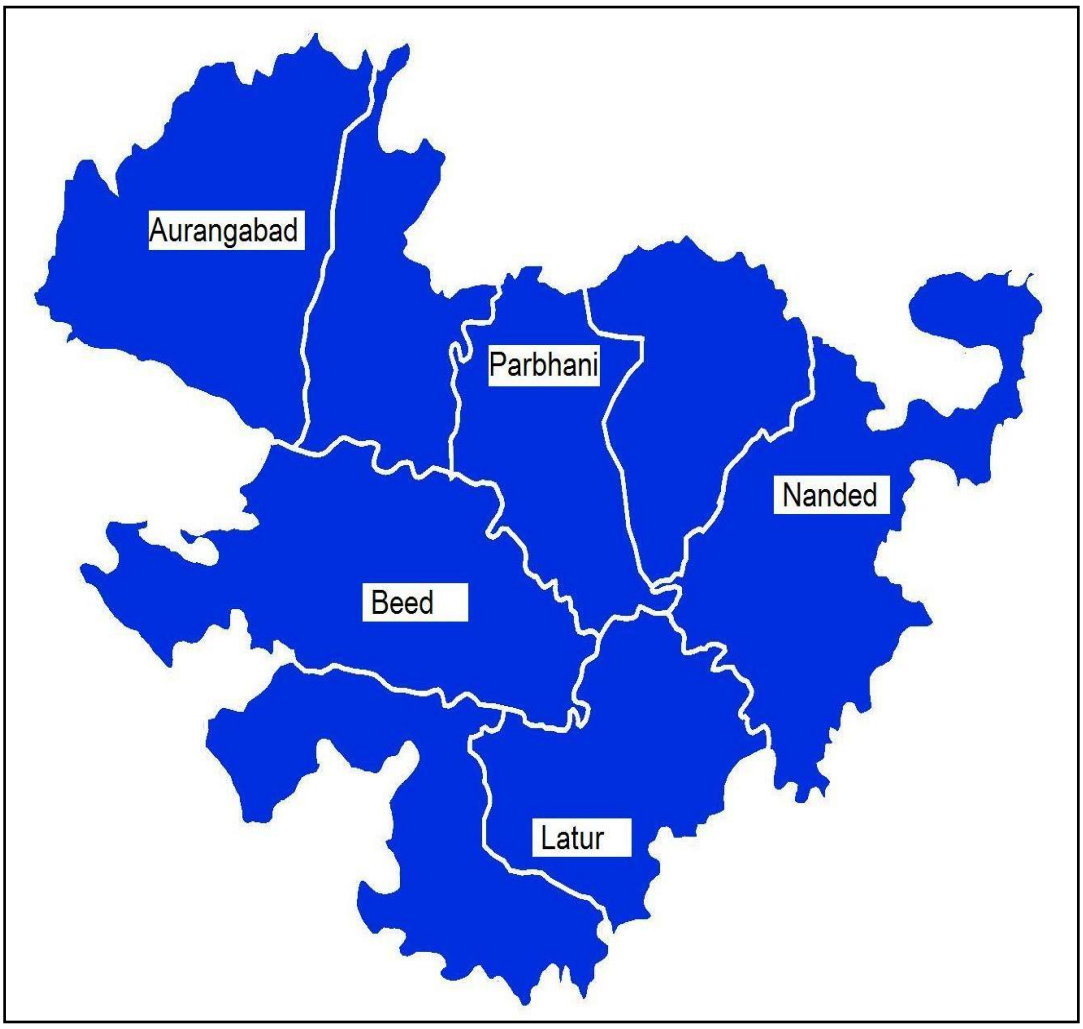


Fig. 3.4 Collection sites of Marathwada region

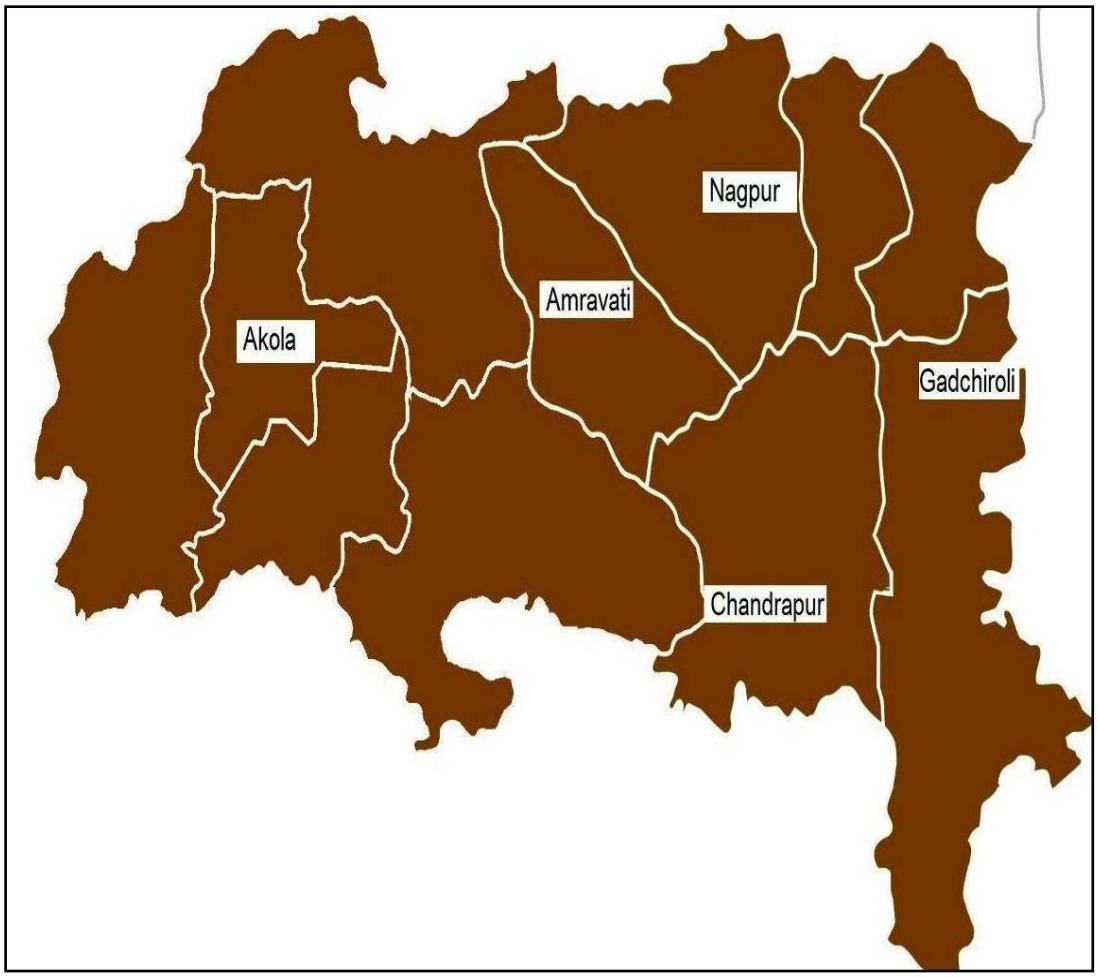


Fig. 3.5 Collection sites of Vidarbha region

3.1.1 c. Marathwada

Central part of Maharashtra comprises Aurangabad, Latur, Nanded, Parbhani and Beed in this region, ranging from 18^0 10'-19^0 53' to 75^0 20'-96^0 46' E (Ref. fig 3.4). The soils in this region are made up from basalt rock, and are deep black cotton soils, with moderate drainage. The annual rainfall varies from 887 to 1386mm. The average minimum and maximum temperature vary between 21^0 C to 46^0 C.

3.1.1 d.Vidarbha

The estern parts of Maharashtra are known as Vidarbha. It includes Nagpur, Amravati, Akola, Chandrapur and Gadchiroli, region ranging from 18^0 51'-21^0 06'N to 77^0 04'-79^0 58'E (Ref. fig. 3.5). The soils encountered are made up from basalt rock and are of red and yellow colour. The average annual rainfall varies from 552 to1317mm. The average minimum and maximum temperature vary between 24^0 C to 46^0 C.

3.1.2 Evaluation of genetic diversity

Evaluation of genetic diversity is one of the important steps involved in improvement of the genetic quality, adaptability and utilization of *Jatropha curcas* to improve its utilization. Over the past two decades, the plant has come under close scientific screening, worldwide, as source of bio-diesel. Despite the wide spread studies to find various applications, the programme of evaluation and improvements of genetic resources of these species have not been carried out in the state of Maharashtra. The material presently used for the study was chosen in a random manner.

The review of literature (Ginwal, 2004; Garnayak, 2008; Sbramanyam, 2010) suggests that, environmental stress might have caused a considerable impact on the genetic structure. Quantitative genetic studies serve as useful tool in making important decision. It will help in clustering the available germplasm in different groups indicating the extent of divergence in the population.

3.1.3 Coefficient of variation

Coefficient of variation provides a measurement for comparing the extent of variation between different genetic characters measured in the population. It is estimated at the phenotypic and genotypic level. The phenotypic coefficient of variation gives the

variability at phenotypic level including both genotypic and environmental variabilitys in addition to the genotype, environmental interactions.

3.1.4 Heritability

Heritability is defined as the proportion of total variance to the total phenotypic variance (Lush, 1937). The genotypic coefficient of variation together with heritability estimates give a better idea of advanced phenotypic characters or variability that is heritable. High heritability indicates the presence of additive gene action for the character and helps in the genetic improvement of species through selection (Panse, 1957).

3.1.5 Genetic Divergence

Genetic divergence is the genetic distance between two populations because of their genetic make up. In genetic diversity studies, identification of genetically divergent group is essential to understand the genetic variability, which could help in the improvement programmes. Several workers have reported the importance of genetic studies in plant improvement programmes (Hayes et al., 1955). Many statistical procedures have been developed to measure the divergence between two populations. The most commonly used technique is the D^2 analysis by Mahalanobis (1936). The D^2 analysis calculates the genetic distance between individuals in a population, based on their intra and inter cluster distance, they will cluster together.

The assessment of genetic divergence between population is vital and the utility of multivariant analysis and use of generalized distance (D^2**)** as a quantitative measure of genetic divergence are reported in number of plants like Banana, Sugarcane, Rubber, Areca, Teak, Eucaluptus, etc. In *Jatropha*, the PCV, ECV, GCV and Heritability in seed length, seed breadth, 100 seed weight, germination percentage, seed survival percentage, etc. were studied for the seeds collected from four agro-climatic regions of Maharashtra and significant difference was observed.

3.2 Materials Methods

3.2.1 Materials: *Jatropha curcas* L., commonly known as physic nut, belongs to the Family Euphorbiaceae. In present study, its seed and seedling characters were studied

using samples collected from four agro-climatic regions of Maharashtra. Botanical Survey of India, Pune, authenticated the identification of selected plant specimens.

3.2.2 Methods:

a) Bibliographic investigation: Literature pertaining to the taxonomy, distribution, seeds and seedlings were collected from various florae (Hooker, 1875-1897; Gamble 1915-1936). The literature available in the libraries of University of Pune, Botanical Survey of India (BSI) Pune, Research institutes like National Chemical Laboratory (NCL), Agharkar Research Institute (ARI), Pune, were also collected periodically updated.

b) Study location and sample collection: An extensive survey was conducted to collect the seeds of *J. curcas* from 20 different locations belonging to four agro-climatic regions of Maharashtra viz. Kokan (Ref. fig. 3.2), Western Maharashtra (Ref. fig. 3.3), Marathwada (Ref. Fig. 3.4) and Vidarbha (Ref. fig. 3.5). The selection was made on morphological assessment of characters i.e. yield potential, crown potential, girth, diseases resistance, etc. Each zone consisted of five collection sites, which uniformly represented each zone with considerable geographic isolation. The area sample ranged from Latitude: 15°52'-21°.20' N, and Longitude: 72°.15'-79°-58'E (Ref. fig. 3.1 and Table 3.1). The seeds were collected from the locations, which were minimum 150 km apart from each other, in order to avoid narrowing down of the genetic base due to relatedness or interbreeding, as per the method suggested by Turnbull (Ginwal et al., 2005). The seeds were selected according to their condition, where damaged seeds were discarded and seeds in good condition were cleaned, de-shelled and dried by sun drying technique.

c) Morphological study: The colour of fresh seeds was determined by matching them with the standard colour chart published by the Royal Horticulture Society (1966). Seed length, width and breadth were measured by using vernire caliper.

d) Germination and viability study: Germination trials were conducted in laboratory and nursery conditions. The seeds were pre-soaked before sowing in water for 24hrs, by taking four replicate of 100 seeds each. Seeds were sown in polybags, which was filled with substrate soil during February 2009, in the Botanical garden of Modern College of Arts, Science and Commerce, Pune 5. The potting media consisted

of Soil:Sand mixed in the ratio of 1:2 (volume basis). The seeds were treated with 10% BHC to avoid any chance of insect attack. Irrigation and weeding was carried out regularly and whenever required. In above cases, seeds were considered germinated when the radical had emerged about 1cm above the surface of the sowing media. Seed quality were recorded and quantified as viability percentage, germination percentage and germination value (Czabator, 1962). Seedling traits in nursery were recorded for plant survival percentage, seedling height (cm), collar diameter (mm), above and below ground biomass and total biomass (g/seedling). For each parameter, 25 plants per seed source were selected.

Following parameters were studied:

I. Viability percentage: Viability of collected seeds was tested using tetrazolium test as suggested by Agrawal (1980). The living cells were made visible by reduction of an indicator dye. The indicator dye used is colourless solution of Tetrazolium salt (2, 3, 5 Triphenyl tetrazolium chloride – TTC). Within the seeds, the dye interferes with the reduction process of living cells and accepts hydrogenases. By hydrogenation of 2, 3, 5-Triphenyl tetrazolium chloride, a red stable and nondiffusible compound, Triphenyl formazon is produced in living cells which makes it possible to distinguish the red coloured tissue of seeds from the colourless dead ones.

The solution –I was prepared by dissolving 9.078 gm of KH_2PO_4 in one liter of distilled water. Solution – II was prepared by dissolving 11.876 gm of Na_2HPO_4 in one liter of distilled water. 400 ml. of solution – I was mixed with 600 ml. of solution II. To this mixture, 10 gm of Tetrazolium salt added and dissolved. The p^H adjusted to 7.0. The preapared solution was stored in amber colored bottle.

A sample of 100 seeds was used for the test. The sterilized and presoaked seeds were dipped in Tetrazolium salt solution and placed in dark and warm place(in laboratory oven) at 40^o C for 8 to 10 hrs. Tetrazolium solution was removed and seeds were kept submerged in water. The seeds were examined under dissection microscope and viable seed were counted and expressed in percentage.

I. **Germination Percentage** : Every 10^{th} day after sowing, the number of germinated seeds were counted and the germination percentage was calculated as

Photo Plate 3.1 **a)** Collection of fruits from forest area. **b)** Collected fruits kept in plastic tray. **c)** Stored seeds of *J. curcas.* **d)** Germinated seed of *J. curcas.*

Table 3.1 Geographic information of the seed sources of *J. curcas* L. in Maharashtra and their seed-coat colour pattern.

Sr. No.	Agro climatic Zones	Seed sources	Latitude (^{0}N)	Longitude (^{0} E)	Altitude (Meter)	Rainfall (mm)	Relative Humidity %	Mean Temp. ^{0}C	Seed colour
1	Western Maharashtra	Pune	18^0 32’	73^0 51’	559	1259	38	31.4 – 34.0	Brown 200A
2		Nashik	20^0 08’	73^0 55’	608	1159	35	31.7 – 34.9	Brown 200B
3		Kolhapur	16^0 42’	74^0 14’	570	1726	37	31.3 – 34.5	Brown 200B
4		Solapur	17^0 40’	75^0 54’	479	798	33	34.0 – 37.2	Brown 200A
5		Dhule	21^0 20’	74^0 15’	206	549	29	32.3 - 36.9	Brown 200C
6	Kokan	Kalyan	18^0 64’	72^0 15’	011	2519	43	31.9 -34.5	Brown 200A
7		Alibag	18^0 38’	72^0 52’	007	2030	56	31.1 – 34.3	Black 202B
8		Dapoli	17^0 46’	73^0 12’	250	2546	52	31.4 – 34.6	Black 200C
9		Ratnagiri	16^0 59’	73^0 20’	092	3114	59	31.6 – 34.4	Black 202A
10		Sindhudurga	18^0 64’	73^0 38’	016	2901	63	31.9 – 34.5	Black 202A
11	Mahrathwada	Beed	19^0 00’	75^0 43’	519	1007	34	33.4 – 36.5	Black 202A
12		Latur	18^0 10’	76^0 03’	630	887	30	32.6 – 35.9	Brown 200A
13		Parbhani	19^0 16’	96^0 46’	423	1386	29	33.4 – 36.5	Brown 200C
14		Nanded	19^0 05’	77^0 20’	358	1008	28	34.7 – 38.3	Brown 200C
15		Aurangabad	19^0 53’	75^0 20’	581	956	32	32.6 – 35.4	Brown 200B
16	Vidarbha	Akola	20^0 42’	77^0 04’	309	552	33	35.2 – 38.6	Brown 200B
17		Nagpur	21^0 06’	79^0 03’	310	1159	29	34.6 – 37.5	Brown 200A
18		Yavatmal	20^0 24’	78^0 09’	451	1291	27	32.5 – 36.0	Brown 200B
19		Chandrapur	19^0 58’	79^0 18’	193	592	35	34.7 – 38.5	Brown 200C
20		Gadchiroli	18^0 51’	79^0 58’	123	1317	36	36.4 – 37.3	Brown 200C

$$\text{Germination \%} = \frac{\text{Number of Seeds germinated}}{\text{Total Number of Seeds sown}} \times 100$$

II. **Germination energy**: The interest in germination energy is based on a theory that only those seeds which germinate rapidly and vigorously under the favorable laboratory conditions, are likely to be capable of producing vigorous seedlings in field conditions.

$$\textbf{Germination energy} = \frac{\text{Germination periods}}{\text{Total Number of Seeds}} \times 100$$

III. **Survival of seedlings**: On the 60^{th} day of sowing seeds the survival of seedlings were recorded and expressed as percentage of total seeds germinated.

IV. **Seedling growth**: Seedling growth was measured on 30^{th} day. The shoot and root length was measured in centimeter from the transition zone to the shoot tip for shoot length and from transition zone to root tip for root length.

V. **Biomass of seedling**: Five seedlings of average height were taken for measurement of biomass of above ground level and below ground level. Seedlings were washed to remove all soil particles and fresh weight of both above and below ground biomass were recorded separately. The shoots and roots were dried in a laboratory oven at 30ºC and weight was measured at an interval of 24hrs, until the weight becomes constant. The final weight was recorded as the dry weight and expressed in mg.

VI. **Statistical Analysis**:

a) **Analysis of variance (ANOVA)** :- Analysis of variance was carried out for all the characters studied as per the procedure prescribed by Panse and Sukhatme (1954)

b) **Phenotypic variance (Vp) :**

$$Vp = Vg + Ve$$

Where, Vp = Phenotypic variance

Vg = Genotypic variance

Ve = Environmental variance

c) Genotypic variance (Vg) :

$Vg = Va + Vd + Vi$

Where, Va = additive variance

Vd = dominance variance

Vi = epistatic variance

d) Environmental variance (Ve) :

$Ve = Veg + (Vg \times E) + Ves$

Where, Ve = Environmental variance

Ves = Specific environmental variance

Veg = General Environmental variance

(Vg x E) =Genotype and environmental interaction

e) PCV (Phenotypic Coefficient of Variation) :

$$PCV = \frac{\sigma p}{\bar{x}} \times 100$$

Where,

σp = Phenotypic standard deviation

$\bar{x}$ = Grand mean for population characters

f) GCV (Genotypic Coefficient of Variation) :

$$GCV = \frac{\sigma g}{\bar{x}} \times 100$$

Where,

σg = Genotypic standard deviation

$\bar{x}$ = Grand mean for population characters

g) ECV (Environmental Coefficient of Variation):

$$ECV = \frac{\sigma E}{\bar{x}} \times 100$$

Where,

σE = Environmental standard deviation

$\bar{x}$ = Grand mean for population characters

h) Heritability: Heritability (H^2) in the broad sense means the fraction of the total variance, which is heritable. It was estimated in percentage by using following formulas:

$$H^2 = \frac{\sigma^2 g}{\sigma^2 p} \times 100$$

Where,

$\sigma^2 g$ = Genotypic variance

$\sigma^2 p$ = Phenotypic variance

i) Genetic advance: A Heritability estimate along with expected genetic gain is more useful than the heritability value alone in predicting the resultant effect for selecting the best genotypes (Johnson et al., 1955).

$$GA = (k) \times \sqrt{Vp} \times (H)$$

Where,

k = the selection differential in standard deviation units (the value of 'k' at 5% selection intensity (2.06) was used in the calculations),

Vp= the phenotypic variance of the base population for the trait in question, and

H = the heritability of the trait expressed in fraction.

j) Genetic gain: Genetic gain is the amount of increase in performance that was achieved through artificial genetic improvement programs. This is usually used to refer to the increase after one generation has passed.

The expected Genetic Gain (GE) was calculated by the following formula:-

$$GE = \frac{\text{Genetic Advance} \times 100}{\text{Population Mean}}$$

3.3 Result and Discussion

Our investigation revealed that, seeds and seedling characters varied among four agro-climatic regions, and provenances within those regions. Initially, regional variations for different characters was explored; then within those particular agro-climatic region (in which variations were observed), provenance variation for different characters was studied. Regional means were compared with appropriate LSD, and it was found that seed and seedling characters differed significantly among zones. Seed length was highest for Marathwada region while it was minimum for Kokan region (Fig.3.6a). Seed width was maximum for Marathwada region, followed by Western Maharashtra and Vidarbha. It was minimum for Kokan region (Fig. 3.6b). Seed thickness was the highest for Marathwada while it was minimum for Kokan (Fig. 3.6c). Maximum seed weight was recorded for Marathwada and minimum for Kokan region (Fig. 3.6d). The highest seed germination percent was recorded for Vidarbha region and minimum for Kokan region (Fig. 3.6e). The highest seedling height was observed for Western Maharashtra region and minimum for Kokan region (Fig. 3.6f). Collar diameter was maximum for Western Maharashtra (Fig.3.6g). Maximum weights of seed kernel were found for Marathwada region and minimum for Kokan region (Fig.3.6h). Total biomass was maximum recorded for Marathwada region (Fig. 3.6i).

3.3.1 Seed morphology: Significant difference ($P \leq 0.05$) occurred among the seed sources for seed size (Ref. Table 3.3). Maximum seed length (18.35 mm) was observed in Marathwada region followed by vidarbha region. Minimum seed length (17.10 mm) was recorded in Kokan region. There are no significant differences among the provenances within agroclimatic regions (Intrapopulation). Seed width varied from 10.11 to 11.62 mm. with maximum width in Marathwada region followed by Western Maharashtra. Minimum was recorded for Kokan region. Similarly, Seed thickness varied from 8.40 to 9.20 mm.

Seed sources of *Jatropha curcas* revealed slight difference in seed colour pattern of their seed-coat (Ref. Table 3.1). Seed collected from Ratnagiri, Sindhudurga, Beed and Latur matched to the black group (202A) colour pattern, while remaining matched to three brown groups, i.e. 200 A, B, C. Weight of the whole seeds and its components revealed maximum weight in Kokan region followed by Western Maharashtra region. It was significantly low for Marathwada and Vidarbha region.

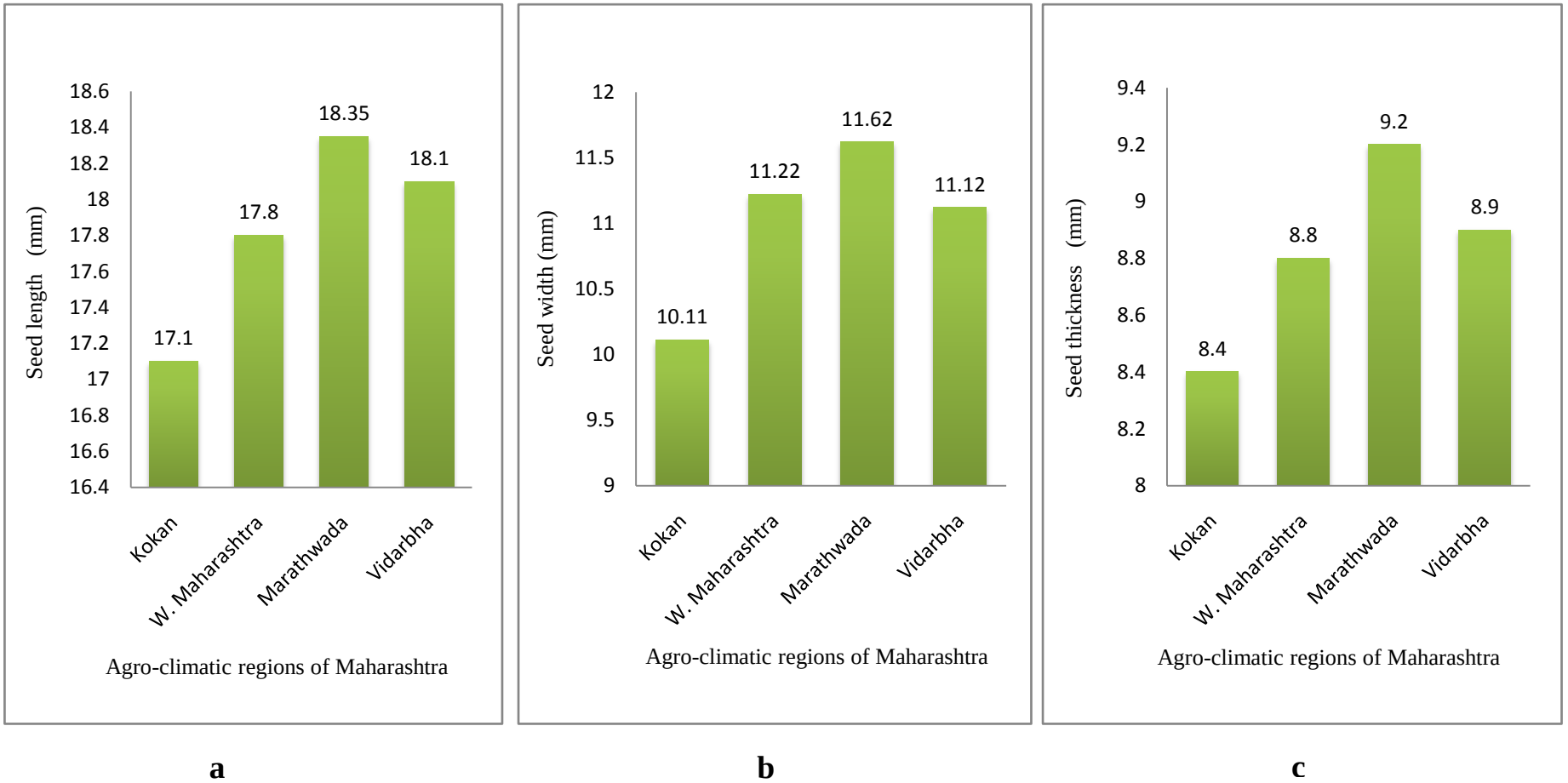


Fig 3.6 A Agro-climatic regional variation for **a)** seed length, **b)** seed width, **c)** seed thickness

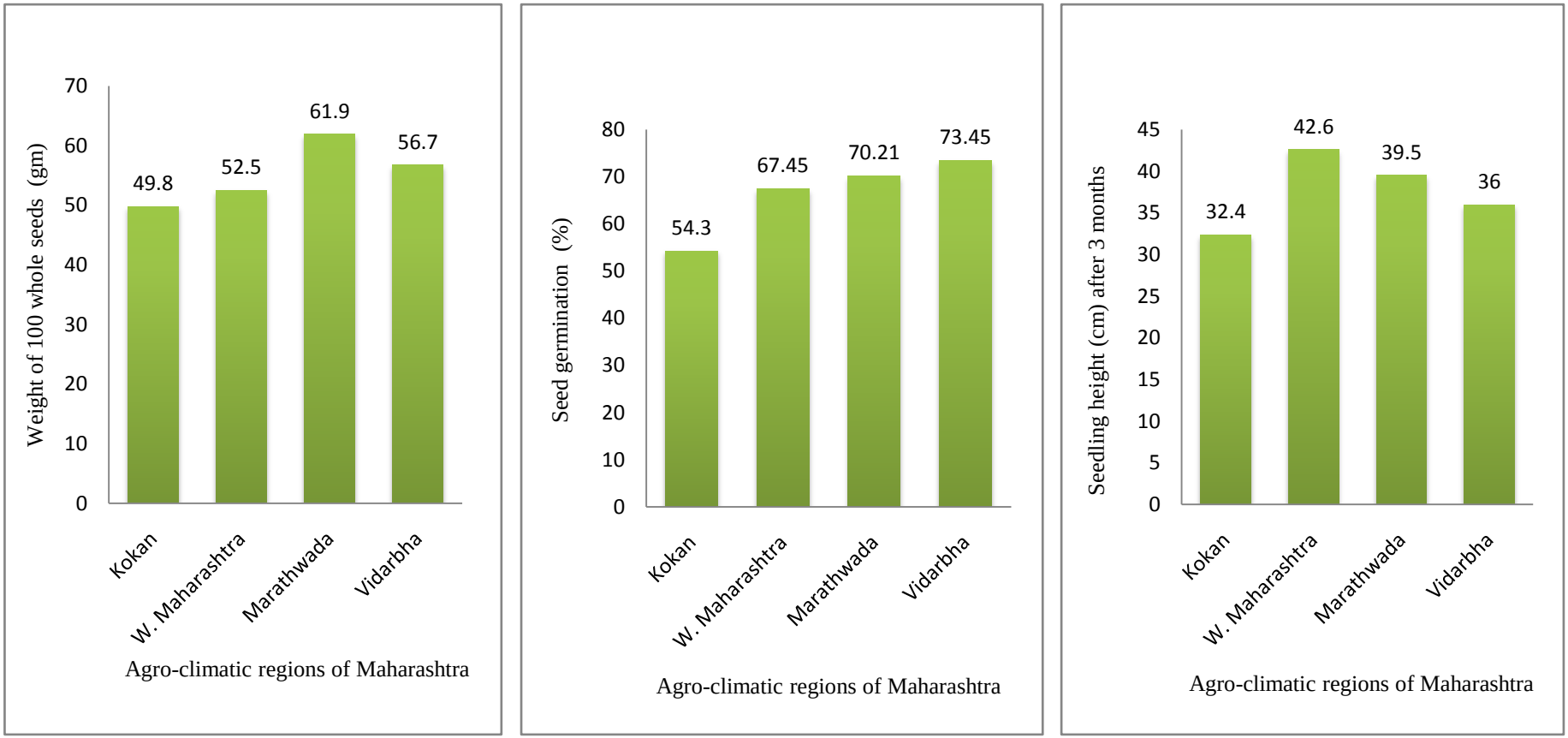
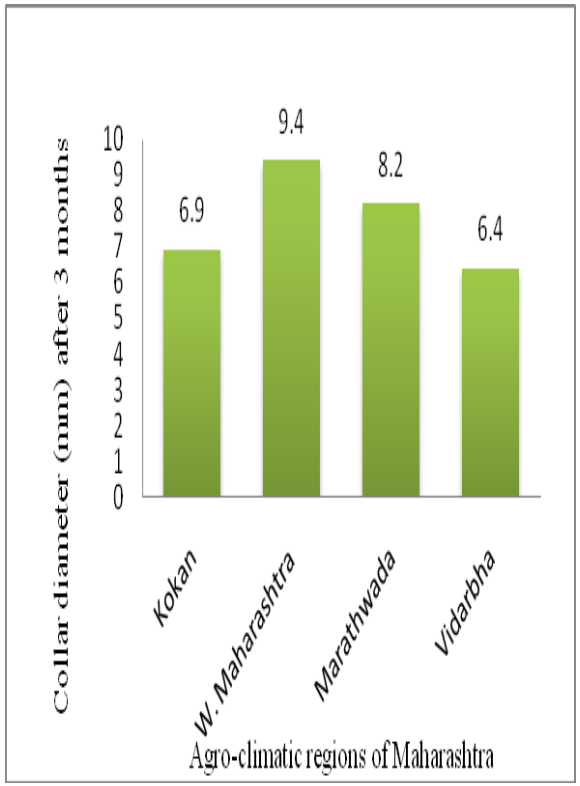
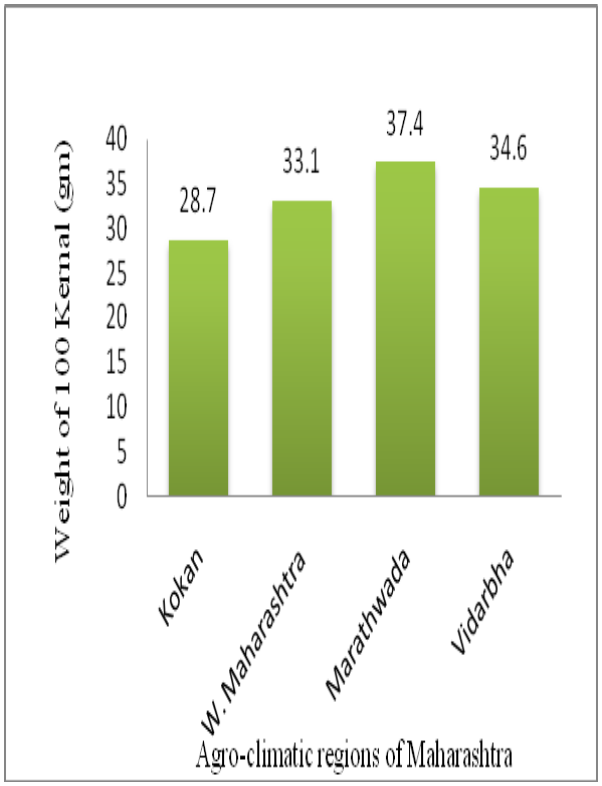


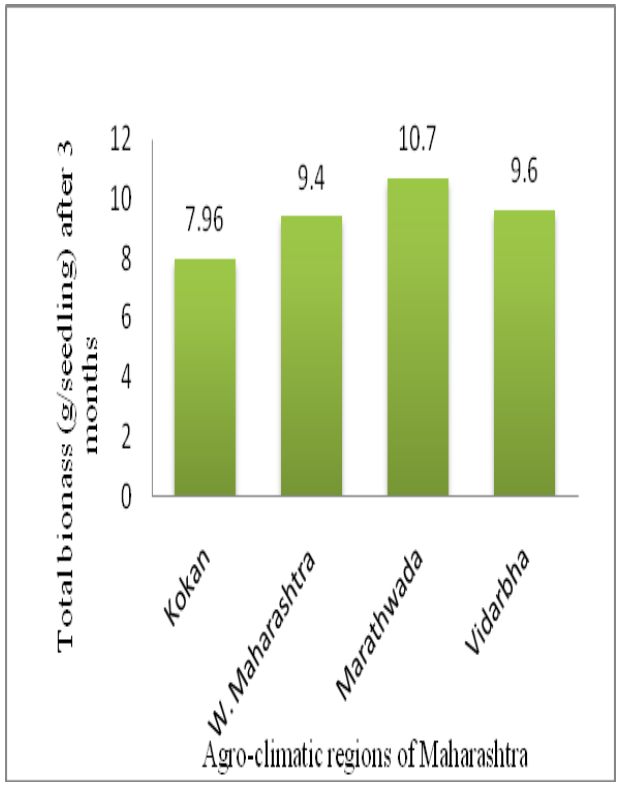
Fig 3.6 B Agro-climatic regional variation for **d)** seed weight, **e)** seed germination, **f)** seedling height,



g



h



i

Fig 3.6 C Agro-climatic regional variation for **g)** collar diameter, **h)** weight of kernel **i)** Total biomass.

Table 3.2 Variation in *J. curcas* L. seeds and seedling traits in laboratory and nursery conditions.

Sr. No.	Parameters	Range	Mean	CD	CV %
1	Weight of100 whole seeds (g)	49.8 - 61.9	55.85	0.267	12.67
2	Weight of 100 seed coat (g)	1.21 - 1.46	1.31	1.06	7.05
3	Weight of 100 Kernel (g)	28.7 – 37.4	33.05	0.291	10.25
4	Seed length (mm)	17.10 - 18.35	17.72	0.401	1.76
5	Seed width (mm)	10.11 - 11.62	10.86	0.265	1.00
6	Seed thickness (mm)	8.40 - 9.20	8.80	0.475	2.67
7	Seed viability % (Lab)	78.20 - 95.6	86.90	2.84	8.49
8	Seed germination % (L)	58.42 - 83.52	70.97	2.15	12.54
9	Seed germination % (N)	54.30 -73.45	63.87	1.85	9.64
10	Germination energy (N)	41.67 - 62.35	52.01	1.67	10.31
11	Seedling survival % after 1 months (N)	97.46 - 99.45	48.73	2.50	0.92
12	Seedling survival % after 3 months (N)	95.27 - 98.64	56.49	3.46	0.89
13	Seedling height (cm) after 3 months	32.40 - 42.60	37.5	3.22	6.58
14	Collar diameter (mm) after 3 months	6.4 - 9.4	7.9	2.61	3.45
15	Above ground biomass(g/seedling) in 3 months	5.49 - 7.58	6.53	1.57	15.52
16	Below ground biomass (g/seedling) in 3 months	0.98 - 1.86	1.42	0.90	10.57
17	Total biomass (g/seedling) after 3 months	7.96 - 10. 7	9.21	2.64	8.42

(* L = Laboratory, N= Nursery)

The coefficient of variance (CV) for weight of whole seeds, seed coat and kernel were recorded 12.67, 7.05 and 10.25 respectively (Ref. Table 3.2). Seed size, i.e. length, width and thickness has also shown similar trend. The CV for these traits was found 1.76, 1.00 and 2.67 respectively. The seed sources of *Jatropha curcas* exhibited considerable amount of variation in morphological traits of the seed. Analysis of variance of the data on seed weight and seed size showed that the difference between seed sources were significant ($P \leq 0.05$). The co-efficient of variation observed was very low with regard to seed size (≤ 2.7), while moderate for seed weight (10.25 to 12.67).

3.3.2 Seed germination and seedling growth traits: Variation in seed viability, germination percentage and germination energy of seeds grown in laboratory was found significant among different seed sources with CV of 8.49, 12.54 and 10.31% respectively. Maximum values for these parameters were recorded for Marathwada region seed sources followed by Vidarbha. Minimum values for these parameters were recorded for Kokan followed by W. Maharashtra. Almost similar trend was noticed in nursery. The survival percentage of seedlings after one and three months of seed sowing in nursery revealed a maximum of about 99% survival for Marathwada region seed sources. The CV was recorded very low, viz, 0.92 and 0.89% respectively at both the age interval (Ref. Table 3.2). Growth parameters were determined through measurement of seedling height, collar diameter, above and below ground biomass and total biomass after three months of sowing under nursery condition.

Height growth varied significantly, across the sources, maximum seedling height (42.60 cm) was recorded for Marathwad source, while minimum (32.40 cm) for Kokan. The coefficient of variation (CV) between the sources of this trait was 6.58%. The collar diameter, above and belowground biomass has also revealed more or less similar pattern. The CV for this parameter was recorded 3.45, 15.52, 10.57 and 8.42 respectively (Ref. Table 3.2).

Present study revealed that seed and seedling characters of *J. curcas* differed significantly among different agro-climatic regions and provenances. Regional differences showed that environmental factors play a vital role in changing seed and seedling characters. Each provenance represented a particular locality, soil and climatic condition. The provenances possessed genotypic characters according to their

locality, but when grown in different agro-climatic regions, they were adjusted by local climatic conditions and this adjustment affected their performance. On an average, provenances from Marathwada, Vidarbha and Western Maharashtra performed best whereas, provenances from Kokan region proved poorest. Because, this study was conducted at Pune (Western Maharashtra), the provenances belonging to this particular region experienced less stress, whereas other provenances faced very different climate.

The highest 100 seed weight and germination per cent of Vidarbha provenance is due to the fact that it belongs to the estern parts of Maharashtra, where annual rainfall is 552-1317mm and annual temperature range is 24-46°C (July). The lowest 100 seed weight and germination of Kokan provenance could be due to attributed to the origin of seeds from an area experiencing low temperature ranges. Maximum rainfall within two months (July and August) and low water holding capacity of soil.Within regions, differences might be due to local variations in bioclimate, soil fertility and topographic conditions, as zones are themselves characterized by well distinguished annual rainfall, temperature range and major soil groups. Various climatic factors influence the vegetation collectively but not individually. Thus, the vegetation of a place is the result of various climatic factors acting together. While affecting vegetation collectively, these factors modify the influence of each other to certain extent. Total rainfall of a place influences vegetation, but the effect of total rainfall is modified by the number of rainy days. Considering these facts, provenances may possess climatic and edaphic features different from their agro-climatic regions. These factors ultimately caused provenance variation within zones.

Manga and Sen (1995), observed that germination percent in *Prosopis cineraria* can be improved by selecting large and heavy seeds. The performance of seed immediately after germination is governed by seed size (Willan, 1985). Heavy and large seeds contain more food reserves than smaller ones, which is helpful in germination by providing more energy (Lusk, 1995). Ponnamal et al. (1993) also reported similar findings, for example, within the Marathwada and Vidarbha region, seeds had maximum seed weight and the highest germination percent.

Seed size and weight are two important characters for improving seedling productivity and reducing nursery cost through selection of quality seeds, apart from selecting and delineating provenances (Armstrong and Westoby 1993; Isik 1986 and Uniyal et al., 2002). The purpose for provenance testing is to measure the pattern of genetic variation and to aid in selection of well-adapted and highly productive seed sources.

Within the four agro-climatic regions, highest seed weight was observed for Vidarbbha and Marathwada regions respectively, at the same time both provenances produced highest seedling length in their respective zones. Hence, it is clear that seeds with greater seed weight produced seedlings with higher shoot length. This may be due to greater nutrient reserves in larger seeds (Kathju et al. 1978). Similar trend was also reported for *Virola koschyni* (Gonzales 1993), *Hardwickia binata* (Ponnamal et al. 1993) and *Albizia lebbek* (Roy 1985). Thus, it can be concluded that seed size has operational importance. Provenances with higher seed weight also possessed higher length and vice versa. Similar results were also reported in Albizia lebbeck (Bhat and Chauhan 2002; Luna et al., 2006). Seed weight is also related to oil content. Agro-climatic regions with higher seed weight also had higher oil per cent.

3.3.3 Genetic analysis: Wide differences have been also recorded for variance components of seed germination and seedling growth parameters of *J. curcas* seeds (Ref. Table 3.3). The highest phenotypic variance was found for the laboratory germination value (123.58) while least for below ground biomass (0.034). The genetic variance varied from 0.029 to 116.66 for the same parameters. For the environment variance the maximum value was recorded for seed viability percentage in laboratory conditions (5.41) while minimum for below ground biomass (0.016).

The seed vigour, i.e. germination energy in nursery exhibited the highest value of PCV (16.52) and GCV (16.24). While, ECV was recorded maximum for (9.647) below ground biomass (Ref. Table 3.3). The phenotypic and genotypic variance and their coefficient of variability for most of the parameters of seed germination and seedling traits were found to be higher than corresponding environmental variance and environmental coefficient variability (ECV).

Table 3. 3 Variances, coefficient of variability and estimate of genetic component in *J. curcas* L. for seed and seedlings traits observed in laboratory and nursery conditions

Sr. No.	Parameters	Variances			Co-efficient of variability			Genetic component		
		Vp	Vg	Ve	PCV	GCV	ECV	Heritability	Genetic advance	Genetic gain
1	Seed viability % (Lab)	40.62	35.21	5.41	6.38	6.11	3.05	85.346	10.643	9.065
2	Seed germination % (Lab)	11.5	108.32	3.18	12.42	10.45	1.22	98.520	14.65	22.543
3	Germination energy % (lab)	120.2	116.66	3.54	16.52	16.24	2.15	98.560	20.643	35.438
4	Seedling survival % after 1 months (Nry)	0.807	0.413	0.394	0.764	0.762	0.735	56.381	1.143	1.437
5	Seedling height (cm) in 3 months	1.064	0.543	0.521	1.124	1.120	0.935	69.244	1.243	1.419
6	Collar diameter (mm) in 3 months	3.24	2.514	0.726	10.51	9.816	4.125	75.146	1.983	12.543
7	Above ground biomass(g/seedling) in 3 months	1.301	1.153	0.148	10.66	9.824	3.416	83.456	1.543	19.041
8	Below ground biomass (g/seedling) in 3 month	0.05	0.034	0.016	9.651	9.647	9.647	99.728	0.349	16.943
9	Total biomass (g/seedling) in 3 months	1.837	1.643	0.194	10.64	9.843	3.753	87.564	2.456	20.254

Heritability values were recorded over 75% for majority of the traits except for survival percentage. The genetic advance ranged from 20.643 (Germination energy in lab) to 0.349 (below ground biomass) while genetic gain were recorded maximum of 1.419 for seedling height after 3 months (Ref. Table 3.3). The populations of this species collected from the Maharashtra vary in growing habitat in respect of altitude, temperature, soil conditions and rainfall. The sources used under this study had mean annual rainfall range from 549 to 1726mm. In addition, the source varied considerably in respect to site and soil condition viz. slope, soil depth and soil texture. Variation in sources of *J. curcas* with respect to growth performance is mainly because this species grows over wide range of rainfall, temperature and soil types in Maharashtra. Population might have experienced marked difference in selective pressure.

The seed sources of *J. curcas* exhibited considerable amount of variation in morphological traits of the seeds. Analysis of variance of the data on seed weight and seed size showed that the difference between seed sources were significant ($P \leq 0.05$). The co-efficient of variation observed was very low with regard to seed size (≤ 2.7) while moderate for seed weight (2.90 - 3.70). Marathwada seed sources showed an excellent and stable performance in respect of almost all traits of seed morphology, seed germination and seedling growth. The location of Marathwada seed sources is at fairly high altitude in comparison to others and is characterized with less rainfall, moderately high temperature and better soil depth as compared to the other sources (Ginwal et al., 2005). Perhaps, this might have lead to a better growth of seedlings and improvement in seed characters.

The variance registered in *J. curcas* for seed germination, seedling growth and biomass parameters showed considerable variation. All the variability estimates for seed behavior have expressed high genotypic variability and heritability. The genotypic coefficient of variability and heritability were recorded to be high for viability of seed, germination percentage and germination energy. Heritability in broad sense may give useful indication about the relative value of selection of the material at hand (Ginwal and Gera, 2000). Under nursery condition, genotypic variance was recorded very close to phenotypic variance accompanied by very high heritability. However, the pattern has been observed to be the same for genotypic co-

efficient of variability. Therefore, selection based on these parameters holds good for nursery condition on seed behavior.

Since germination energy is a measure speed of germination, it gives an idea of the vigour of the seed and of the seedling, which it produces. The interest in germination energy is based on the theory that only those seeds which germinate rapidly and vigorously under favorable conditions are likely to be capable of producing vigorous seedling in field condition, whereas weak or delayed germination is often fatal (Ginwal et al., 2005).

The difference observed for germination parameters are genetic in nature because, the environmental deviation were negligible for experimental site, while the randomization and replication must have further reduced the chance of site effects (Ginwal et al., 2004). In present study, the seed sources are evenly distributed resulting in lack of large climatic differences within the sampling ranges. However, small and distinct pockets of *J. curcas* plants scattered within different seed sources may have restricted gene flow from population to population and therefore, resulted in a discontinuous type of variation in germination characteristics, which are genetically controlled (Whittington, 1973). The significant difference in various seed morphological and seedling characters of *J. curcas* provenances is indicative of the possibility of selecting large and heavier seeds for further improvement work. Significant agro-climatic regional impact revealed that environmental factors contribute in changing external appearance as the species grows in a wide range of ecological conditions and hence population can be expected to experience markedly selective pressure on seed characters. The agro-climatic regional (Inter) and provenance (Intra) variation could partly arise from genetic diversity. The results on morphological diversity of *J. curcas, of* Maharashtra obtained during present study, have been duly published (Gopale and Zunjarrao, 2010).

3.4 Conclusion

The seed sources in most of the cases were significantly different in growth variables. It has showed a considerable amount of genetic variability within the distribution range indicating a good scope of genetic gain through selection. *J. curcas* is found

mostly in Maharashtra as small discrete populations, which are basic resources for future improvement and breeding programmes. The results of the study will be valuable for seed zone delineations, strategies for conservation of genetic variation prospects of improvement and assessment of the potential of locally adapted seed source.

The patterns of variation exhibited for various characters were substantially exhibited for various characters were substantially different. The presence of such difference among populations has probably produced by different intensities of natural selection acting upon these traits in their natural habitat. Some of the variation found associated with the discrete populations from which seeds were collected. The Kokan seed source in the present study ranked comparatively low in comparison to the growth producing sources i.e. Marathwada and Vidarbha. It is indicative that there is a better choice of using selected promising genotypes and population from the range of distribution.

Tree breeding strategy is largely dependent upon the extent of variability in the base population, which measured by different parameters viz. genotypic and phenotypic variance, genotypic and phenotypic coefficient of variation. Heritability in broad sense gives useful indication about the relative value of selection of the material at hand to arrive at a more reliable conclusion. If heritability and genetic advance considered jointly, the present study showed that there exists considerable amount of genetic variability in this species in Maharashtra with respect growth performance, which offers scope to breeder for selection and breeding. It is advisable that this seed sources should be used for collection of bulk quantity of seeds to achieve better productivity. It is quite clear that Marathwada source is good in growth performance particularly in the prevailing conditions in the Maharashtra. The Western Maharashtra, Vidarbha sources also performed satisfactory in respect to growth.

This work further gives a direction to effect and practice studies for genetic improvement of this species. Perhaps, this is a first attempt and report, which surveys and assesses growth performance of *J. curcas* populations from four agro-climatic regions of Maharashtra.

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4. Biochemical Diversity

4.1 Introduction

Jatropha curcas is perhaps currently the plant of greatest importance for the extraction of oil for production of bio-diesel. Various authors have mentioned the advantageous characteristic of this plant from the family euphorbiaceae, in comparioson with other plants, highlighting particularly its adaptation to marginal environment (Berchmans and hirta, 2008). Even though *Jatropha* has been scientifically investigated earlier for useful secondary metabolites, the kind of comprehensive research and development efforts are necessary to generate profitability and the critical information for the different climatic and edaphic regions have only recently started (Jorge et al., 2010). The success of these ventures rests on the continuous inflow of relevant information from research into practice (Francis, 2005).

One of the most important inputs for successful cultivation of *J. curcas* is the selection of elite planting material. Observations in upcoming plantations indicate that the productivity of the individual plants show high variations. The major limitation is promotion of the crop in its undomesticated condition (Basah et al., 2009). Gaining insights into the genetic variability of *J. curcas* provenances and revelation of correlations between the genetic parameters and biochemical characteristics of seed provenances collected from different regions of the world would be a critical input for the selection of appropriate genotypes for cultivation and breeding purposes (Basah et al., 2009). Recent interest in promotion of *J. curcas* as a bio-diesel crop demands genetic improvement of the crop for increased seed yield and oil content. Germplasm characterization is necessary to enhance germplasm management and utilization. Information regarding the extent and pattern of genetic variation in *J. curcas* population is limited.

Many efforts are being diverted towards utilization of agricultural by-products as source of animal feed. In this regard, *Jatropha* has been paid a special attention as by-products of the plant hold potential for use as animal feed (Goel et al., 2007). The seed cake remaining after oil extraction is an excellent source of plant nutrients (Foidl et al., 1996). Due to its large number of potential utilizations, this tropical plant is

cultivated in many latin American, Asian and African countries. The seeds contain 30–35% oil that can be converted into good quality bio-diesel by transesterification (Foidal et al., 1996). However, very less work on genetic improvement aspects of this species has taken up so far in India.

In last decade, the number of studies and publication on *J. curcas* has grown geomatricaly, driven by the growing intrest of governmental agencies in many parts of the worlds. The majority of these reports used Asian accessions to study the agronomic performance, the genetic variation through molecular markers and content of seed oil (Sujatha et al., 2005; Basha and Sujatha, 2007; Behra et al., 2010). On the other hand, little attention has been paid to the composition of fatty acids of the seeds as chemical markers for estimating the genetic diversity of this plant.

Systematic provenance trials at different locations have not yet been carried out with *J. curcas* in India and to the necessary extent in the world. The genetic background of the *J. curcas* grown in Africa and Asia is unclear (Heller, 1996). At the global level, the information on genetic improvement of *J. curcas* is restricted to few publications. Heller (1996), reported a significant difference in the vegetative development among 13 provenances of *J. curcas* in multilocation field trials in two countries viz. Senegal and Cape Verde, a significant genotype-environment interaction (G x E) in Senegal has been observed. Owing to its importance, the species has gained popularity and is being scaled up in different parts of India on large-scale. Being a naturalized species with a wide range of distribution, *J. curcas* hold a considerable scope of variation, which suggests a high potential of this genus in India. The screening of existing populations for growth and oil yield could be utilized profitably for selection of best sources for a forestation and productions of oil. There is a good reason for developing *J. curcas* as a new energy crop as it does not compete with conventional food crops for land, water and manpower resources and also it has the ability to make a significant contribution to the nation's growing need for energy through large-scale cultivation (Srivastava, 1999).

The oil mainly used by the soap, pesticides and pharmaceuticals industries. It consist of four important fatty acids, two of them, palmitic and stearic acid, are saturated fatty acids. Oleic acid is a mono and linolenic acid is a poly-unsaturated fatty acid. The

proportion of each fatty acid in oil may vary from region to region because of variable genetic make-up. Studies based on molecular markers have also shown the great genetic diversity existing in *Jatropha curcas* population (Ranade et al., 2008). Thus, there is great scope to assess the diversity of fatty acid composition of the *Jatropa* seeds for selection of ecotype based on their fatty acid composition.

The term 'Phorbol esters' is used today to describe a naturally occurring compound widely distributed in plant species of the families *Ephorbeaceae* and *Thymelacea.* These compounds are esters of tigliane diterpanes (Evans, 1986a). Phorbol esters (Phorbol-12-myristate 13-acetate) has identified as the major toxic principle in *Jatropha* (Makkar et al., 1997). The presence of high levels of antinutrients prevents its use in animal feeding. The oil from the nontoxic Mexican varieties of *Jatropha* reported to have negligible amount of phorbol ester i.e.0.27mg/ml. of oil, whereas the toxic varieties contain 2.49 mg/ml phorbol ester of oil (Goel et al., 2007).

In the present study the content of oil in the seeds was investigated along with its composition of fatty acids, phorbol esater content (toxic componenet) and protein contetnt of the of the oil cake, in four agroclimatic region of Maharashtra, state of India. It is useful to understand the genetic richness and agro-climatic effects on germplasm. They are also useful to generate basic information for initiating the wide range provenance tests, including provenances of wide geographical origin, involving many countries.

4.2 Materials and Methods

4.2.1 Collection of seed material: An extensive survey was conducted to collect the seeds of *J. curcas* from 20 different locations belonging to four agro-climatic regions of Maharashtra viz. Kokan, Marathwada, Western Maharashtra and Vidarbha. The selection was made on morphological assessment of characters i.e. yield potential, crown potential, girth, diseases resistance, etc. Each zone consisted of five collection sites, which uniformly represented each zone with considerable geographic isolation. The area sample ranged from Latitude: 15°52'-21°.20' N, and Longitude: 72°.15'-79°-58'E. The seeds were collected from the locations, which were minimum 150 km apart from each other, in order to avoid narrowing down of the genetic base due to

relatedness or interbreeding, as per the method suggested by Turnbull (Ginwal et al., 2005). The seeds were selected according to their condition, where damaged seeds were discarded and seeds in good condition were cleaned, de-shelled and dried by sun drying method. Seeds were grounded using grinder prior to extraction of oil.

Table 4.1. Site and Soil Characterization of *Jatropha curcas* L. seed sources regions

Agroclimatic Zones	Geology /Parental Material	Relief	Drainage	Texture	Soil Texture
Western Maharashtra	Basalt	Hilly/Plain	Moderately drained	Clay	Regur/Black cotton
Kokan	Laterite	Hilly/Costal Plain	Well drained	Clay	Latenit
Marathwada	Basalt	Plain	Moderately drained	-	Regur
Vidarbha	Granik, Gneist Schist	Plain	Moderately drained	-	Red and yellow

4.2.2 Equipments

I. **Spectrophotometer** (Chemito, India): Used for quantifying proteins in a oil cake. The reading is taken at wavelengths of 650nm (Lowry's, 1951).

II. **High Performance Liquid Chromatography: (**Shimadzu) Used for quantifying the amount of phorbol ester present in the *jatropha* seed, With HPLC, a pump (rather than gravity) provides the higher pressure required to move the mobile phase and analyte through the densely packed column. The increased density arises from smaller particle sizes. This allows for a better separation on columns of shorter length when compared to ordinary column chromatography.

III. **Gas Liquid Chromatography:** (Chemito HP 8610) It is used for the quantifying fatty acids, where, fatty acid methyl esters were separated out in Dichloromethane. In gas chromatography, the *mobile phase* (or "moving phase") is a carrier gas, usually an inert gas such as helium or an unreactive gas such as

nitrogen. The *stationary phase* is a microscopic layer of liquid or polymer on an inert solid support, inside a piece of glass or metal tubing called a column (a homage to the fractionating column used in distillation). The instrument used to perform gas chromatography is called a *gas chromatograph* (or "aerograph", "gas separator").

IV. **Soxhelt apparatus:** An apparatus for extracting components from a solid (oil from a *Jatropha* seeds). The material used is placed in a thimble made of thick filter paper and this is held in a specially designed reflex condenser with a suitable (Petroium ether) solvent. The chamber holding the thimble fills with warm solvent and this is led back to the source via a side arm. The apparatus can be operated for long periods, with components concentrating in the source vessel. It is named after Franz Soxhlet, who devised it in 1879.

4.2.3 Oil extraction: The seeds were grounded using mechanical grinder and defatted in a soxhlet apparatus using petroleum ether (Boiling point 40-60ºC). Ether was removed using water bath, until no odour of ether remains. Extracted seed oil was stored in freezer for subsequent physicochemical analysis *viz*, quantification of fatty acids, Estimation of 'Phorbo ester' and bye-product after extraction was used to quantify protein content.

4.2.4 Quantification of fatty acids

Preparation of Methyl esters: (IS: 548 (Part III) 1976): In a clean dry round bottom flask, accurately weighed 0.150 g of a oil sample was taken and 5ml of methanolic sodium hydroxide solution was added in it. The reaction mixture was refluxed for about 30 min, followed by neutralization with dil. HCL. Fatty acids formed were extracted by using 10-15 ml petroleum ether (40-60 ºC) and extraction was repeated thrice. All the ether extracts were combined and washed with water and dried over 5 gm anhydrous sodium sulphate (Na_2SO_4). Petroleum ether was evaporated by using rotatory vacuum evaporator. Traces of ether were removed by applying high vacuum. In the round bottom flask, 10 ml of pure methyl alcohol (CH_3OH) and 2 drop of conc. H_2SO_4 were added into the obtained fatty acids. The contents of the flask were refluxed by attaching water condenser for about 3 Hrs. Cooled reaction mixture was extracted with petroleum ether and extraction was repeated thrice. Combined extracts

washed with water and dried with 5 gm anhydrous sodium sulphate (Na_2SO_4). Petroleum ether was evaporated and the methyl esters formed were further analysed.

GLC conditions for quantification of fatty acids: The fatty acid methyl esters were separated out in Dichloromethane. GLC analysis of fatty acid methyl esters was achieved on a Chemito HP 8610. GC equipped with and FID (Flame Ionization Detector) and a VF-WAX ms column (0.32mm x 30m). Temperature programming was done to achieve the separation of stearic and oleic acids. Operation conditions used were: oven temperature 150-250ºC at 20ºC for 11min; Injector temperature 240ºC and detector temperature 250ºC. The sample (0.5µl) was injected into GC, the result was processed on HP chem station software.

4.2.5 Quantification of phorbol esters: Seed cakes (500mg) were weighed; ground with a small amount of sand using a pestle and mortar, then 20ml dichloromethane was added. The mixture was again ground for about 5min with mortar. The material was allowed to settle and the liquid phase was filtered. Similar procedure was repeated thrice with residue, the liquid phase was again collected. The filtrate was dried under vaccum at 40ºC. The dried residue was dissolved in 5ml tetrahydrofuran, passed through a 0.2µm filter paper and injected (20µl) into the HPLC.

HPLC conditions for quantification of Phorbol esters: The HPLC equipment used consisted of a Shimadzu 20A, L-7100 hplc pump, an L.7450 Photo diode array detector, an L-7200 auto sampler, a D–7000 interphase module and an LC organizer. The analytical column was reversed phase C18 (LiChrospher 100, end capped 5um) 250 x 4mm I.D. (Lichrocart) protected with guard column containing the material as in the main column. The procedure outlined by Makkar et. al., (1998b) was followed. The separation was performed at room temperature (25ºC) and flow rate was 1.5ml/min. The four-phorbol ester compound peaks that appeared between 26 and 31min were identified and integrated at 280nm. The results are expressed as equivalent to a standard to Phorbl-12-myristate 13 acetate (obtained from Sigma), which appeared between 52 and 53min.

4.3 Result and Discussion

4.3.1 Topography: The agro-climatic conditions of the regions, from where the seeds were collected, the maximum altitude was recorded for Latur (630m.), followed by Nashik (630m)., and the minimum was at Alibag (007m). As far as rainfall is concerned, the maximum was for Kokan (3114mm) and the minimum value (592mm) was recorded for Marathwada. The climatic classification of different regions, according to the Indian meterological department is listed in table 3.1, while site and soil characterization is given in table 4.1. It is observed during present study, that the period for seed collection of *Jatropha* in general is July to December in the state of Maharashtra. In places such as Kokan and Western Maharashtra, the plants shed leaves in October and November, whereas in Marathwada and Vidarbha, they keep on shedding leaves till December. It was also observed that fruiting begins earlier in Kokan region as compared to other; one can collect seeds during August to October in this region, whereas in remaining regions seeds can be collected up to November. Variation in fruiting season could be due to differences in environmental conditions.

4.3.2 Oil content: Seed sources of *J. curcas* varied significantly in respect of oil content in whole seed (Ref. Table 4.2). Data revealed that the oil percentage of whole seed was highest in Vidarbha region followed by Marathwada region and Western Maharashtra region. The minimum oil percentage was recorded for Kokan region (Ref. fig. 4.5).

Among the Vidarbha region, Amravati seed source recorded highest (38.20) oil percentage, followed by Nagpur (37.87) and Akola (35.80). The minimum oil percentage recorded for Chandrapur (32.64) seed source.Within, Marathwada region seed sources viz, Latur, Aurangabad and Beed recorded 35.87, 45.43 and 34.26 respectively and minimum recorded for Parbhani (32.43) and Nanded, (33.37). Among the Western Maharashtra region Dhule (37.18) and Kolhapur (35.86) recorded more than 35% oil content while Pune, Nashik and Solapur recorded 34.95, 31.18 and 32.67% of oil content respectively. Kokan region was recorded lowest oil percentage (Ref. fig. 4.5). Among these regions highest oil percentage were recorded for Alibag (30.03), followed by Dapoli (28.41) and Ratnagiri (28.89). The least oil percentage was recorded for Sindhudurga (22.64) and Kalyan (26.24) seed sources.

The storage of oil in seeds is generalized feature in higher plants, which serve as source of energy during the heterotrophic stage (Pujara et al., 2006). This stage is crucial in the success or failure of embryo to germinate, emerge and establish itself as new plant (Bewely, 1994). Therefore, the content of the endosperm, at least in part determines reproductive success of plant. As results, the total content and composition of seed oil should be considered characters subject to natural selection. The present study in microevolutionary context showed that the variation in oil content of the seed seed in population of *J. curcas* in Maharashtra is considerably high (Table 4.2). There is positive correlation with altitude confirms the findings of Pant et al. (2004), who determine the variation in yield and oil content of Indian accessions of *J. curcas* in two soil condition and and three altitudinal ranges. They reported that, there is a higher content of oil when plants are found in non-arable soils and high elevation.

It has been documented that there is considerable variation in oil content of this species that can be generated by genetic and environmental factors, including rainfall and soil fertility (Mishra, 2008). The first study to look relationship between ecological parameters and accumulation of oil was that of Levin (1974), who studied the relationship between the oil content and that of habit and habitat of angiosperms. He was found that oil content, evolutionary has increased with the development of woody stems and shade tolerance.

J. curcas is being explored for its oil yield potential throught the world. Attempts are therefore aimed at screening out *J. curcas* seed sources, which can provide better oil yields (Ginwal et al., 2004). In present study, the oil yield was comparable to other report (Ginwal et al., 2004, Kaushik et al., 2007). Variation in seed sources of *J. curcas* with respect to oil content is mainly because this species grows over a wide range of rainfall, temperature and soil types in Maharashtra. Population might have experienced marked difference in selective pressure. The Vidarbha, Marathwada and Western Maharashtra regions seed sources is at high altitude in comparision to Kokan region seed sources and characterized with less rainfall and moderately high temperature. According to Jones and Miller (1991), drier climate is supposed to improve the oil yield in *J. curcas* seeds. Soil conditions also play significant role in causing variation in oil yield (Srivastava, 1999).

Table: 4.2 Variation in the Oil content (%), Phorbol ester and Protein content of *Jatropha curcas* L. seed samples collected from four agro-climatic regions of Maharashtra.

Regions	Collection Sites	Oil in %	Average (%)	Phorbol ester (mg/g)	Average (mg/g)	Protein mg/g	Average (mg/g)
Western Maharashtra	Pune	34.95 ± 0.2	34.25	1.80 ± 0.1	2.12	68.13 ±0.8	75.44
	Nashik	31.18 ± 1.4		2.38 ± 0.3		70.20 ± 1.7	
	Kolhapur	35.86 ± 0.6		3.32 ± 0.5		74.87 ± 3.8	
	Solapur	32.67 ± 0.7		1.48 ± 0.3		58.46 ± 3.5	
	Dhule	37.18 ± 1.7		1.83 ± 0.1		60.04 ± 2.8	
Kokan	Kalyan	26.24 ± 0.4	27.22	0.63 ± 0.6	2.10	42.26 ± 2.4	47.65
	Alibag	30.03 ± 1.2		3.41 ± 0.5		57.33 ± 4.3	
	Dapoli	28.41 ± 0.5		1.74 ± 0.1		39.66 ± 3.5	
	Ratnagiri	28.89 ± 0.7		1.02 ± 0.4		42.53 ± 2.2	
	Sindhudurga	22.64 ± 2.0		3.80 ± 0.7		56.47 ± 3.9	
Marathwada	Beed	34.26 ± 0.0	34.37	0.66 ± 0.6	2.02	40.13 ± 9.6	61.75
	Latur	35.87 ± 0.6		2.60 ± 0.2		76.93 ± 6.7	
	Parbhani	32.43 ± 0.8		1.20 ± 0.3		58.46 ± 1.4	
	Nanded	33.87 ± 0.2		3.76 ± 0.7		78.06 ± 7.2	
	Aurangabad	35.43 ± 0.4		2.06 ± 0.1		55.36 ± 2.8	
Vidarbha	Akola	35.80 ± 0.8	35.78	4.40 ± 0.9	2.31	87.86 ± 2.4	82.36
	Nagpur	37.87 ± 1.8		0.79 ± 0.6		100.1 ± 7.9	
	Amravati	38.2 0 ± 2.5		1.04 ± 0.5		49.97 ± 4.5	
	Chandrapur	32.64 ± 0.5		4.16 ± 0.8		77.86 ± 2.0	
	Gadchiroli	34.59 ± 0.3		1.17 ± 0.5		96.26 ± 6.1	

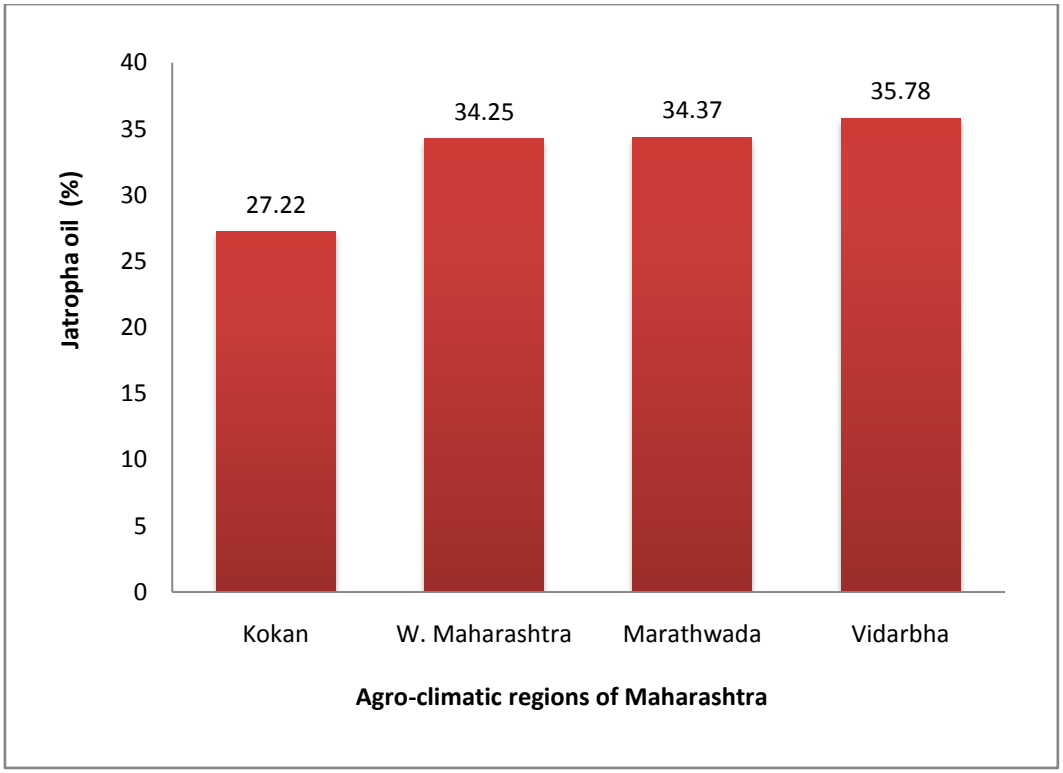

Fig. 4.1 Average percentage of *Jatropha curcas* L. oil in four agro-climatic regions of Maharashtra

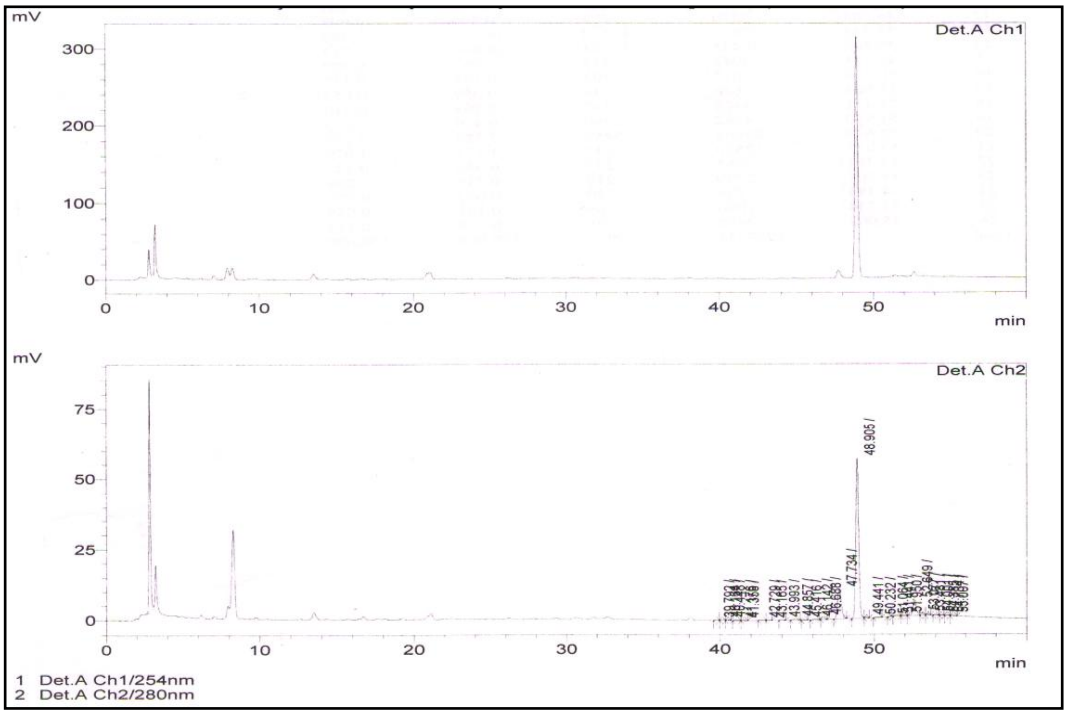

Fig. 4.2 Phorbol ester analysis (Standard)

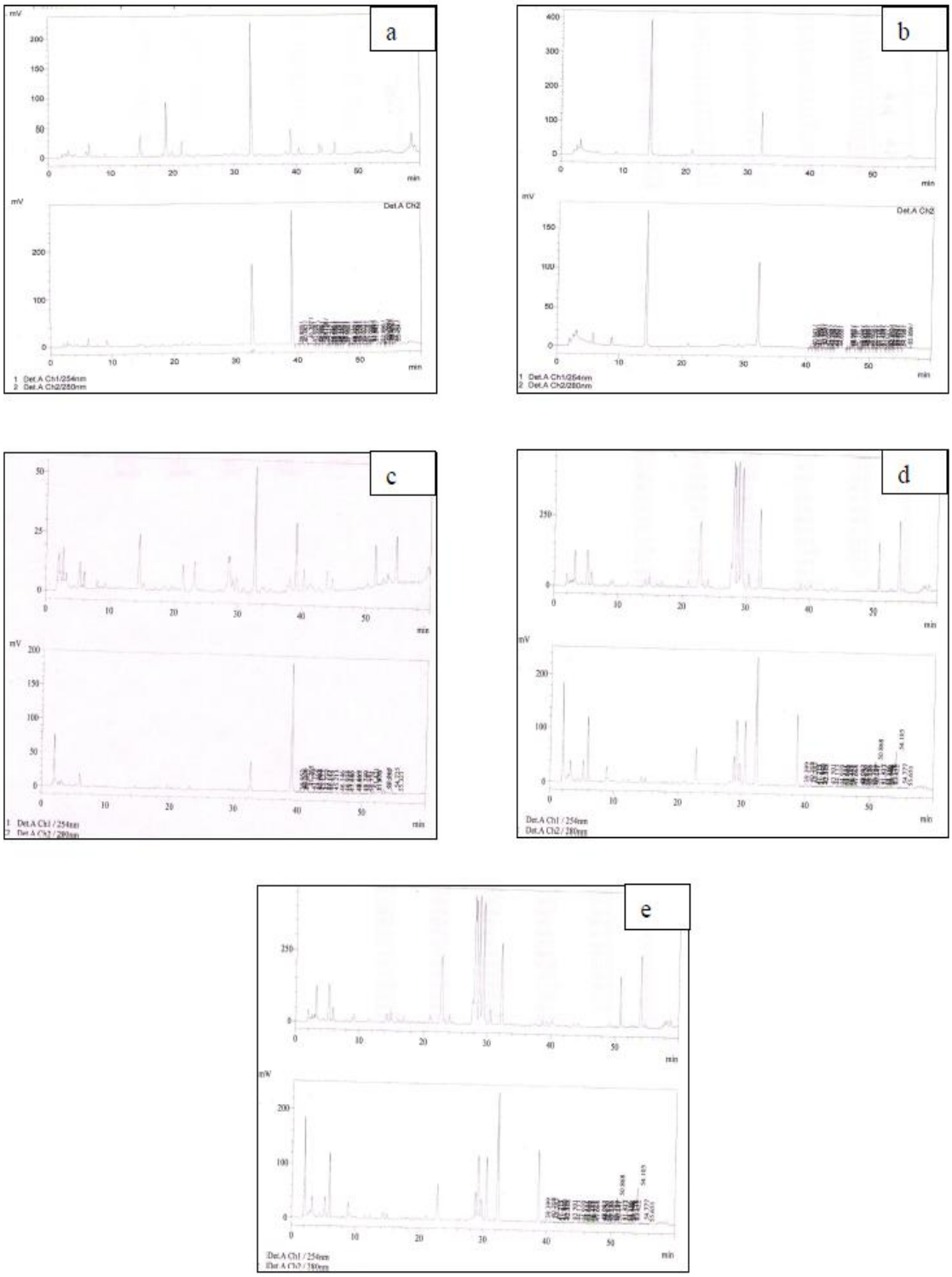

Fig. 4.3 Phorbol ester analysis of Kokan region: a) Sindhudurga, b) Ratnagiri, c) Alibag, d) Dapoli and e) Kalyan

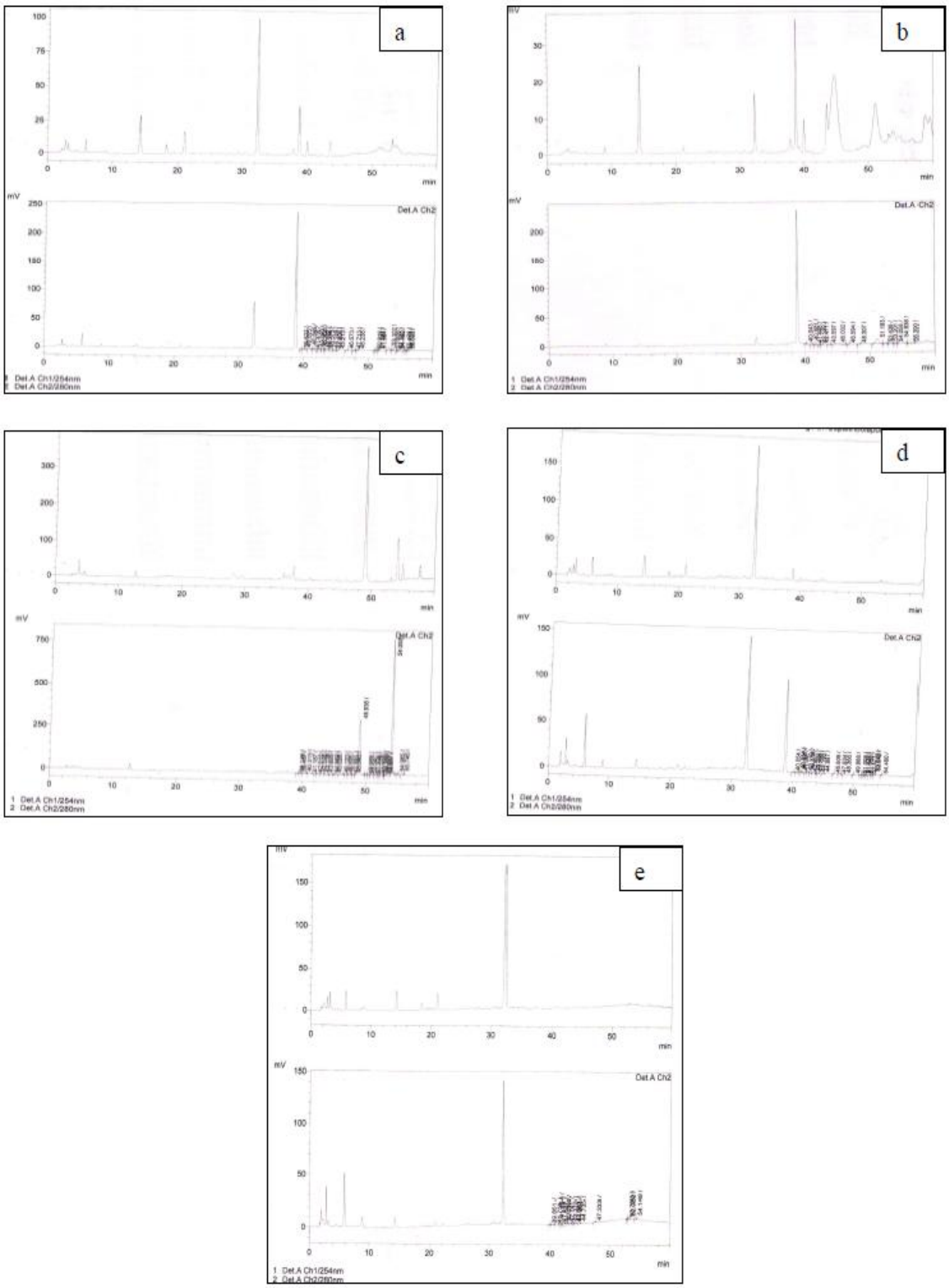

Fig. 4.4 Phorbol ester analysis of Western. Maharashtra region: a) Pune, b) Nashik, c) Kolhapur, d) Solapur and e) Dhule

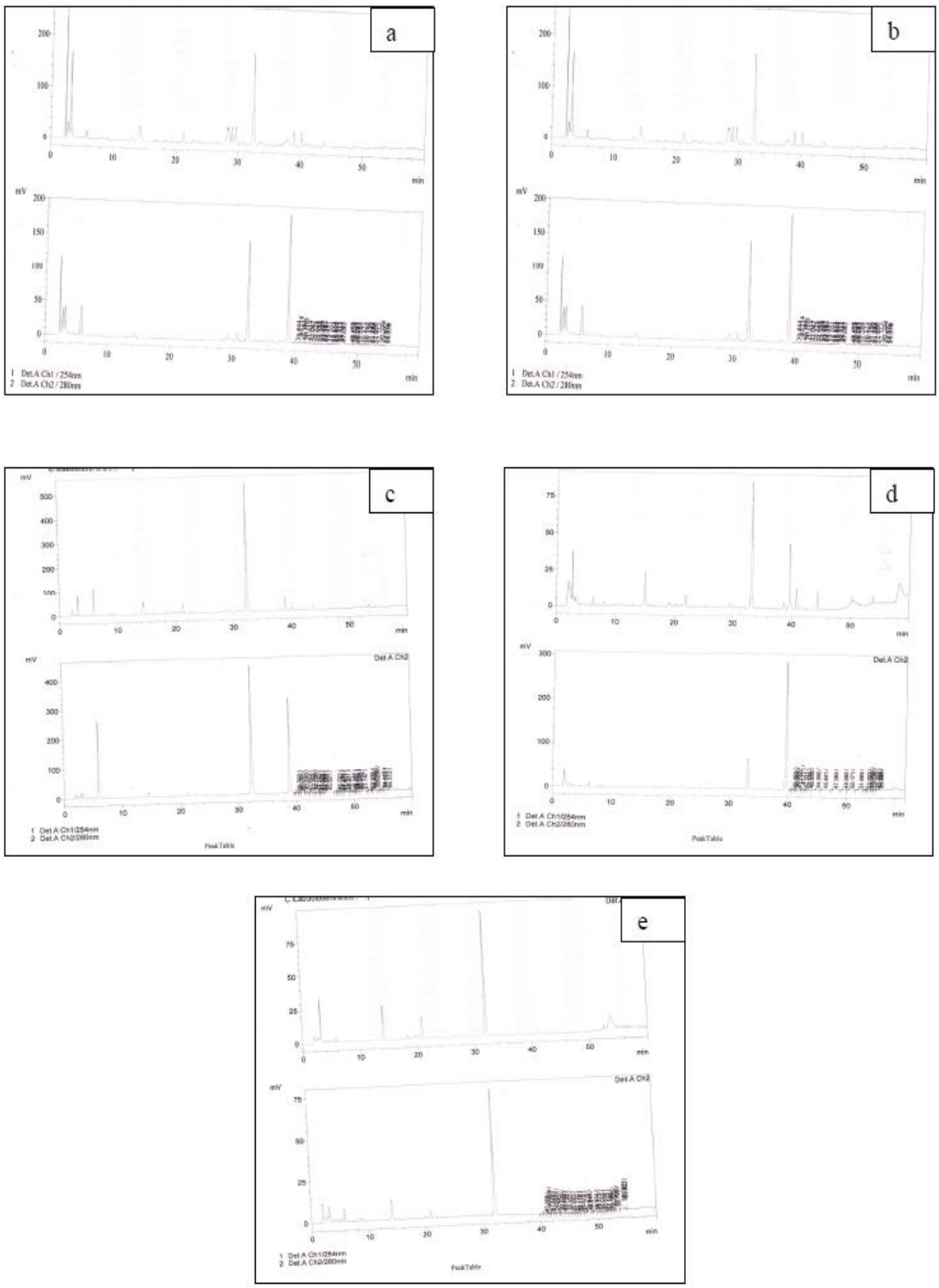

Fig. 4.5 Phorbol ester analysis of Marathwada region: a) Aurangabad, b) Beed, c) Nanded, d) Latur and e) Parbhani

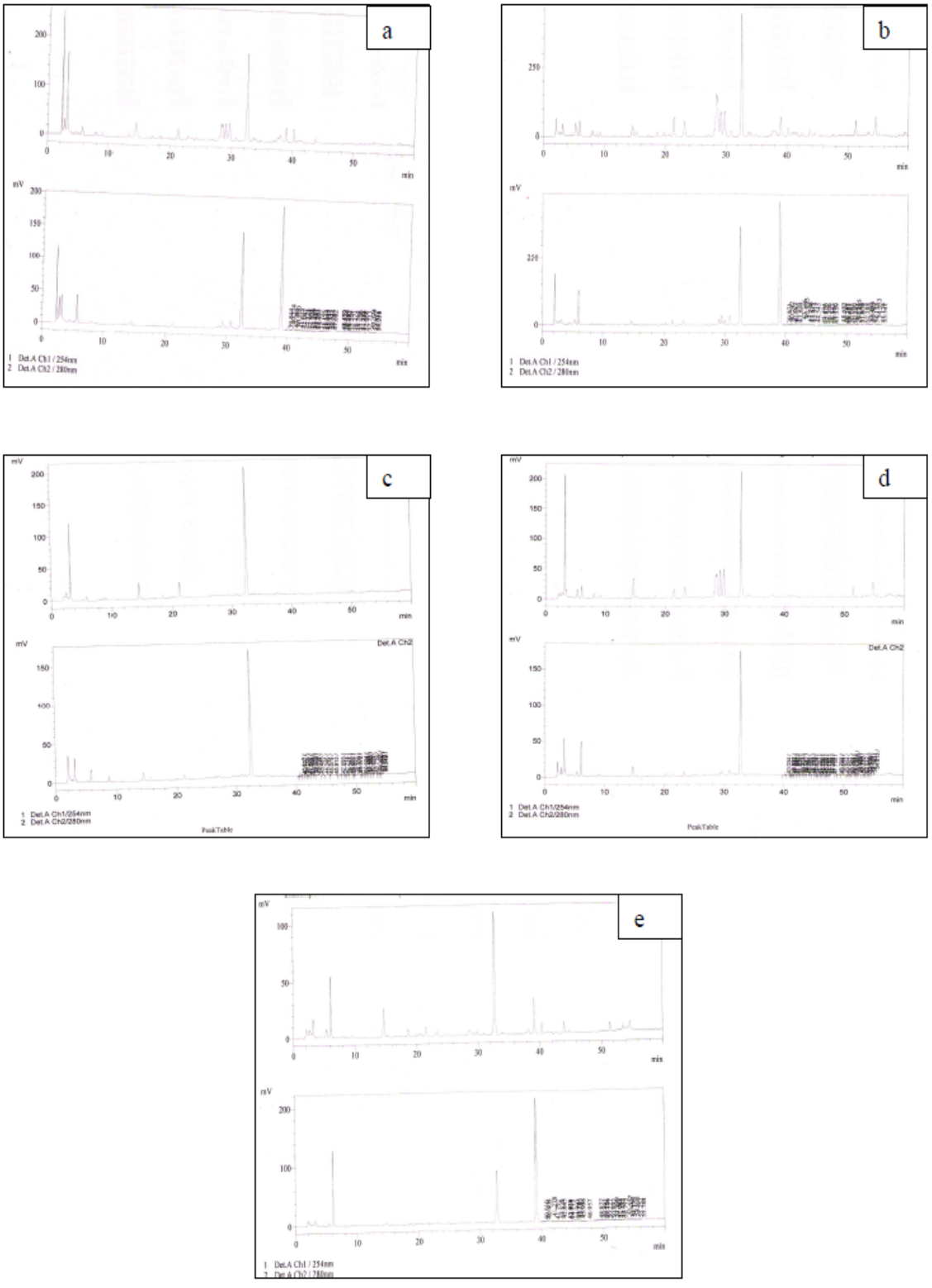

Fig. 4.6 Phorbol ester analysis of Vidarbha region: a) Akola, b) Amravati, c) Nagpur, d) Gadchiroli and e) Chandrapur

4.3.3 Phorbol ester and protein content: The seed cake remaining after oil extraction is an excellent source of plant nutrients (Foidl et al., 1996). However, the presence of high levels of antinutrients prevents its use as animal feed. Phorbol ester (Phorbol–12–myristate 13– acetate) has been identified as the major toxic principle in *J. curcas* (Makkar et al., 1997).

The present investigation also revealed that seed source of *Jatropha* varied significantly in respect to Phorbol ester and protein content (Ref. Table 4.2 and fig.4.2 to 4.5). The phorbol ester does not show regional variation trend unlike oil content. It suggests that synthesis of phorbol ester does not influenced by environmental factors like temperature, humidity, rainfall, soil types etc. for variation within twenty ecotypes. The highest value of this compound recorded for Chandrapur, Nanded, Alibag and Kolhapur 4.16, 3.76, 3.41 and 3.32mg/g. of seed cake respectively. The minimum value recorded for Kalyan, Beed and Nagpur 0.63, 0.66 and 0.79mg/g of seed cake respectively. The remaining seed sources show intermediate value (Ref. Table 4.2).

The phorbol ester was reported to mimic the action of diacyl glycerol. Activator of protein kinase C is regulates different signal transduction pathways, interefere with the activity of protein kinase C. It affects number of processes including, phospholipid and protein synthesis, enzyme activities, DNA synthesis, phosphorylation of proteins, cell differentiation and gene expression (Goel et al., 2007).There is a significant variation in protein content of seedcake of *Jatropha curcas* seeds obtained from four agro-climatic regions of Maharashtra. Out of four agro-climatic regions Vidarbha region seed sources showed maximum protein content (82.36mg/g) followed by Western Maharashtra (75.44mg/g) and Marathwada (61.75 mg/g) respectively. The lowest value 47.65mg/g recorded for Kokan region. Within Vidarbha region maximum value 100.1mg/g was recorded for Nagpur source followed by Gadchiroli, Akola and Chandrapur 96.26, 87.86 and 77.86mg/g respectively. The least amount recorded for Amravati region. In Marathwada region highest value for protein content was recorded for Nanded followed by Latur 78.06, 76.93mg/g respectively. The lowest value was for Beed, Aurangabad and Parbhani 40.13, 55.36 and 58.46mg/g respectively.

Among Western Maharashtra, Pune (68.13), Nashik (70.20) and Kolhapur (74.87) were recorded highest value for protein content. The least value was for Solapur followed by Dhule 58.46 and 60.04mg/g respectively. The average protein content was least for Kokan region, in which Sindhudurga (56.47) and Alibag (57.33) shows maximum content of protein. The lowest within this region was recorded for Dapoli, Kalyan and Ratnagiri 39.66, 42.26 and 42.53 respectively.

4.3.4 Fatty acid composition: The fatty acid composition determination was another important experiment carried out in this study. Results obtained are given in table 4.2. The properties of the bio-diesel fuel are determined by the amounts of each fatty acid that are present in the molecules, chain length and number of double bonds determine the physical characteristics of both fatty acids (Mittelbach and Remschmidt, 2004). Transesterification does not alter the fatty acid composition of the crude oil and this composition plays an important role in some critical parameters of the bio-diesel, as cetane number and cold flow properties (Ramos et al., 2008).

There are three main types of fatty acids that are present in a triglyceride, which are saturated (Cn:0), monounsaturated (Cn:1) and polyunsaturated with two or three double bonds (Cn:2,3). Various vegetable oil is a potential feedstock for the production of a fatty acid methyl ester or bio-diesel but the oil composition affects the quality of the fuel. Ideally, oil should have high monounsaturated fatty acids (Gunstone, 2004). Oils that are rich in polyunsaturated fatty acids such as linolenic acids tend to give methyl ester fuels with poor oxidation stability. Oil with high degree of unsaturation tends to have high freezing point. This oil has poor flow characteristic and may become solid at low temperature (Gunstone, 2004).

The present study revealed that oleic acid content (41-49%) was dominant in the seeds collected from all four agroclimatic regions, followed by linolenic (26-31%) and palmitic (15-19%). The lowest value recorded was of stearic acid (6-8%). The average fatty acid content in four agro-climatic regions remains similar for all fatty acids while, significant variation was observed within the provenances.

Table 4.3 Variation in the fatty acid (%) profile of samples collected from the agro-climatic regions of Maharashtra.

% of Fatty acids									
Agro-climatic regions	**Oil sources**	**Palmitic acid $C_{10:0}$**		**Stearic acid $C_{18:0}$**		**Oleic acid $C_{18:1}$**		**Linolenic acid $C_{18:2}$**	
Western Maharashtra	Pune	15.11	19.79	6.01	7.81	38.96	41.29	39.92	31.49
	Nashik	16.48		6.52		40.33		36.68	
	Kolhapur	14.31		4.84		37.70		43.16	
	Solapur	14.22		5.92		43.78		36.08	
	Dhule	36.86		15.77		45.72		1.64	
Kokan	Kalyan	22.05	16.92	10.41	7.91	53.70	46.06	13.85	29.11
	Alibag	15.33		7.30		41.59		35.78	
	Dapoli	15.84		7.15		44.69		32.33	
	Ratnagiri	15.75		7.38		45.13		31.73	
	Sindhudurga	15.67		7.33		45.09		31.90	
Marathwada	Beed	18.84	19.32	4.89	6.99	56.70	47.46	19.57	26.26
	Latur	33.69		13.39		51.20		1.73	
	Parbhani	14.71		6.89		47.75		30.65	
	Nanded	14.58		5.03		41.72		38.67	
	Aurangabad	14.79		4.75		39.94		40.70	
Vidarbha	Akola	15.27	15.33	7.59	8.22	46.67	49.11	30.47	27.41
	Nagpur	14.23		7.43		44.77		33.56	
	Amravati	14.06		7.80		47.80		30.35	
	Chandrapur	14.14		8.21		47.80		30.35	
	Gadchiroli	18.99		10.11		58.55		12.35	

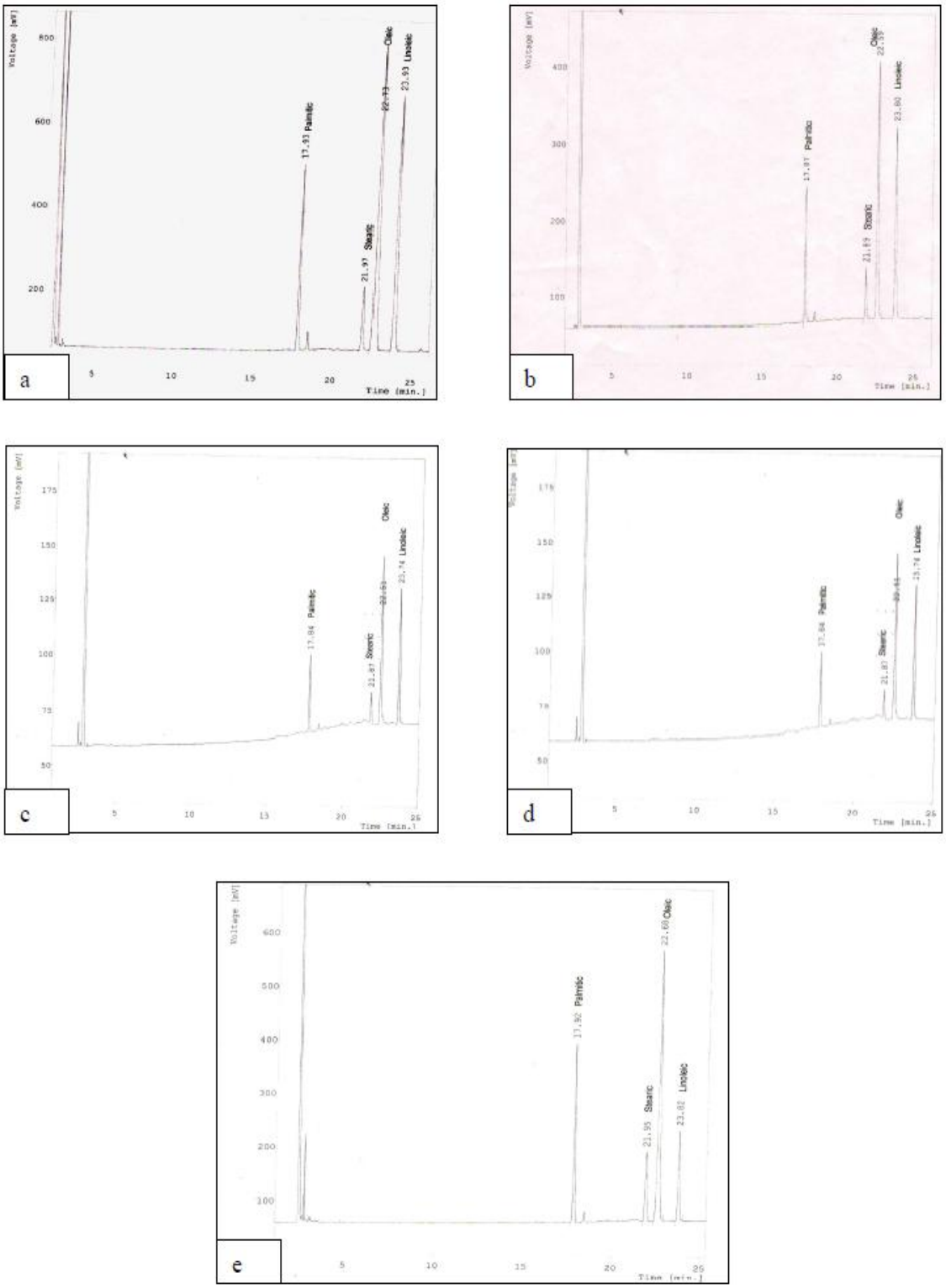

Fig. 4.7 Fatty acid analysis of Kokan region: a) Sindhudurga, b) Ratnagiri, c) Alibag, d) Dapoli and e) Kalyan

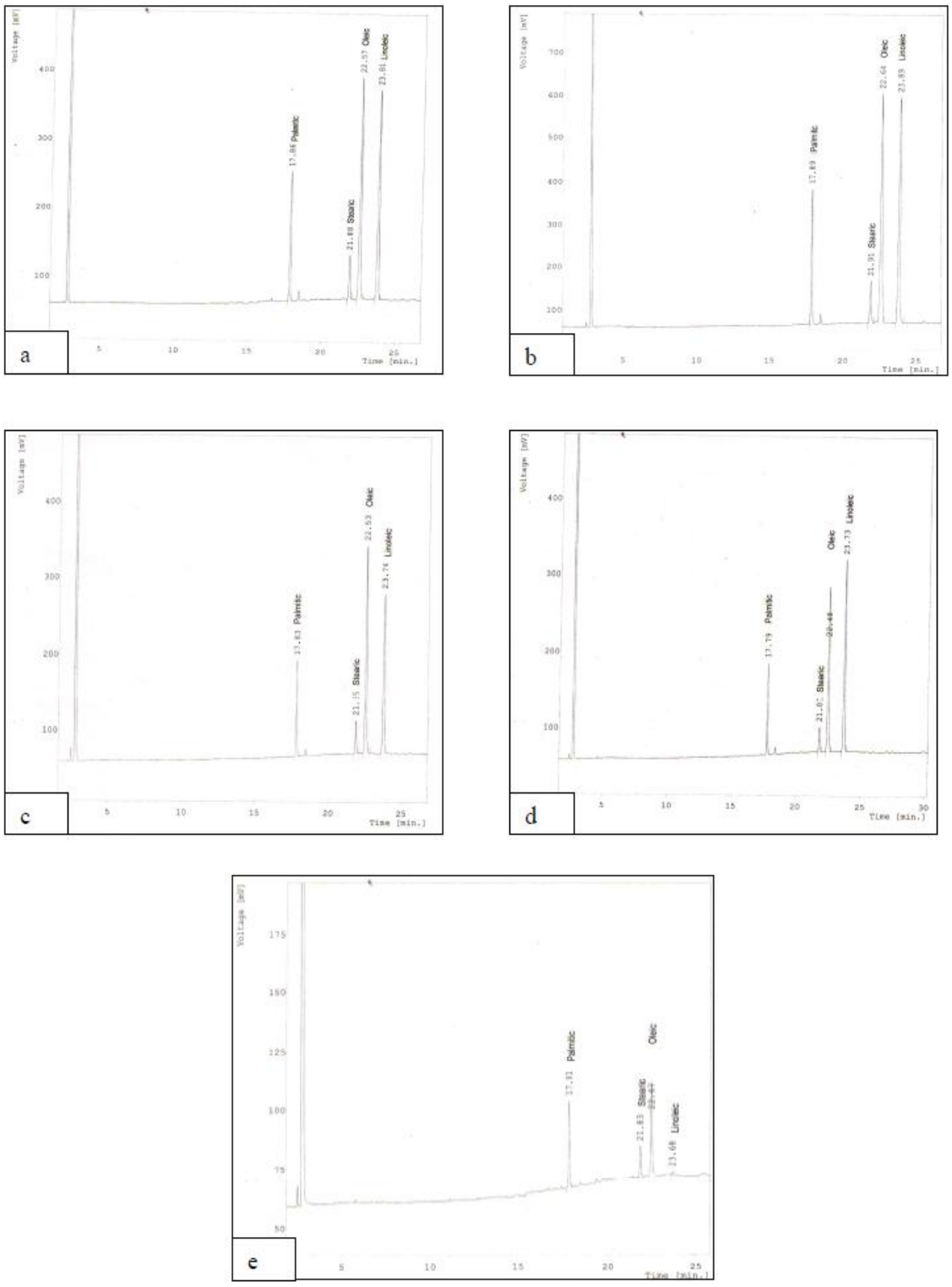

Fig. 4.8 Fatty acid Analysis of Western Maharashtra region: a) Pune b) Nashik c) Kolhapur d) Solapur e) Dhule

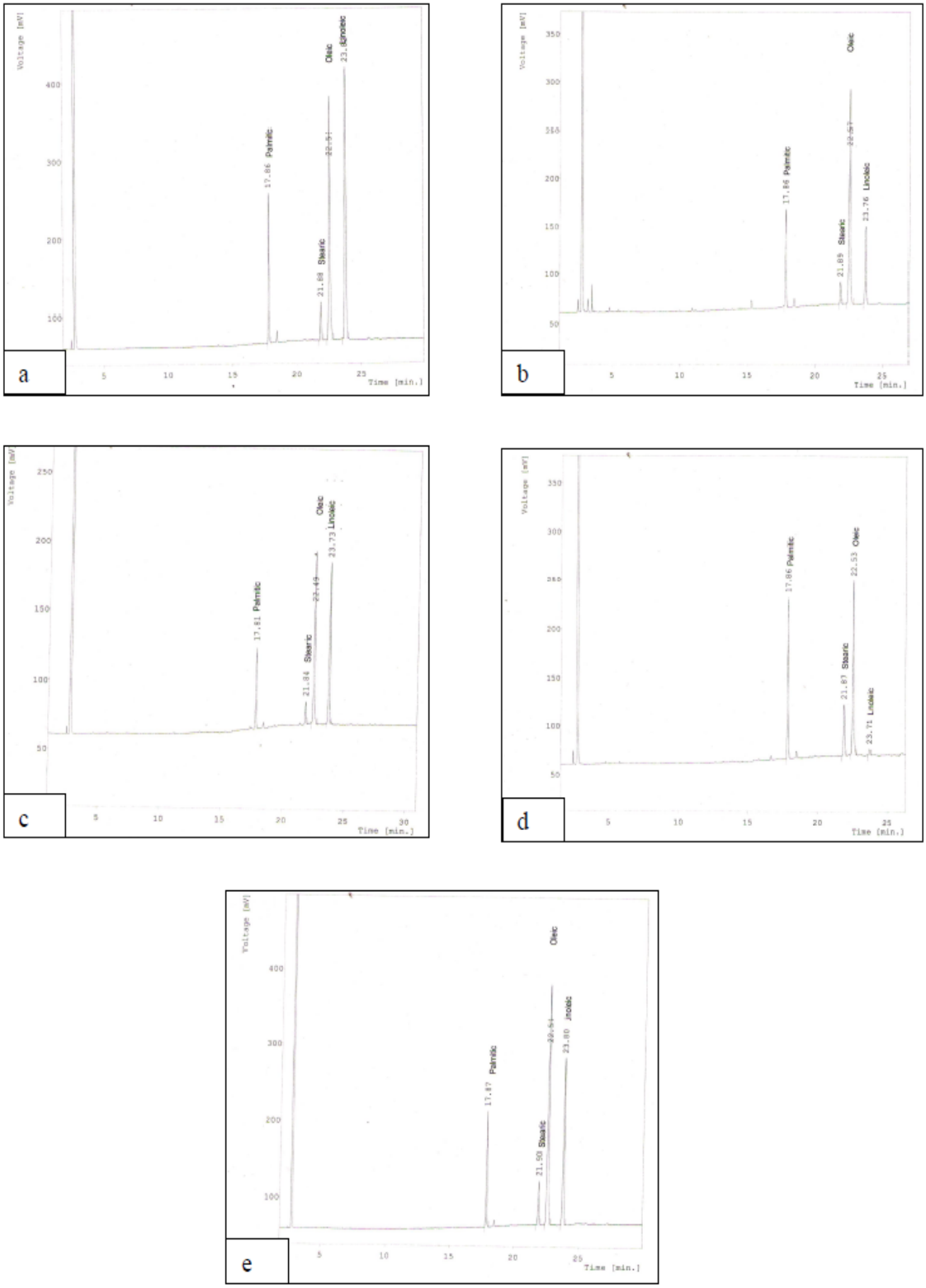

Fig. 4.9 Fatty acid analysis of Marathwada region: a) Aurangabad, b) Beed, c) Nanded d) Latur and e) Parbhani

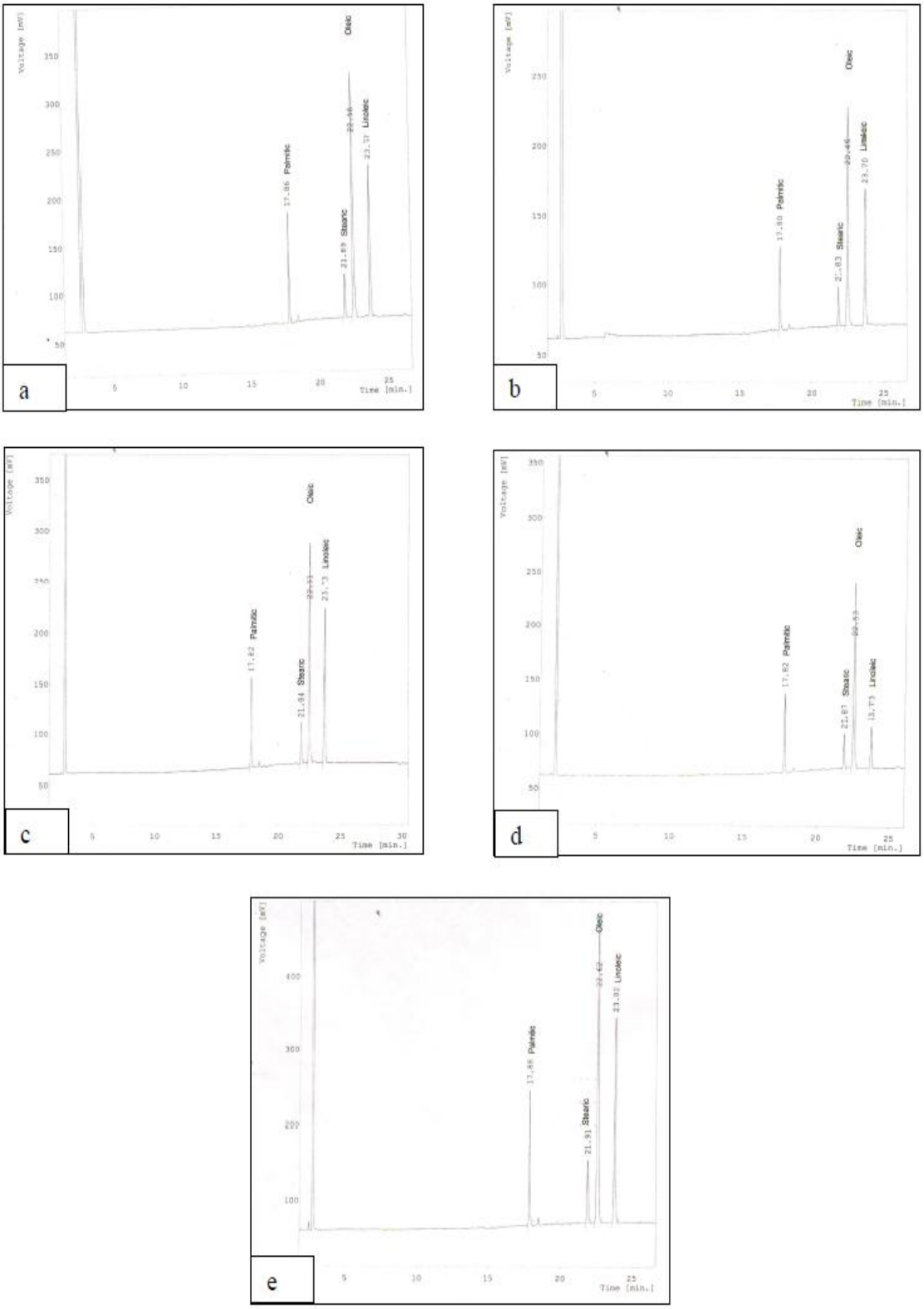

Fig. 4.10 Fatty acid analysis of Vidarbha region: a) Akola, b) Amravati, c) Nagpur d) Gadchiroli and e) Chandrapur

The highest value for oleic, linolenic, palmitic and stearic fatty acids were recorded for Gadchiroli, Kolhapur, Dhule and Latur respectively. The Gadchiroli sample contains highest oleic acid content (58%).

The proportion of unsaturated fatty acids are an important variable in the bio-diesel manufacturing, since quality standards indicate it must be minimized (Conley, 2006). Interestingly, our study found that oil content and proportion of unsaturated fatty acids are negatively correlated, which , if confirmed in larger study of genetic association with outstanding genotypes will permit genetic improvement for increased oil yield while decreasing unsaturated fatty acids. To date great progress has been made in molecular understanding of the origin of the fatty acids in seed and diversification mechanisoms (Barker, 2007). However, it is surprising how little attention has been given to the selection factors thatdrive the evolution ogf fatty acid composition of seeds. In this regard, Linder (2000), suggesated that the temperature of germination is an important selective agent that causes the seed oils of plants native to high latitudes (or altitudes) to have a higher proportion of unsaturated fatty acids is more feasible compared to saturated. Thus, the seeds with more unsaturated fatty acids germinate and grow faster at lower temperature. On the other hand, in warm environments, such as the tropical seeds with more saturated fatty acids are selectively favoured because they have more energy and do not need to germinate quickly, since the conditions in the tropics are more or less stable throught the year.

Based on the previous study, it is hypothesized that, in case of *J. curcas,* soil moisture has exerted selection pressure to select a higher proportion of unsaturated fatty acids. It is well documented that this species is tolerant to drought, although adapting to different environments, from humid to semi-arid. However, the plant is susceptible to flooding, for example according Dehgan and Schutzman (1994). *J. curcas* is found in South America in seasonly dry tropical area, but is completely absent in the always wet Amazon region. In Mharashtra and sub tropical region, the onset of flowering and seed production coincides with the onset of the rainy season, so seeds, which do not exhibit dormancy, should germinate quickly, usually within five days (for which they require higher propotion of unsaturated fatty acids) and establish themselves as seedlings before soil moisture level increases to water logging.

Oils rich in polyunsaturated such as linolenic acids, tend to give methyl ester fuels with poor oxidation stability. Vegetable oil with high degree of unsaturation tends to have high freezing point. This oil has poor flow characteristic and may become solid (e.g palm oil) at low temperatures, though they may perform satisfactorily in hot climates. (Gunstone, 2004). Monounsaturation of *Jatropha* seed oil is higher than other vegetable oil such as palm kernel, sunflower and palm oil (Marketta et al., 1989). The major fatty acids in *Jatropha* seed oil are oleic, linoleic, palmitic and the stearic fatty acid. Highest percentage of fatty acid (42.8%) was for oleic acid. It was followed by linoleic acid (32.8%). Thus, *Jatropha* seed oil can be classified as oleic–linoleic oil due to higher percentage of both i.e. oleic and linoleic fatty acids. Compared to other vegetable oils *Jatropha* seed oil has highest oleic content than palm oil, palm kernel, sunflower and coconut and soybean oil.

It is interesting to note wide variation in seed proteins (39.66–100.10mg/g), oil content (22.64 – 37.87 %) and phorbol esters (0.63–3.41mg/g) in twenty provenances from four agro-climatic regions of Maharashtra. The *Jatropha* kernel meal has high crude protein content (order of 60%) and good amino acid composition (Martinez-Herrera et al., 2006). It has the potential for use as animal feed. The kernel meal from the non-toxic genotype would be an excellent animal feed and the oil could possibly be used as edible oil (Goel et al., 2007). Sujatha et al. (2008), have reported that, toxic mother plants produce toxic seeds, independent of the phenotype of father. It could be a single suppressor gene that blocks production of all phorbol esters (Goel et al., 2007) drives that non-toxic genotype. The removal of phorbol ester from the toxic genotypes is imperative in order to make this by product of bio-diesel industry a valuable livestock feed (Basha et al., 2009).

In the present investigation, biochemical variation has been assessed in *J. curcas* germplasm collected from twenty provenances belonging to four agro-climatic regions of Maharashtra. The variability observed in the present study is useful for developing hybridization programmes. Higher oleic acid and lenolenic acid content containing ecotype can be utilized for improvement of the fatty acid profile of those having comparatively lower oleic acid and linoleic acid. The results on biochemical analysis observed during the present study have been duly published (Gopale and Zunjarrao, 2011).

4.4 Conclusion

The result of the present investigation has revealed that, the seed oil content, protein of oil cake and fatty acids significantly vary in four agro-climatic regions of Maharashtra. There was linear relationship between protein and oil content. Vidarbha, Western Maharashtra and Marathwada regions seed sources possess high amount of oil and protein content. It can be concluded on the basis of present study of biochemical diversity that, Vidarbha and Western Maharashtra were more suitable for the plantation of *Jatropha curcas*.

The observed biochemical variations demonstrate that they are useful markers in estimating the genetic diversity of *J. curcas* and selection of elite plant. The populations of *J. curcas* from Marathwada, Vidarbha and Western Maharashtra regions represent valuable genetic resources for the feature establishment of extensive plantation of this oil producing plant. There are no significant variations in phorbol ester in four agro-climatic regions of Maharashtra. It indicates that, the synthesis of 'Phorbol ester' is under the strong influence of genetic characters, rather than environmental conditions.

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5. Molecular Diversity

5.1 Introduction

The genus *Jatropha* of Euphorbiaceae family is one of the prospective bio-diesel yielding tree crops. It is morphologically a diverse genus comprising 172 species of shrubs, rhizomatous shrubs, herbs and small trees (Pamidiamarri et al., 2008). About five species of *Jatropha* have been recorded in India. Out of these important ones are *Jatropha curcas*, *Jatropha gossypifolia*, *Jatropha glandulifera*, *Jatropha multifida,* and *Jatropha podagrica.* Out of these five species *Jatropha curcas* is one of the most important bio-diesel yielding crop. *Jatropha curcas* commonly called as ratanjyot, chandrajyot, Jamal gota, Jangli arandi, Mogali arandi, Kala aranda and physic nut etc, is multipurpose tree of significant economic importance. It is native of Mexico and tropical South America. The plant was reported to have been introduced in Asia and Africa by Portuguese as an oil yielding plant. Now it is occurring throughout India including Andaman Island in semi wild condition. It is found throughout most tropics and is known nearly by 200 different names indicating its significance and various possible uses (Kumar et al., 2008). It adapts well to semi arid marginal site, waste land and dry environment.

The oil is non-edible due to the presence of a toxic substance phorbol ester. It is renewable resource a safe source of energy and a viable alternative to diesel, kerosene, LPG, furnace oil, coal and fuel wood (Martin and Mayeux, 1984). Such a multiple utility biofuel crop needs genetic improvement in order to alter its status of wild perennial form to a cultivable crop with higher yield and oil content. Currently crop improvement work in this species is very limited. The species has a wide range of adaptability for climatic and edaphic factors and grows well even on the marginal lands, enduring drought, alkalinity/salinity of soil and thus serve as best source to green up barren wastelands (Tewari, 1994). It is also suitable for preventing soil erosion and shifting sand dunes. The wide geographical and climatic distribution is indicative of the fact that there was tremendous genetic diversity (Ginwal et al, 2004).

The preogramme has been sponsored by various agencies in different countries have a common mandate of survey of *Jatropha curcas* plantations. Selection of candidate plus phenotypes, establishment of seed production area, standardization of cultivation

practices and progeny trait of high yielding genotypes was essential for the successful implementation of the programme (Sujatha, 2006).

Jatropha species are essentially cross-pollinated, which result in a high degree of variation and offers the breeder ample scope to undertake screening and selection of seed sources for the desired traits (Ginwal et al., 2005). Selection is the most important activity in all tree-breeding programmes (Zobel and Talbert, 1984). Since, variability is a prerequisite for selection programme, it is necessary to detect and document the amount of variation existing within and between populations. Traditional methods using morphological characteristics for establishment of genetic diversity and relationship among accessions were largely unsuccessful due to the strong influence of environment on highly heritable seed traits like 100 seed weight, seed protein and oil content in *Jatropha curcas* (Heller, 1996). Reports available showing tremendous variability in oil content (28-42 %) of seed of *Jatroppha curcas* accessions (Ginwal et al., 2004; Pant et al., 2006; Kaushik et al., 2007) from different agro-climatic regions of India. Hence, selection based on genetic informatation using neutral molecular markers is essential, as it is more reliable and consistent.

DNA marker based fingerprinting can distinguish species rapidly using small amounts of DNA and therefore can assist to deduce reliable information on their phylogenetic relationships. DNA markers are not typically influenced by environmental conditions and therefore can be used to describe patterns of genetic variation among plant populations and to identify duplicated accessions within germplasm collections. Various approaches are available for DNA fingerprinting such as amplified fragment length polymorphism (AFLP) (Zabeau and Vos, 1993), restriction fragment length polymorphism (RFLP) (Botstein et al., 1980), simple sequence repeats (SSRs) (Tautz, 1989) and randomly amplified polymorphic DNA (RAPD) (Williams, 1990). Among these, RAPD is an inexpensive and rapid method not requiring any information regarding the genome of the plant, and has been widely used to ascertain the genetic diversity in several plants (Belaj et al., 2001; Deshwall et al., 2005). RAPD analysis requires only small amount of genomic DNA, can produce high levels of polymorphism, and may facilitate more effective diversity analysis in plants and it provides information that can help to define the distinctiveness of species and phylogenetic relationships at molecular level. Use of such techniques for germplasm

characterization may facilitate the conservation and utilization of plant genetic resources, permitting the identification of unique genotypes or sources of genetically diverse genotypes (Ganeshram et al., 2008). These molecular markers have been successful used in *Jatropha curcas* for detecting diversity and relationship of inter and intra populations (Gneshram et al, 2008; Basha and Sujatha, 2007). RAPD markers were employed to confirm hybridity of inter-specific hybrids (Sujatha and Sujatha, 2003) and to determine the similarity index between accessions of the genotypes.

The present study was undertaken to investigate the extent and distribution of genetic diversity in *Jatropha curcas* from four agro-climatic regions of Maharashtra i.e. Western Maharashtra, Central Maharashtra, Kokan and Vidrabha by using RAPD markers, which can be used to identify the redundancy in germplasm collections.

5.2 Materials and Methods

5.2.1 Glasswares and Plasticwares: Micropipette of different precision measurements (1000, 200, 100, 20, 10 & 2µl) was procured from Gilson medical Electronics, France. Micro tips, eppendorff tubes (0.05, 1.5 and 2 ml) and microtip-boxes procured from Axygen scientific Pvt. Ltd. (India). While centrifuge tubes (50ml) was purchase from Tarson products Pvt. Ltd, (India). Storage vials (Laxbro, India) were use for storage of crushed leaf sample at -70°C.

5.2.2 Reagents and Chemicals:

- Liquid Nitrogen
- Tris-HCl pH 8.0 (1M); EDTA pH 8.0 (0.5M); NaCl (5M); CTAB (20%); $NaCH_3COO$ (3M); Chloroform:Iso-amylalcohol (24:1 v/v); β-mercaptoethanol; cold isopropanol and ethanol (70%)
- Extraction buffer: 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl, 2.0% CTAB, 0.2% β-mercaptoethanol (v/v) (added immediately before use).
- TE buffer: 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA.
- Agarose
- Electrophoresis buffer: Tris-borate-EDTA (1x)
- Loading buffer: Bromophenol blue (0.25%) and glycerol (30%)
- Fluorescent dye: Ethidium bromide (10 µg/mL)

- Marker: Low range DNA ladder (3 Kb) (Bangalore Genei, India)
- Enzymes: RNAase A (10 mg/mL) and Taq DNA Polymerase (Bangalore Genei, India)
- Buffers: *Taq* DNA Polymerase buffer with $MgCl_2$ (Bangalore Genei, India)
- Nucleotides: dNTPs (G, A, T, C) (Bangalore Genei, India)
- Primers: RAPD Primer obtained from Bangalore Genei, India was used (Table 5.1)

Table 5.1 List of RAPD primers used in diversity analysis of *Jatropha curcas* L.

No.	Primer Name	Sequence (5'-3')
01	OPB 02	TGATCCCTGG
02	OPA07	GAAACGGGTG
03	OPB10	CTGCTGGGAC
04	OPB07	GGTGACGCAG
05	OPC18	TGAGTGGGTG
06	OPC02	GTGAGGCGTC
07	OPN-07	CAGCCCAGAG
08	OPE20	AACGGTGACC
09	OPR14	CAGGATTCCC
10	OPE01	CCCAAGGTCC

Table 5.2 List of collection sites of *Jatropha curcas* L. seeds and details of collection sites

Sr. No	Accession No.	Agro climatic Zones	Collection sites	Latitude (^{0}N)	Longitude (^{0} E)	Altitude (Meter)	Rainfall (mm)	Relative Humidity %
01	JC 01	Western Maharashtra	Pune	18^0 32’	73^0 51’	559	1259	38
02	JC 02		Nashik	20^0 08’	73^0 55’	608	1159	35
03	JC 03		Kolhapur	16^0 42’	74^0 14’	570	1726	37
04	JC 04		Solapur	17^0 40’	75^0 54’	479	798	33
05	JC 05		Dhule	21^0 20’	74^0 15’	206	549	29
06	JC 06	Kokan	Kalyan	18^0 64’	72^0 15’	011	2519	43
07	JC 07		Alibag	18^0 38’	72^0 52’	007	2030	56
08	JC08		Dapoli	17^0 46	73^0 12	250	2546	52
09	JC09		Ratnagiri	16^0 59	73^0 20’	092	3114	59
10	JC 10		Sindhudurga	18^0 64’	73^0 38’	016	901	63
11	JC 11	Mahrathwada	Beed	19^0 00’	75^0 43’	519	1007	34
12	JC 12		Latur	18^0 10’	76^0 03’	630	887	30
13	JC 13		Parbhani	19^0 16’	96^0 46’	423	1386	29
14	JC 14		Nanded	19^0 05’	77^0 20’	358	1008	28
15	JC 15		Aurangabad	19^0 53’	75^0 20’	581	956	32
16	JC 16	Vidarbha	Akola	20^0 42’	77^0 04’	309	552	33
17	JC 17		Nagpur	21^0 06’	79^0 03’	310	1159	29
18	JC 18		Yavatmal	20^0 24’	78^0 09’	451	1291	27
19	JC 19		Chandrapur	19^0 58’	79^0 18’	193	592	35
20	JC 20		Gadchiroli	18^0 51’	79^0 58’	123	1317	36

5.2.3 Equipments

I. **Horizontal electrophoresis unit** (Tarson, India): The basic principle of electrophoresis is, charged ions or molecules migrate when placed in an electric field. The rate of migration of a substance depends on its net charge, size, shape and the applied current. It consists of a power pack and electrophoresis unit. The power pack supplies a stabilized current at controlled or required voltage and current output. The electrophoresis unit contains the electrodes, buffer reservoirs and gel casting assembly.

II. **UV Transilluminator**: For gel visualization under ultraviolet radiation.

III. **Gel Documentation System** (Alphaimager, USA): It is a powerful, flexible package including the hardware and the software for imaging and analyzing 1-D electrophoresis gels, dot blot arrays and colonies. The lane-based functions can be used in determination of molecular weights and other values.

IV. **Water bath**: It is used for maintaining the constant temperature, in which, temperature setting, temperature indicator, and cooling effect are also available.

V. **SpinWin** (Tarson, India): It is a mini centrifuge equipped with continuously variable electronic speed control, speed indicator, Amp meter, timer, dynamic break, zero starting switch and fuse safety device for 230 V 50 Hz AC mains.

VI. **Spectrophotometer** (Chemito, India): Used for quantifying DNA in a solution. The reading is taken at wavelengths of 260 and 280 nm and the ratio between them provides an estimate of the purity of the sample DNA (Maniatis, 1982).

VII. **PCR Robocyler** (Corbett, USA): It is a microprocessor which utilizes a robotic arm to quickly move from one temperature block to another based on user defined program. This system has four separate ranodized aluminum temperature blocks (3-heating block and 1-cold block) containing 96 precision cut wells that remain at set temperature, where thermal cyclers have a single block that changes temperature during each cycle. In the robocycler, tubes are moved from one block to next by the robotic arm. The four-block design

decreases cycling time by up to 30% and achieves a well-to-well temperature uniformity of ± 0.1 °C for amplification process.

5.2.4 Source of plant material

A representative set of 20 seed sources/ accessions of *Jatropha curcas* from four agro-climatic regions of Maharashtra (Ref. Table 5.2) were selected for assement of diversity. The five accessions were selected randomly from each agro-climatic region and the seeds were collected and maintained at Department Of Botany, Modern College of Arts, Science and Commerce, Shivajinagar, Pune 411005, Maharashtra. Fresh young leaves from nursery-raised plants of individual genotypes/accession progeny collected. These leaves kept in zip lock polythene bags and these bags were sealed. The bags labeled with the location and date of collection. Bags were stored in thermacol box containing Stay Cool Packs to maintain the cold temperature for 4-5 days to avoid DNA damage. In laboratory, leaves taken out from polythene bags, washed with distilled water, dried on filter paper and then used for crushing with liquid nitrogen. Crushed leaf samples kept in storage vials and stored at -20^0C refrigerator until further use.

5.2.5 Extraction and purity of Genomic DNA: DNA was isolated using modified CTAB method (Khanuja *et al*., 1999). The protocol is as follows:

i. Fresh young leaves from nursery raised plants of individual accessions progeny were collected in icebox. 2.0g of leaves were grind using mortor and pestle in the liquid nitrogen.

ii. Added preheated (at 65^0 C for 40 min) 20.0 ml of extraction buffer to the ground leaves and mixed in the mortar.

iii. Poured the slurry into clean 50 mL polypropylene centrifuge tubes rinsed the mortar and pestle with 1 mL of extraction buffer and added to the original extract. 2% (v/v) β-mercaptoethanol was added to the mixture and mixed gently.

iv. Incubated at 60°C for 60 minutes and cooled to room temperature.

v. Added equal volume of 24:1 (v/v) chloroform:isoamyl alcohol and mixed gently by inverting the tubes 20 to 25 times to form an emulsion.

vi. Added equal volume of 24:1 (v/v) chloroform:isoamyl alcohol and mixed gently by inverting the tubes 20 to 25 times to form an emulsion.

vii. Spinned at 1000 rpm at room temperature for 10 min.

viii. Transferd the top aqueous phase to a new 50 mL centrifuge tube with a wide-bore pipette tip. A second chloroform:isoamylalcohol extraction was performed.

ix. Added 0.5 volumes of 3M $NaCH_3COO$ to the aqueous solution recovered from the previous step and mixed well.

x. Added equal volume of cold (-20°C) isopropanol and cooled at 4 to 6°C for 15-20 minutes or until DNA strands begin to appear.

xi. DNA was spooled out and washed with cold (0 to 4°C) 70% ethanol. Ethanol was then completely removed without drying the DNA pellet by leaving the tubes uncovered at 37°C for 20 to 30 minutes.

xii. Dissolved the DNA in 200 to 300 µL TE.

xiii. Treated with 1 µL RNAaseA per 100 µL DNA solution and incubated at 37°C for 15 minutes.

xiv. Extracted with equal volume of choloroform: isoamyl alcohol (24:1).

xv. Transferred the aqueous layer to a fresh 1.5 ml microfuge tube and added equal volume of isopropanol.

xvi. Spinned at 10,000 rpm for 10 min at 25-30ºC.

xvii. Washed the pellet with 70% ethanol. Ethanol was then completely removed without drying the DNA pellet by leaving the tubes uncovered at 37°C for 15 to 20 minutes.

xviii. DNA was dissolved in 200 µl of TE water and stored at 4 ºC until required.

xix. DNA concentrations were determined either by running aliquots of DNA samples on a 0.8% agarose gel electrophoresis or by taking the absorbance at 260 nm. The ratio between 260 and 280nm provided an estimate of the purity of the DNA sample. DNA samples with a ratio of approximately 1.8 under spectrophotometer and producing an intact single band without smear on 0.8% Agarose gel electrophoresis were considered as good quality DNA.

5.2.6 DNA amplification: The PCR protocol described by Mullis *et al*. (1986), was followed and carried out in a total volume of 25 µl containing following components. The optimized PCR reaction as follows.

i. A low range DNA ladder (3Kb) was use for band sizing.

ii. The RAPAD products were loaded on 2% agarose gel stained with ethidium bromide for electrophoresis in 1X TBE at a constant current 60 mA, < 150 V for 2 h. Visualization and photography of the gel was done in Gel Documentation system. 1X Taq Buffer (Banglore GeNei, India, Mg^{++} free)

iii. 2.5 mM MgCl2 (Banglore GeNei, India)

iv. 0.2 mM dNTPs (Banglore GeNei, India)

v. 0.6 U Taq polymerase enzyme (Banglore GeNei, India)

vi. 0.25 μM primer (Table 5.1)

vii. 50 ng DNA template.

viii. Amplification was carried out in 25 μl reaction volume containing 1X PCR assay buffer (50Mm KCl, 10Mm Tris HCL, 2.5Mm $Mgcl_2$) and 0.2Mm dNTPs, 20 Mm of primer, 0.6 Units of Taq DNA polymerase and 50ng DNA template.

ix. Amplification reaction carried out in PTC-Strategene USA programmable thermal cycler.

x. The cycle programme was initial denaturation at 94^0 C for 10min.

xi. Followed by 45 cycles, each cycle consisted of denaturation at 94^0 C for 60 sec. and annealing at 50^0 C for 1 min. and extension at 72^0 C for 1.5 min. and final extension at 72^0 C for 10 min.

xii. PCR amplifications performed on a Stratagene Corbett Cycler under the following conditions (Figure 5.1).

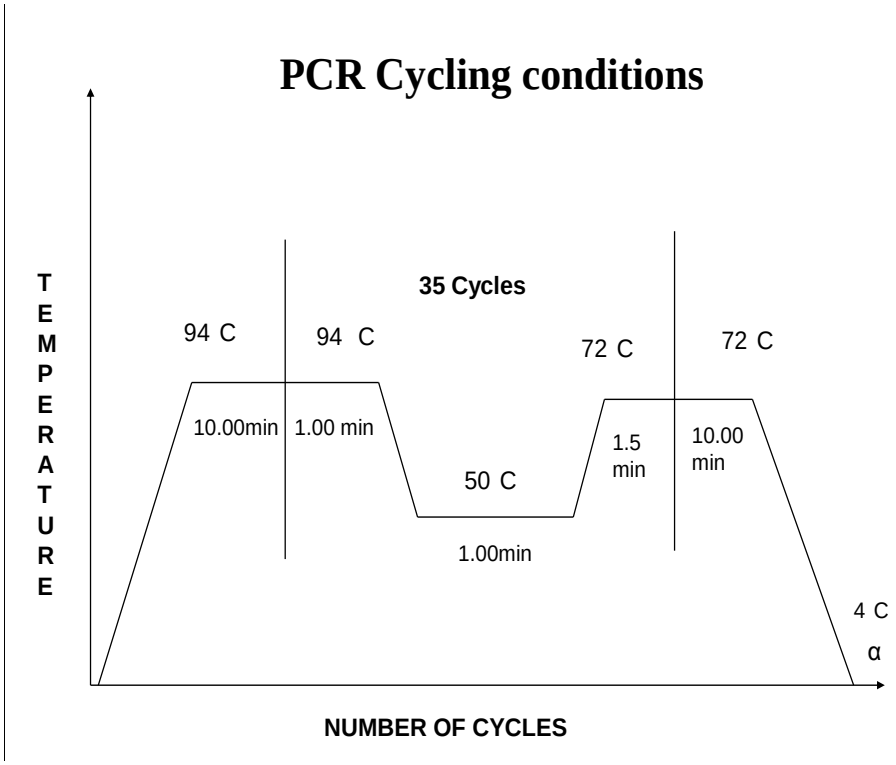

Fig. 5.1 Schematic diagram of PCR reaction

5.2.5 Data Analysis: Amplified products for RAPD analysis were scored based on the presence (taken as 1) or absence (taken as 0) of band for each primer. Banding pattern for each primer scored by visual observations, where only clear and unambiguous bands scored. The size (nucleotide base pair) of amplified bands was determined based on its migration relative to molecular size marker (DNA ladder from Banglore Genie Pvt. Ltd., India). The data entry was done into a binary data matrix as discrete variables. Jaccard's coefficient of similarity was measure and a dendrogram based on similarity coefficients was generated by using Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

5.3 Result and Discussion

Through critical analysis of earlier reports (Reddy et al., 2007; Basha and Sujata 2007; Gupta et al., 2008; Ranade et al., 2008; Pamidiammari 2008 and Ganeshram et al., 2007) 10 primers were selected for analysis of 20 accessions. They reported to produce reproducible bands in *J. curcas* species. Twenty accessions were uniformly represented four agro-climatic regions of Maharashtra, each region was uniformly

Table 5.3 List of polymorphic RAPD primers and number of PCR amplified bands generated from *J. curcas* L. accessions

No.	Primer	Sequence (5'-3')	Polymorphic bands	Monomorphic bands	Total Bands	% of polymorphisom	% of Monomorphisom
01	OPB02	TGATCCCTGG	10	3	13	76.92	23.07
02	OPA07	GAAACGGGTG	12	1	13	92.30	7.69
03	OPB10	CTGCTGGGAC	9	5	14	64.28	35.71
04	OPB07	GGTGACGCAG	11	2	13	84.61	15.38
05	OPC18	TGAGTGGGTG	6	3	9	66.66	33.33
06	OPC02	GTGAGGCGTC	8	4	12	66.66	33.33
07	OPD20	ACCCGGTCAC	10	4	14	71.42	28.57
08	OPE20	AACGGTGACC	12	2	14	85.57	14.28
09	OPR14	CAGGATTCCC	10	2	12	83.33	16.66
10	OPE01	CCCAAGGTCC	6	5	11	54.54	45.45
Total			94	31	125	74.62	25.34

represented by five accessions each. The details of the nucleotide sequences of ten RAPD primers shown in table 5.3 were used to amplify DNA from 20 accessions. The details of the names and nucleotide sequences of primers used to generate 125 PCR products and summary of the total number of polymorphic and monomorphic DNA fragments and percentage of polymorphism and monomorphism shown in Table5.3. A total 125 bands were scored of which 94 (75.2%) were polymorphic and 31 (25.347%) were monomorphic across the genotypes. On an average, total number of bands per primer was 12.5 bands of which 9.4 were polymorphic. A wide variation in the number of bands were ranging from 6 -12. The primer produces 12 bands are OPA 07, OPE20 while 10 bands produce by OPB 02, OPN 07 and OPR 14. It indicates potentiality of various primers in resolving variations in genotypes studied (Ref. fig. 5.2, 5.3 5.4 and 5.5).

Based on RAPD polymorphism (Ref. Table 5.3), the primers namely; OPA 07 and OPE 20 s*h*owed maximum polymorphic bands of 12, OPB 07 showed 11 while OPB 02, OPN 07 and OPR 14 showed 10 bands. The average number of polymorphic bands per primer is 9.4 and the percentage of polymorphism ranges from 54.54 (OPR 14) to 92.30% (OPA 07) (Ref, Table 5.3). Recently Basha and Sujatha (2007), had reported low levels of molecular diversity among Indian accessions of *J. curcas* germplasm indicating a narrow genetic base.Ganeshramet al. (2008), detected polymorphism in *Jatropha* species with 26 RAPD primers (80.2%) across eight species, which were considerably higher. In the present study, 20 *J. curcas* accessions from Maharashtra showed high percentage of 75.2%. Jaccard's genetic similarity co-efficient varied from 0.14 to 0.98 (Ref. Table 5.6).

The highest genetic similarity (More than 80%) between JC01 and JC 02, JC01and JC05, JC08 and JC13, JC11and JC15, JC16 and JC12, JC09 and JC12, JC13 and JC11. The lowest of 0.14 were between JC05 and JC11, JC12 and JC11. UPGMA cluster analysis of the Jaccard's similarity coefficient generated a dendrogram (Ref. figure 5.6) which illustrated the overall genetic relationship among the accessions studied. UPGMA dendrogram showed two main clusters split at Jaccard's similarity co-efficient of 0.10. Cluster I include JC01 to JC05. Cluster 2 indicated the four sub clusters (Ref. Fig. 5.6). Association among the 20 accessions was also resolve by PCA (Ref. Fig. 5.7). The overall grouping pattern of PCA corresponded well with the

clustering pattern of the dendrogram (Ref. figure 5.6). In agreement with the dendrogram, JC 18, JC 01 and JC 62 did not group with any other accessions in the PCA, also confirming their genetic distinctness from all other *J. curcas* accessions.

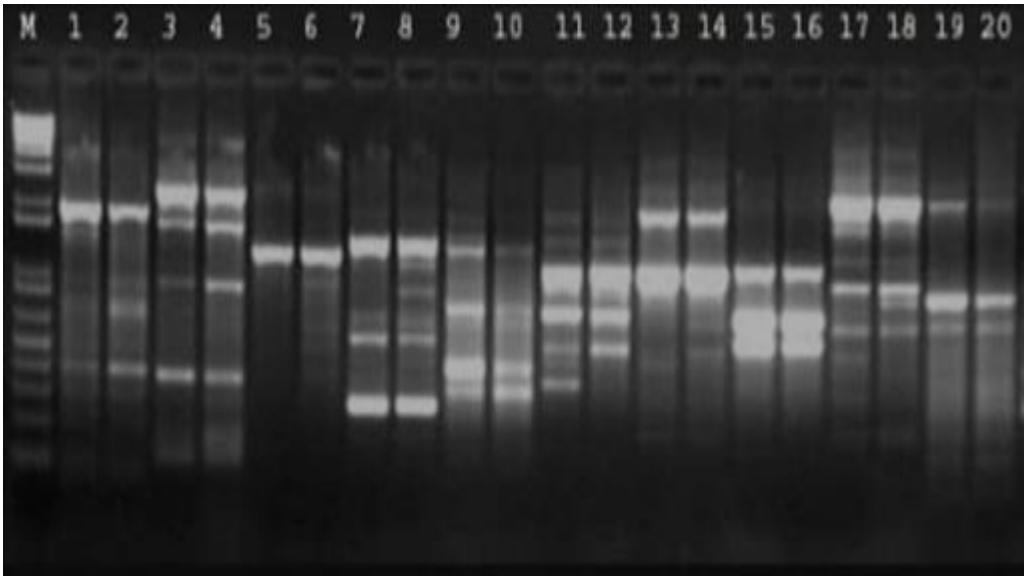

Fig. 5.2 RAPD polymorphisom for *J. curcas* genotypes detected with OPB 07

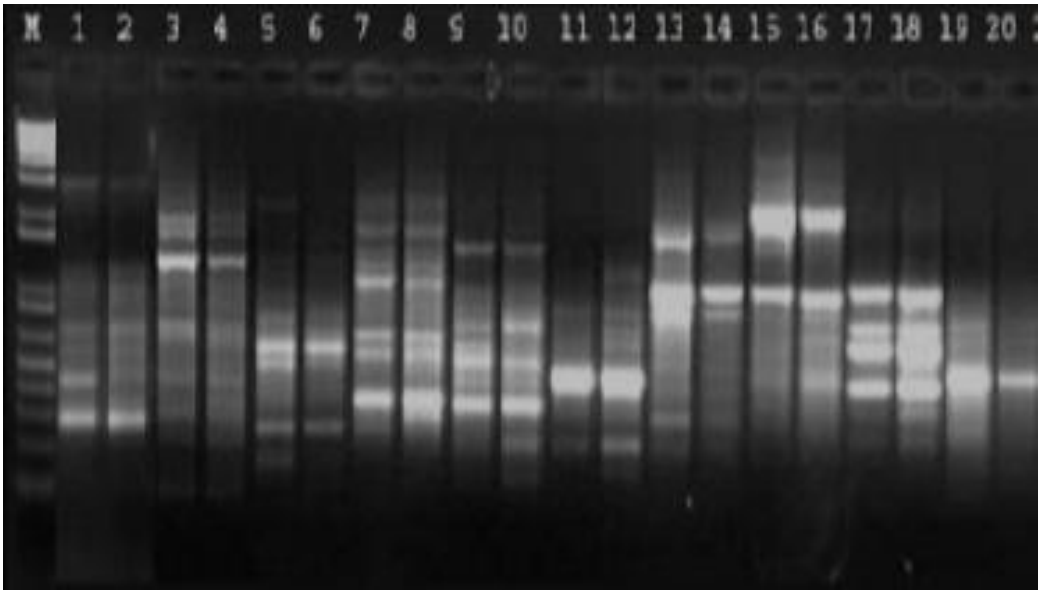

Fig. 5.3 RAPAD polymorphisom for *J. curcas* genotypes detected with OPE 20

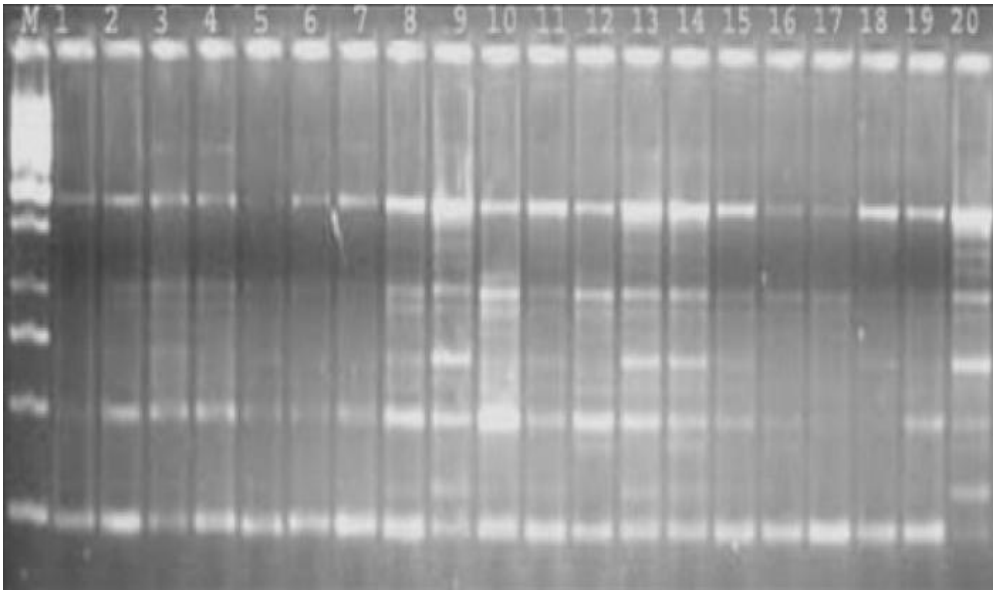

Fig. 5.4 RAPAD polymorphisom for *J. curcas* genotypes detected with OPA 07

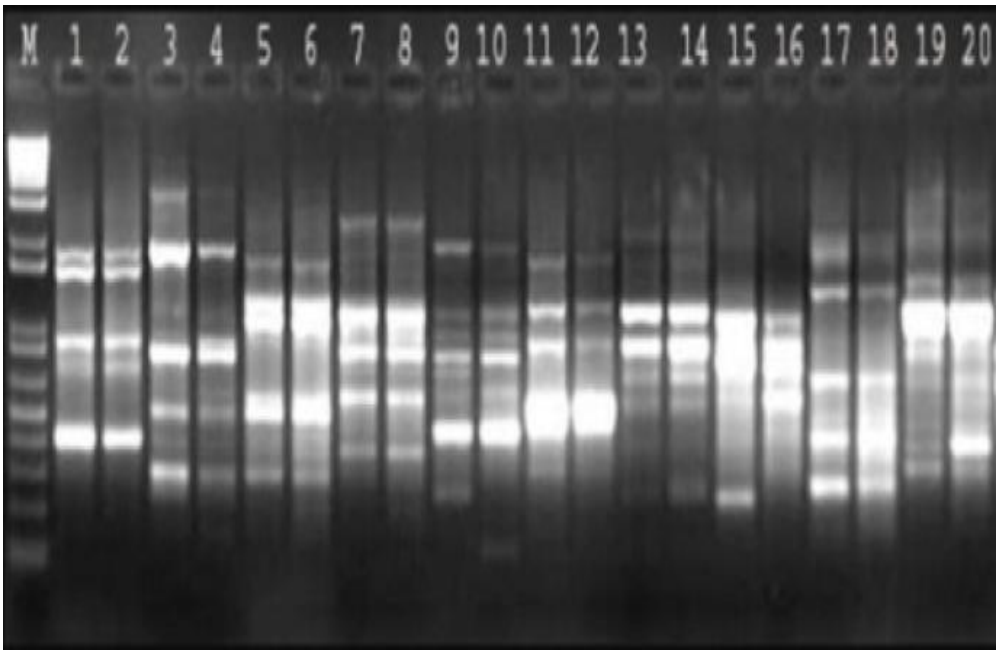

Fig. 5.5 RAPAD polymorphisom for *J. curcas* genotypes detected with OPR 14

Table 5.4 Jaccards similarity coefficient of 20 *Jatropha curcas* L. accessions.

	JC01	JC02	JCO3	JC04	JC05	JC06	JC07	JC08	JC09	JC10	JC11	JC12	JC13	JC14	JC15	JC16	JC17	JC18	JC19	JC20
JC01	1																			
JC02	0.98	1																		
JC03	0.63	0.72	1																	
JC04	0.54	0.45	0.62	1																
JC05	0.81	0.72	0.45	0.66	1															
JC06	0.22	0.34	0.17	0.75	0.71	1														
JC07	0.25	0.43	0.13	0.29	0.5	0.78	1													
JC08	0.42	0.33	0.65	0.45	0.26	0.64	0.67	1												
JC09	0.41	0.52	0.61	0.43	0.24	0.72	0.69	0.63	1											
JC10	0.25	0.38	0.38	0.25	0.57	0.63	0.27	0.38	0.29	1										
JC11	0.63	0.22	0.48	0.24	0.14	0.58	0.43	0.51	0.43	0.78	1									
JC12	0.56	0.42	0.14	0.33	0.57	0.61	0.48	0.67	0.8	0.71	0.73	1								
JC13	0.33	0.29	0.61	0.53	0.61	0.68	0.67	0.81	0.58	0.61	0.81	0.32	1							
JC14	0.29	0.33	0.33	0.38	0.43	0.29	0.68	0.46	0.71	0.69	0.67	0.47	0.57	1						
JC15	0.43	0.56	0.14	0.38	0.64	0.67	0.58	0.61	0.58	0.46	0.82	0.69	0.61	0.72	1					
JC16	0.33	0.43	0.51	0.39	0.46	0.63	0.75	0.37	0.59	0.67	0.73	0.86	0.43	0.29	0.44	1				
JC17	0.28	0.29	0.22	0.29	0.43	0.38	0.42	0.71	0.67	0.57	0.21	0.38	0.62	0.47	0.35	0.39	1			
JC18	0.48	0.57	0.29	0.43	0.71	0.67	0.35	0.48	0.37	0.55	0.56	0.44	0.34	0.67	0.59	0.33	0.39	1		
JC19	0.33	0.41	0.51	0.37	0.41	0.68	0.65	0.27	0.33	0.67	0.67	0.41	0.64	0.72	0.67	0.49	0.33	0.71	1	
JC20	0.21	0.29	0.38	0.44	0.57	0.67	0.37	0.61	0.48	0.37	0.52	0.72	0.58	0.37	0.43	0.71	0.68	0.48	0.67	1

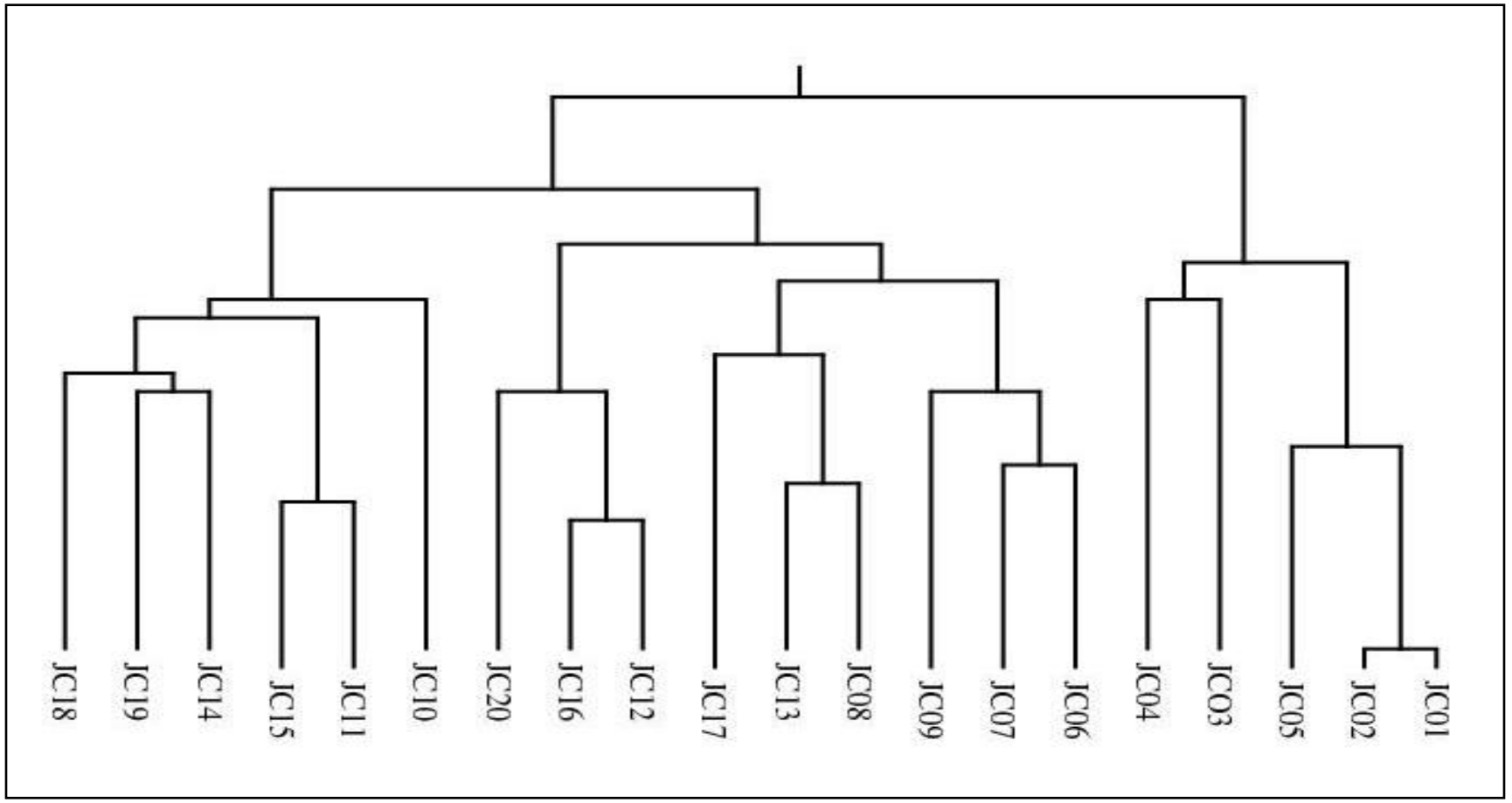

Fig 5.6 RAPD based phylogenetic tree for 20 *Jatropha curcas* L.accessions constructed according to Jaccard coefficient

All genotypes from different geographical regions showed close resemblance and fall under single sub-populations. This association between genotypes from neighboring regions may be the result of similar agro-climatic conditions or due to seed movement and gene flow (Padmesh et al., 1999). In practice, better under-standing of the distribution of genetic variation at the intra specific level would help to identify superior genotype(s) for cultivar up-grades and as well as to evolve strategies for the establishment of effective in situ and ex situ conservation programmes (Bhutta et al., 2006; Basha and Sujatha, 2007). Although such empirical determination of diversity can be obtained by evaluating morphological, physiological and biochemical traits, the study also reveals the limitations of conventional taxonomic tools in resolving the taxonomic confusion prevailing in plant classification. The technical simplicity of the RAPD technique has facilitated its use in the analysis of genetic relationships in several genera (Wilikie et al., 1993; Demeke, 1992; Nair et al., 1999). The major concern regarding RAPD generated phylogenies includes homology of bands showing the same rate of migration and cause and origin of sequence in the genome (Stammers et al., 1995). In spite of this limitation, RAPD markers have the greatest advantage of its capability to scan across all regions of the genome hence its suitability for phylogenetic studies at species levels (Wilikie et al., 1993; Demeke, 1992).

The diversity among the *Jatropha curcas* genotypes in the present study ranged from 0.14 to 0.98 percent. These results agree with the findings of Sudheer et al. (2008). Highest genetic polymorphism was found in *Jatropha curcas* by RAPAD analysis. Basha et al. (2009), and Ganesh et al. (2007), observed similar results. In present study RAPD analysis showed that accessions from Kokan region are most divergent among the genotypes studied. Accessions from Kokan region were also found to be divergent phenotypically with larger seed size, significantly lower percentage of oil and protein.

Ecological and geographical differentiations are two important factors, which influence breeding and sampling strategies of tree crops (Matyas, 1996). This was further help in understanding the population distribution. Variation in genetic diversity within the species is usually related with geographic range, mode of reproduction, mating system and seed dispersal (Loveless, 1992). Gupata et al. (2008), while assessing genetic variation in 14 accessions of *J. curcas* from different

agro-climatic regions of India reached similar conclusions. Howevere, in another study (Basha and Sujatha, 2007) modest level of genetic variability was reported in *J.curcas* germplasam from India. The results from the present study showed that *J. curcas* germplasam from Maharashtra constitutes a broad genetic base, from clustering pattern and genetic relationship obtained using RAPD markers, breeders can identify the diverse genotypes from different clusters and employ them in future breeding programmes.

In present study, we could identify polymorphic RAPD markers that could distinguish geographically isolated genotypes in four agro-climatic regions of Maharashtra. The *Jatropha* plant is a new system and only recently exposed to molecular investigations, mainly due to its increasing popularity as a bio-diesel feedstock and valuable co-products (Kohli et al., 2009). This study constitutes the first successful attempt to assess genetic diversity of *J. curcas* using molecular markers in Maharashtra provenances when compared with inter and intra population variations. RAPD technique has been successfully use in variety of taxonomic and geneticdiversity studies of *Jatropha* and the present results corroborate this conclusion (Rodriguez et al., 1999). The number of alleles per locus was a potentially more sensitive measure of genetic diversity. Studies show that, dominant markers predictably can underestimate genetic diversity (Wu et al., 1999) and therefore, true diversity of *J. curcas* in the present study might be higher than reported here.

5.4 Conclusion

The genetic distances estimated based on 10 primers exhibited a wider range (Ref. Table 5.3), suggesting that *J. curcas* germplasm collection represents genetically diverse populations. This may attributed to the high level of cross-pollinated nature of this species and interaction of materials from different genetic sources (Ikbal et al., 2010). The high diversity revealed by RAPD markers in this study is in agreement with the general belief that out-breeding plant species always exhibit considerable diversity, same as in the species of the present study. Subramanyam et al. (2009), was found similar results. It is also generally believed that, availability and maintenance of higher genetic diversity within population favores is generally due to the genetic systems of the species such as gene flow, mating systems, mutations, etc. Therefore,

the out crossing nature of *J. curcas* might have promoted higher diversity observed (Ikbal et al., 2010).

Modest to high genetic diversity reported using RAPD markers. The results of the present study showed that, *J. curcas* germplasm from Maharashtra constitute a broad genetic base rich for a breeding and improvement program. From the clustering patterns and the genetic relationship obtained, selection for breeding programmes can be done from the different clusters realized to capture in total the available gene pool. Due to the high genetic variation observed, random seed collection for plantation establishment are likely to result in varied seed yield, hence, affects uniform production likely in a plantation.

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6. *In vivo* Propagation

6.1 Introduction

Jatropha curcas L. is a multipurpose plant that belongs to the family Euphorbiaceae and is not only valued for its medicinal properties and resistance to various stresses, but also for its use as an oil seed crop (Openshaw, 2000). It has drawn attention in recent years, since the demand for fuel (diesel) has increased drastically. *Jatropha* produces seeds with an oil content of 30-48% by weight (Kochhar et al., 2005). *Jatropha* plants raised from seeds reach to fruiting within three to four years after planting. However, *Jatroha* plants propagated by stem cuttings, yield fruits in about one year from planting (Jones, 1992). Seeds of *Jatropha* have limited viability and can only be stored for 15 months, after which their viability is reduced to 50% (Kochhar et al., 2008). At present, good improved strains of *Jatropha* are available at different centers in our country for large-scale planting. However, the required quantity to genetically pure seed material for commercial planting is still not available. A lot of genetic variations exist in the available seed material (Pant et al., 2006). This obviously necessitates vegetative reproduction for the multiplication of desired strains.

To meet the large-scale demand and ensure easy supply of elite planting material, there is a need to establish mass multiplication technique. The propagation through seeds is dependent on good rainfall, moisture condition, sowing time and depth of sowing. Tissue culture technique offers rapid and continuous supply of planting material but the reports were not promising as the multiplication rate was low. Propagation by stem cutting is traditional and promising method for the multiplication of this plant. Many internal factors such as auxins, rooting co-factors, carbohydrate and nitrogrn levels have been shown to influence rooting of stem cuttings (Hertman and Kestar, 1975). Adventitious root formation has lot of commercial interests because there are many plant species of which cuttings are difficult to root. In some plant species, adventitious root formation initiates without any treatment. Others required treatments of different growth regulators usually auxins (Syros, 2004). Auxin induces root formation by breaking root apical dominance induced by cytokinin (Cline, 2004). Indole Butyric Acid (IBA) is a synthetic rooting chemical that has been found to be reliable for root induction. IBA is widely used because it is non-toxic to

most plants over a wide range and promotes root growth in large number of plant species (Hartmann et al., 1990).

Different planting media were also used to initiate healthy roots because planting medium is considered to be an important factor for the growth and development of plants. According to Larson (1980), the best planting medium must have a pH conducive to plant growth, a structure that will permit gaseous exchange to provide aeration for the rooting and permit water infiltration and movement. To standardize the methodology for propagation of this plant, the present experiments were designed with the objective to determine most suitable concentration of growth hormone, ideal length of stem cuttings and proper substrate for the propagation of *Jatropha curcas* on large-scale.

6.2 Materials and Methods

6.2.1 Auxin treatments and seasonal variations: The present study was carried out at the Botanical garden of Modern College of Arts, Science and Commerce, Pune 5. Maharashtra. Elite plants of *Jatropha curcas* were selected from natural populations near Sinhagad fort near Pune.

Seasonal studies were carried out for propagation, in which the effects of growth hormone on rooting of stem cutting have been evaluated. Healthy and uniform stem cuttings (20-30cm long and 2-3cm thick) of *J. curcas* were obtained from the middle portion of one year old branches of three to five year old plants during Mansoon (June/July), Winter (December/January) and Spring (March/April) in the year 2008 - 2009. Stem cuttings were dipped in 0.1% bavistin fungicide for 2-3 minutes and subsequently washed in distilled water before giving hormonal treatment (200, 400, 800, 1400, 2000, 4000 and 8000mg/l IBA). The basal portions of cuttings (1-2cm) were dipped in each concentration of IBA for 5min. Then the stem cuttings were planted in polythene bags filled with substrate (garden soil).

The experiments were designed in randomized complete block design (RCBD), with factorial 3x6. Each replication consisted of three cuttings. The polybags were then kept in nursery beds and irrigated regularly depending on weather conditions.

Sprouting, rooting and root character were recorded after 20th, 40th and 60th days in three season viz. Monsoon, Winter and Spring.

6.2.2 Length of stem cuttings and seasonal variations: To determine the optimum length of the stem cuttings for vegetative propagation, healthy cuttings of the branches were collected from selected locality as prescribed in 6.2.1. After removal of side branches and leaves, stem cuttings of 10, 20, 30, 40 and 50 cm length were made, with uniform thickness i.e. 2.5- 3.0 cm, planted in polythene bags (9"x 6" size) containing loam soil and farm yard manure as substratum. This experiment was performed in triplicate for three seasons. Observations were recorded on 20th, 40th, and 60th days, for survival percentage, root length, root fresh weight and root dry weight were recorded.

6.2.3 Effect of substrates and seasonal variations: To study the effect of different growing media on rooting of cuttings, the experiment was set up in RCBD (Random Column Block Desigen) with three replications and one variable factor i.e. different growing media viz, Sand:Soil(1:1), Sand:Soil (1:2), Sand:Soil:FYM(1:2:1), Sand:Soil:Cocopeat (1:2:1), and Sand:Soil:Vermicompost (1:2:1). Five cuttings per treatment were inserted in black polythene bags filled with the selected substrate and in this way a total 150 cuttings were planted in five different substrates with one control. For assessing survival percentage, seedlings that survived after two months were counted and the data was expressed in percentage.

6.3 Results and Discussion

6.3.1 Auxin (IBA) treatments and seasonal variations: Present study revealed that, rooting percentage of stem cuttings show significant difference. Stem cuttings that were treated with 2000mg/l in Spring season rooted faster than control (Ref. Table 6.1 and figure 6.1). It showed highest percentage of rooting and survival. It was 78, 62 and 54% in Spring, Monsoon and Winter season respectively (Ref. Table 6.1). It was observe that higher concentration of IBA i.e. 4000 and 8000mg/l. have inhibitory effect on rooting and survival percentage of stem cuttings. It was 64, 52 and 49% for Spring, Monsoon and Winter season respectively with 8000mg/l IBA treatment.

Similarly, for 4000mg/l concentration treatment, it was 57, 50 and 66% for the said three seasons (Ref. Table 6.1).

Cuttings treated with 200, 800 and 1400 mg/l concentration of IBA showed 62, 68 and 63% rooting in spring season respectively, but poor response to rooting during Winter. It was 32, 36 and 45% respectively, while, it was moderate during Monsoon season (Ref. Plate 6.1). It was 49, 53 and 45% rooting and survival of stem cuttings (Ref. Table 6.1) Untreated cuttings rooted fairly well in Spring season and application of IBA further triggered to enhance rooting significantly. The cuttings showed poor performance to root in spite of IBA treatment during Winter. This could be due to higher meristemstic growth and consequently more endogenous auxins level during the Winter season and thus applied IBA raising the concentration to supra optimal levels that were inhibitory. On other hand, in the Spring season exogenously applied auxins enhanced rooting by raising it to an optimal level. Thus results are indicative of varied stimulus of hormone application with the concentration of hormone in different seasons.

According to Villar (1977), better formation of roots due to auxin treatment may be due to accumulation of metabolites at the site of application, synthesizes of new protein, callus formation, cell division and cell enlargement. Seasonal variation in rooting behavior of branch cuttings could be due to difference in the activities of hydrolyzing enzymes causing seasonal fluctuation in the availability of sugar, which are principal source of metabolic energy required for cell division and differentiation during root initiation in cuttings (Jalani, 1980).

Various workers (Nanda, 1984 and Handa, 2005) have reported that, the seasonal variations in the rooting response of stem cuttings. Such variations were due to varying levels of endogenous root forming substances influence by climatic factors such as temperature, light and humidity (Fernquist, 1966). According to Davis (1996), hormones have been showed to regulate different aspects of plant growth and development including cell division, cell elongation and cell differentiation. Auxin is a substance that is produced in one tissue (Shoot) and migrated to effect the development of another tissue. It promotes cell elongation

Table 6.1 Effect of IBA on rooting of stem cuttings of *Jatropha curcas* L.

Growth Hormone Treatments (mg/l)	Percentage of stem cuttings survived and rooted								
	Monsoon			Winter			Spring		
	Days after planting								
	20	40	60	20	40	60	20	40	60
Control	0.0	20.31±3.9	46.32±4.1	0.0	22.51±2.6	26.32±4.1	0.0	26.4±1.6	58.7±2.6
200	0.0	29.37±4.2	49.21±3.7	0.0	21.47±3.6	32.21±3.7	Callusing	46.6±1.4	62.6±3.1
800	0.0	25.57±3.4	53.69±4.1	0.0	26.51±3.2	36.69±4.1	0.0	42.8±2.3	68.2±3.8
1400	0.0	22.53±2.8	45.06±3.4	0.0	22.59±4.4	45.06±3.4	0.0	39.4±2.6	63.9±2.7
2000	0.0	30.42±2.4	62.72±5.76	0.0	32.72±1.8	54.72±5.76	0.0	64.5±1.8	78.4±3.2
4000	0.0	24.85±3.0	57.61±3.84	0.0	28.95±3.6	50.61±3.84	0.0	42.3±1.4	66.5±2.5
8000	0.0	27.33±2.5	52.84±3.22	0.0	30.83±4.7	49.84±3.22	0.0	41.5±2.5	64.2±2.2
CV(%)	-	14.32	11.95	-	16.86	7.07	-	26.05	9.52
LSD	-	3.12	7.06	-	2.84	7.39	-	4.34	6.88
SE	-	0.48	0.50	-	0.42	0.50	-	0.24	0.35

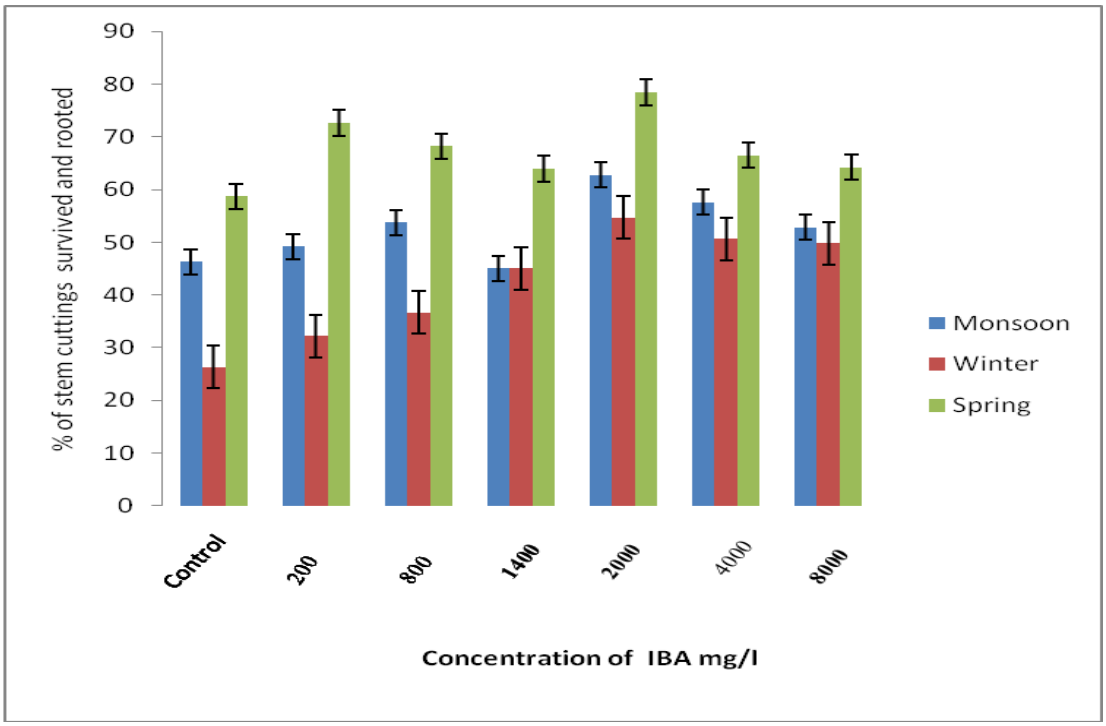

Fig. 6.1 Interactive effect of concentration of IBA and seasonal variation on rooting and survival of stem cuttings after 60 days

and has variety of other growth regulating effects. The effect of auxin is specific in the development of root and shoot by Arora (2006). According to Camellia (2009), the application of IBA increased the number of roots. Cuttings with high number of root have the advantage by enhancing good anchorage when it was planted in the field. The hard woodcutting is the best type of cutting because it gave the best performance compared to the one using semi-hardwood and soft woodcuttings of *Jatropha curcas.* IBA application at 2000 mg/l gave the best rooting performance in Spring season.

6.3.2 Effect of length of cuttings and seasonal variations: The second experiment showed the survivals were correlated with lengths of stem cuttings. It was also noted that, seasonal variation was playing significant role in rooting of stem cuttings. Among the different lengths of cuttings 20 cm, long stem cuttings recorded highest percentage of rooting. It was 76, 64 and 45% in Spring, Monsoon and Winter respectively. The minimum was record for 10 cm, 30cm, 40 cm and 50 cm long stem cuttings, for 50 cm long stem cuttings, rooting percentage was 40, 57 and 32% in the above said three seasons. While, for 10 cm long stem cuttings, it was 47, 32 and 45% in Spring, Winter and Monsoon season respectively (Ref. Table 6.2).

a **b** **c**

Photo Plate 6.1: Effect of IBA on rooting performance on stem cutting of *Jatropha curcas* L., during. **a.** Monsoon, **b.** Winter, **c.** Spring (60 days after planting.)

Similar trend was recorded for number of roots per cuttings. During spring season highest number of roots (14) was recorded for 40cm long stem cutting, followed by 50cm, 20cm and 30cm long stem cuttings, it was significant. During Winter season and Monsoon number of roots per cutting was least for all size of stem cuttings and significant variations were never found for the same (Ref. Table 6.2 and Plate. 6.2).

It is presumed that the medium sized cuttings might have got sufficient food material and hormones for inducrion of root and shoot. Small sized cuttings registered very poor growth, were inferior to the medium, and longer sized cuttings. A reduction in root length was observed with reduction in size of cutting, due to inadequate supply of nutrients and leaching of nutrients in shorter cuttings, resulted in poor performance in rooting (Good et al., 1966). According to Kathiravan et al. (2009), Macro propagation of *Jatropha curcas* L. stem cuttings with 20cm length and 2.5 to 3.0 cm thickness is successful in generating higher survival and biomass productivity. Our findings are in agreement with the result by Kathiravan (2009).

Hedge (1988), recorded that, this might be due to higher adventitious ability of juvenile characters of large cuttings compared to smaller size cuttings, which are having tender tissues, with unsaturated latex and higher content of metabolites like tannin, lignin etc. It was adversely interfereing with sprouting and root development. According to Reinhard (2003), the behavior of stem cuttings varies with age, genotypes and physiological status of mother plant, which is also one of the reasons for good performance of the medium size, stem cuttings. The initial levels of endogenous auxin and its oxidation enzymes IAA-oxidase and peroxidase play a significant part in the rooting process. IAA-oxidase activity is involved in triggering and initiating the root primordial, whereas peroxidase is involved in both root initiation and elongation (Kochhar et al., 2005).

Reductions in number of roots were observed with reduction in size of cuttings (Ref. Table 6.2). According to Good (1966), poor performance of shorter stem cuttings is due to inadequate supply of nutrients and leaching of nutrients in shorter cuttings. According to Hegde ((1988), the poor performance of small size cutting was due to the reason that cuttings are still under maturity and may be devoid of sufficient food material for induction of roots and shoots. The under performance of large sized stem

cuttings may be attributed to reason that these cuttings might have converted most of food material for lignifications, which resulted in over lignified stem caused lower rooting and shooting percentage.

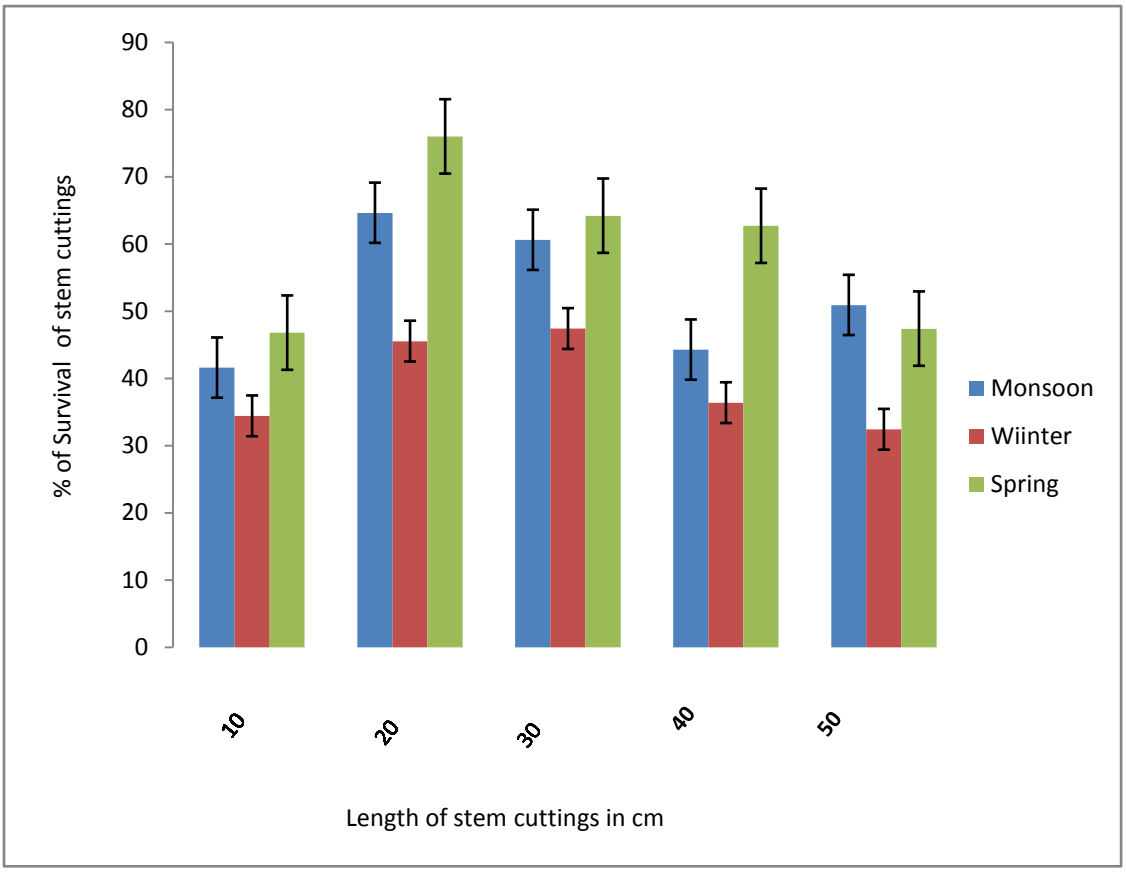

Fig. 6.2 Interactive effects of length of stem cuttings and seasonal variation on % of rooting and survival of stem cuttings

In *Jatropha,* shoots were formed much earlier than roots, shoots thus formed earlier due to reserve carbohydrates. It starts producing auxins that moves downward, thereby acuumulating in the lower portion of the cuttings. When the concentration reaches a threshold value, endogenous auxin at the extreme basal end start being mobilized and signals the process of root initiation (Kochhar et al., 2005).

Between day 7 and day 15, some callus developed at the basal end of cuttings (Ref. Plate 6.2). The basal end was swollen and the colour was changed from green to whitesh green. The formation of callus was continuing to the following days until the 11^{th} day, root primordial were emerging from the callus (Ref. Photo plate. 6.2.).

a

b

c

Photo Plate 6.2 Development of adventitious roots at the basal end of *Jatropha curcas* L.stem *cutting*. **a.** Day 0: No changes occurred, **b.** Day 10: Development of callus at the basal end of stem cutting, **c.** Day 15: Root primordia emerged from the callus.

Table 6.2 Effect of different lengths of stem cuttings of *J. curcas* on the rooting and survival of stem cuttings

Length of cuttings (cm)	Monsoon		Winter		Spring	
	Rooting (%)	No. of roots per cutting	Rooting (%)	No. of roots per cutting	Rooting (%)	No. of roots per cutting
10	41.60	2.6±0.4	34.42	2.1±0.4	46.8	2.8 ± 0.3
20	64.64	2.8±0.6	45.54	2.5±0.4	76.0	12.3 ± 0.4
30	60.61	3.0±0.3	47.41	2.7±0.5	64.2	10.2 ± 0.7
40	44.28	4.9±0.6	36.38	3.3±0.2	62.7	14.3 ± 0.5
50	40.92	4.3±0.5	32.42	3.2±0.3	47.4	12.9 ± 0.2
LSD	-	0.45	-	0.34	-	0.40
CV(%)	-	28.69	-	18.04	-	42.85
SE	-	0.15	-	0.11	-	0.13

LSD: Least significant difference, CV: Critical variance, SE: Standard error

Although the root primordial seems to be emerged from the callus, closer examination from histological analysis showed that the root was emerging from an area near the vascular bundle and not from the callus itself. Cross section of stem cuttings of stem on day 0 showed no changes (Ref. fig. 6.2.a). On day 15th, cells started to dedifferented and develop to be in to root initials. On the 15th day onwards, subsequent development of these root initials into organized root primordial was done as well as the development of root cap. Root primordial elongated towards the epidermis (Ref. Plate 6.2.). The root primordial grew and emerged through epidermis on 16th day the vascular connections between the root primordial and the conducting tissues of the cutting itself was formed.

6.3.3 Role of substrates and seasonal variations: Third experiment revealed that, seasonal variation and substrates have significant impact on rooting of stem cuttings. Among the three seasons Spring season was found better for rooting of stem cuttings and survival. It was 78, 72, 74, 70 and 69% for S_5, S_4, S_3, S_2 and S_1 respectively. Minimum rooting and survival percentage recorded for Winter season. It was 45, 36, 37, 32 and 22% for S_5, S_4, S_3, S_2 and S_1 respectively (Ref. Table 6.3).

a **b** **c**

d **e** **f**

Photo Plate 6.3 Macropropagation of *J. curcas*: **a)** Preapration of 15cm long stem cuttings. **b)** 0.1 % Bavistin treatment. **c)** IBA treatment. **d)** Incubation of stem cuttings inside the mist chamber. **e)** Root growth after 60 days. **f)** Plantlet ready for plantation in field condition after 4 months of planting of stem cuttings.

Table 6.3 Effect of substrate on the rooting of stem cuttings of *Jatropha curcas* L.

Substrate Treatment		Percentage of cuttings survived and rooted								
		Monsoon			Winter			Spring		
		Days after planting								
		20	40	60	20	40	60	20	40	60
S_0	Control (Fine Sand)	0.0	8.45±37	26.27±2.07	0.0	9.54±1.64	26.11±2.94	0.0	41.62±2.74	64±3.7
S_1	Sand: Soil (1:1)	0.0	12.56±1.91	29.47±1.45	0.0	10.40±1.67	22.21±1.15	0.0	46.31±3.47	69.8±2.9
S_2	Sand:Soil (1:2)	0.0	11.94±1.47	36.81±1.26	0.0	10.73±1.57	32.41±1.44	0.0	44.41±4.12	70.8±3.1
S_3	Sand:Soil:FYM (1:2:1)	0.0	19.43±2.01	58.94±0.89	0.0	16.45±2.44	37.34±1.74	0.0	56.34±3.45	74.4±2.6
S_4	Sand:Soil:Cocopeat (1:2:1)	0.0	24.31±2.8	42.54±0.36	0.0	15.94±1.73	36.40±2.73	0.0	54.31±4.27	72.4±3.7
S_5	Sand:Soil:Vermicompost (1:2:1)	0.0	36.47±2.4	56.64±1.64	0.0	22.61±3.14	45.63±1.86	0.0	74.53±4.67	78.2±3.2
CV (%)		-	57.33	32.69	-	35.29	25.21	-	22.74	6.66
LSD (5%)		-	3.64	2.32	-	3.45	3.92	-	8.62	6.09
SE		-	0.24	0.15	-	0.25	0.24	-	0.47	0.40

LSD: Least significant difference, CV: Critical variance, SE: Standard error

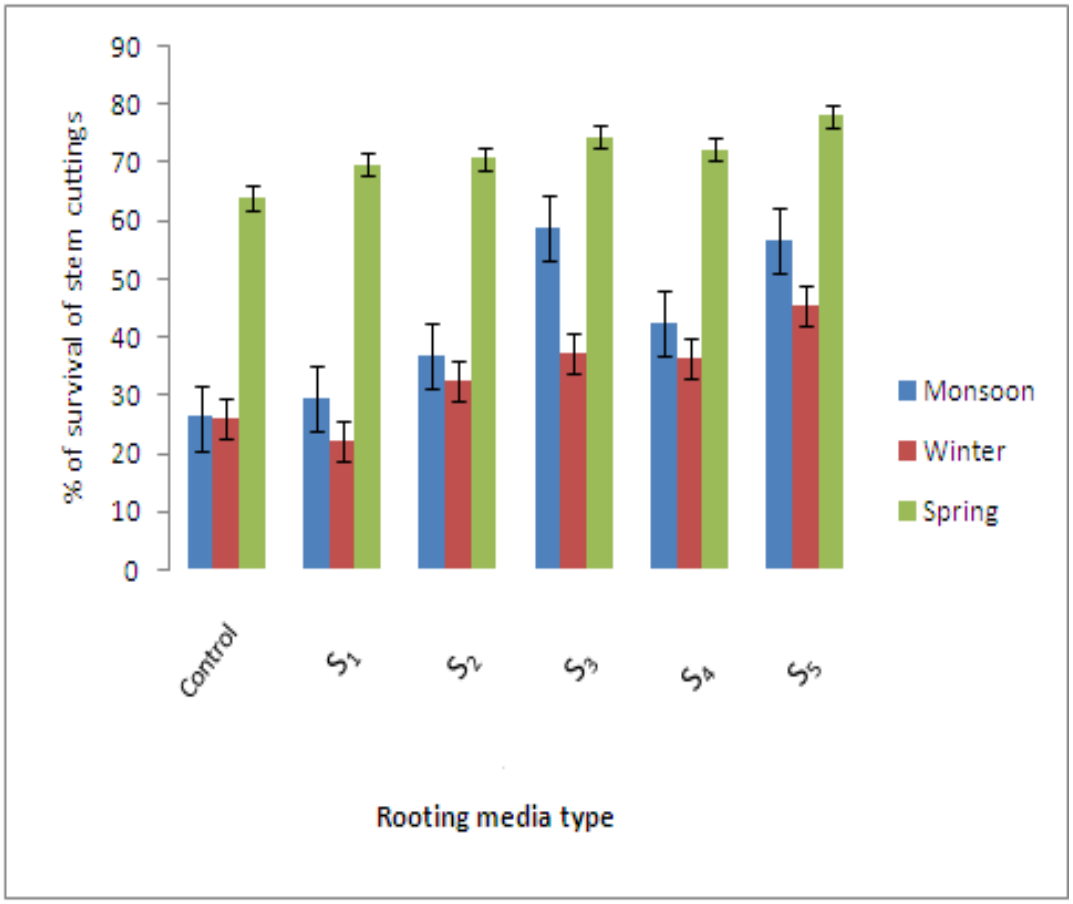

Fig. 6.3 Interactive effect of substrates (media type) and seasonal variation on % of rooting and survival of stem cuttings

The kind of substrate does not show significant difference. However, as compared to the control all other substrates are superior for rooting and survival of stem cuttings of *Jatropha curcas*. Sand: Soil: Vermicompost (1:2:1) showed maximum percentage of survival. It was 78, 56 and 45% in Spring, Monsoon and Winter respectively, followed by S_4, S_3, and S_2 substrates. Minimum was recorded for control followed by S_1. There was no significant difference for rooting and survival of stem cutting between substrate S_2 and S_1. It was 69, 29, 22% for S_1 and 70, 36 and 32% for Spring, Monsoon and Winter respectively (Ref. Table 6.3).

Thus, this experiment has revealed that the substratum and seasons play important role in the survival and sprouting of stem cuttings. The stem cuttings planted in Sand: Soil: Vermicompost (1:2:1) showed 78, 56, and 45% rooting and survival in Spring, Monsoon and Winter seasons, respectively (Ref.Table 6.3 Photo plate 6.3). It has been concluded that Sand: Soil: Vermicompost (1:2:1) is most efficient substrate.

Similarly, 20cm long stem cuttings showed best performance, an IBA planted. It showed that 78 % of rooting with the Fine Sand: Soil: Vermicompost (1:2:1) in spring while, 56 and 45% in monsoon and winter, respectively (Ref Table 6.3). This could be due to the availability of good amount of nutrients in vermicompost and due to seasonal factors. According to Kambooh (1984), organic matter content of the planting medium has a profound effect on its biological, chemical and physical properties. Organic matters provide food and energy to the plants.

6.4 Conclusion

It could be concluded from the present study that for large-scale production of genetically pure and improved planting material of *J. curcas,* vegetative reproduction through 20cm long stem cutting treated 2000mg/l (IBA) is the most effective and economic method in Spring and Monsoon seasons. Dhillon (2009), also recorded a similar observation. Secondly, the present study revealed that, a trend of relatively better survival and rooting of stem cuttings were obtained with Sand: Soil: Vermicompost (1:2:1) as compared to other substrate with 20cm length of cuttings. The observed results could be due to better aeration and high water holding capacity of substrate. The further study should evaluate the field performance of such plant produce by stem cuttings.

Hence, this study implies that, the stem cuttings with above mentioned growth hormone treatment, size of stem cuttings, type of substrate help to develop uniform plant stock in nursery. The results on vegetative propagation of *J. curcas* obtained during present study have been duly published (Gopale and Zunjarrao, 2011)

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7. *In vitro* Propagation

7.1 Introduction

Energy is an important input for development. It aims at human welfare covering household, agriculture, transport and industrial complexes. Like other natural resources, energy sources are also renewable as well as non-renewable. Non-renewable hydrocarbons are being used as major energy sources the world over. Besides, the threat of depleted reserves, excessive use of non-renewable resources can increase greenhouse gases, which are held responsible for global warming and ozone depletion. Biofuels and bioenergy include a wide range of alternative sources of energy of biological origin. These are pollution free, environmentally clean and socially relevant. Many oil producing crops and plants have been considered for the purpose, among these, *Jatropha curcas*, a member of Euphorbiaceae family with several attributes and considerable potential has evoked interest all over the tropics as a potential biofuel plant (Openshaw, 2000).

The use of vegetable oil as biofuel is being considered as a suitable alternative to limited fossil fuel reserves. In this context, non-edible vegetable oil of *Jatropha curcas* has the potential of providing a promising and commercially viable alternative to petro-diesel (Gubitz et al., 1999). Special interest has been shown in the cultivation of *J. curcas* as energy plantations because of its seed oil, which could be easily converted into quality bio-diesel (Ghosh et al., 2007). It contains more oxygen with a higher cetane value increasing the combustion efficiency and it is clean, non-toxic ecofriendly and economic due to its low production cost. It also possesses advantages like easy adaptability to semi-arid marginal lands and non-competitiveness with conventional crop for land (Francis et al., 2005). It is a multipurpose, drought resistant, perennial plant of Latin American origin, but it is now widespread throughout the tropical regions of the world (Openshaw, 2000).

Large-scale cultivation of *J. curcas* remains the single most important issue that will ultimately decide success. Seeds are genetically heterozygous as *Jatropha* is cross-pollinated, which results in a high degree of variation (Ginwal et al., 2005). Low and unpredictable yields were reported from established plantations. Therefore, there is an

urgent need for improvement of this species. Genetic engineering is a powerful tool to aid in the improvement of agronomic traits. Used with classical breeding methods, genetic engineering can accelerate the development of new cultivars with improved traits (Sharma et al., 2007; Lemaux, 2008). Genetic engineering of plants relies on a tissue culture system to regenerate transformants.

Intervention of biotechnological methods to introduce desirable traits in *Jatropha* species is the need of the hour. Various researchers (Srivastava 1974; Srivastava and Johri 1974; Sujatha and Dhingra 1993; Sujatha and Reddy 2000) have reported tissue-culture protocols for endosperm cultures and the rapid propagation of selected genotypes of *Jatropha*. Sujatha and Dhingra (1993), have reported plant-regeneration systems from various explants of *J. integerrima*. Sujatha et al. (2006), developed a method for the differentiation of adventitious shoot buds interspersed with callus from vegetative explants of nontoxic *J. curcas.* Weida et al. (2003), reported callus mediated shoot bud induction from *J. curcas* on Murashige and Skoog's (MS) medium supplemented with 0.5 mg/l 6-benzylaminopurine (BA) and 1.0 mg/l indole-3-butyric acid (IBA). All the above studies reported either callus mediated regeneration or direct shoot morphogenesis with interspersed callus from hypocotyl, leaf, and petioles. Despite sufficient regeneration systems achieved from *Jatropha* leaf explants, the presence of intermediary callus or callus mediated regeneration is least desired for the production of true-to-type plants. However, no report of complete plant regeneration through somatic embryogenesis is available in this species, to date all applied research focus on somatic embryogenesis and it is now considered as the gateway to many more technologies.

Despite the research efforts over the past few years in *J. curcas* tissue culture, no facile protocol of regeneration has been developed so far (Sujatha and Mukta 1996; Wei et al., 2004; Sujatha et al., 2006; Rajore and Batra 2005; Datta et al., 2007; Jha et al., 2007; Deore and Johnson 2008). However, no lab to land transfer protocol of *J. curcas* using leaf disc explants is available. Leaf disc explants is an important source tissue of micro propagation and plants raised from these are comparatively more resistant to genetic variation (Pierik, 1991).

Considering the economical importance of *J. curcas* and critical analysis of earlier reports, to meet the large-scale demand and ensure easy supply of the elite plant material, there is a need to establish mass multiplication techniques. The objective of this study was to develop an *in vitro* plant regeneration method, from leaf disc explants, without intervening callus. Induction of somatic embryogenesis not only helps to obtain a large number of plants but can act as a powerful tool for improvement of any plant species, because of its single cell origin (Bhansali et al., 1991), for subsequent use in genetic transformation. We have investigated *in vitro* adventitious shoot-bud induction from leaf discs of *J. curcas* and induction of somatic embryogenesis. In this process, we have established an efficient protocol for high-frequency direct regeneration of plantlets from leaf discs of *J. curcas.*

7.2 Materials and Methods

7.2.1 Glassware's and Plastic wares: Test tubes (25x150mm), conical flasks (250 ml capacity), bottles with caps (200 ml capacity), pipettes (0.1, 0.2,1,2,5,10 ml capacity) and measuring cylinders (25 ml, 100 ml, 1000 ml capacity) of Borosil, India were used for culturing the tissues and for preparation of media. Autoclavable screw cap bottles (100, 250 and 500 ml) for storing stock solutions were procured from Qualigens, India. Glassware used for histological studies were coupling jar (60ml capacity), slides (Blue Star, India) and cover slips (Micro-Aid, India). Plastic wares including sterile disposable plastic petriplates of 55 and 85 mm diameter were procured from Tarson, India.

7.2.2 Chemicals: Chemicals used for surface sterilization procedures were Bavistin (BASF, India), Savlon (Johnson and Johnson Limited, USA) and Mercuric chloride (Qualigens Fine Chemicals, India). A media additive sterilant, Plant Preservative Mixture (PPM) was procured from Sameer Science Laboratory, India. An antibiotic, Cefotaxime (Alkem, India) was often used for controlling the bacterial growth in plant tissue culture medium. Sucrose was purchased from Hi-Media (India) and Qualigens Fine Chemicals (India). Agar-agar (bacteriological grade), used as gelling agent in the semisolid culture.

Growth regulators including Kinetin (KN), 6-benzyl adenine (BA), N-phenyl-N'-1, 2, 3-thidiazol-5-ylurea (Thidiazuron, TDZ), α-naphthalene acetic acid (NAA) Indole-3-Acetic Acid (IAA), Indole -3-Butyric Acid (IBA) and 2, 4-dichlorophenoxyacetic acid (2, 4-D) were obtained from Sigma (U.S.A.).

Chemicals used for histological studies including formaldehyde solution, glacial acetic acid and xylene were procured from Qualigens Fine Chemicals, India. Ethanol, 2-methyl propan-2-ol (tert butyl alcohol) was from S.D. fine chemicals, India; paraffin wax (m.p.58-60°C) was obtained from (E. Merck, India Ltd.) while Haematoxylin and Eosin stain were procured from Hi-Media Laboratories Pvt. Ltd., Bombay. DPX-4 mountant [189-(2-chloro-N-(4-methoxy-1,3,5-triazin-2-yl amino carbonyl) benzene sulphanamide)] was from BDH, India.

7.2.3 Equipments

The major equipments used include:

I) pH meter: pH is the negative logarithm of hydrogen ion concentration. The measurement of pH in pH meter (Thermo Orion, USA) is based on ion exchange in between hydrated layers formed on glass surface. Change in ion exchange results in emf or voltage difference causing current flow. The current intensity gives the value of pH.

II) Electronic Balance: A manual top loading balance (Contech, India) used for quick weighing and for analytical purposes. This is a single pan balance capacity 100-200gm, sensitivity 0.1mg operating on 230 V 50 H2 AC mains. Precision of ± 0.005g, weighing range 0-1, 200g, and digital read out was used for making stock solutions of growth regulators and for other fine weighing.

III) Autoclave: The horizontal autoclave was used for sterilization of media, glassware, water, dissecting instruments etc. and for decontamination of contaminated cultures in culture vessels. It is based on application of steam under pressure. Autoclaving was carried out at 121°C temperature under 15 lb/inch^2 pressures. Except culture media, all other materials were autoclaved for one hour. The culture media were autoclaved for 20 min.

IV) **Laminar airflow ultra clean unit**: All aseptic manipulations were carried out on this unit. In laminar air flow cabinet (Microfilt, India), with the help of air pump, air is passed through HEPA filters of pore size, 0.22 micron. Due to positive pressure, the entry of any contaminant is restricted from the open side of the bench. The instrument is fitted with UV tubes in addition to the fluorescent tubes.

Apart from these, instruments like Magnetic stirrer (Remi, India), Steamer (Ultradent, India), Temperature controlled oven (Pathak Electricals, India), Light microscope, Microtome, Camera (Nikon), membrane filter sterilizing unit (Laxbro, India) were used.

7.2.4 Preparation of glassware and instruments: Glassware used in our studies was cleaned by boiling in a solution of sodium bicarbonate for 1 hr followed by washing in tap water. These were then immersed in 30% nitric acid solution for 30 min. and washed thoroughly with tap water. After rinsing with double distilled water, these were allowed to dry on a draining rack. Tubes and flasks were plugged with non absorbent cotton. All dissecting instruments were wrapped singly with paper and put in autoclavable plastic bags for sterilization. Ordinary grade filter paper pieces of approximately 10x20 cm were kept in stack alternately with brown paper pieces of similar size. These were packed in autoclavable plastic bags as stack of 20-25 pieces and autoclaved. Dissection and transfer of explants were carried out on these papers under aseptic conditions and disposed after use. Microtips used for aseptic addition by micropipettes were arranged in cases meant for their size, wrapped with brown paper and autoclaved. Sterilization of glassware and instruments was carried out by autoclaving at 121°C for 1 h at 15 lb/inch^2. Stock solutions of the media ingredients were prepared by dissolving weighed amounts of these salts in distilled water. Appropriate aliquots of these solutions were mixed to prepare the media.

7.2.5 Preparation of media: Success of a tissue culture protocol depends on the appropriate composition of the medium. Several basal formulations like MS (Murashige and Skoog, 1962), B5 (Gamborg *et al*., 1968), SH (Schenk and Hildebrandt, 1972), etc. are now available. In the present studies, MS basal media were used. Concentrations of the macro and microelements, salts and organic

constituents of the different media used in the present studies were enlisted in Table 7.1.

Table 7.1 Stock solutions for Murashige and Skoog (MS) (1962) basal medium

Constituent	Concentrations in MS medium (mg/l)	Concentration in the stock solutions (mg/l)	Volume to be taken for One liter of the medium (ml)
Macronutrients (10X)			
NH_4NO_3	1,650	16.5	50
KNO_3	1,900	19.0	
$CaCl_2.2H_2O$	0.440	4.4	
$MgSO_4.6H_2O$	0.370	3.7	
KH_2PO_4	0.170	1.7	
Micronutrients (10X)			
$MnSO_4.4H_2O$	22.3	223.0	50
$ZnSO_4.7H_2O$	8.6	86.0	
KI	0.83	8.3	
$Na_2MoO_4.2H_2O$	0.25	2.5	
$CoCl_2.6H_2O$	0.025	0.25	
$CuSO_4.5H_2O$	0.025	0.25	
Iron source (10X)			
Na_2EDTA	37.3	373.0	10
$FeSo_4.7H_2O$	27.8	278.0	
Vitamins (10X)			
Nicotinic acid	0.5	5.0	10
Pyridoxine HCl	0.5	5.0	
Thiamine HCl	0.1	1.0	
Glycine	2.0	20.0	
Myoinositol	100.0	1000.0	

7.2.6 Growth Regulators: All the growth regulators are not soluble in water. Solubility of different growth regulators is given in the Table 7.2 The compound is dissolved in few ml of solvent and then distilled water is slowly added to make up to the requisite volume. It is preferable to dissolve 10mg/100ml to give a concentration of 0.1mg/ml.

Table 7.2 Solubility of Growth Regulators used in the experiment

Name	Solubility
Indole-3-acetic acid	1N NaOH
Indole-3-butyric acid	1N NaOH
β-Naphthalene acetic acid	1N NaOH
N^6-Benzyladenine	1N NaOH
Kinetin	1N NaOH
Thiadizuron	H_2O
Adenine sulphate	H_2O
Citric acid	H_2O

Stock solutions of growth regulators (GR) were prepared by adding few drops of appropriate solvent in required amount of growth regulator to dissolve. After dissolution, the required concentration was made by the addition of double distilled water and stored in refrigerator in sterilized bottles.

For media preparation, calculated amount of aliquots were added from the stock solutions. Carbohydrate (Sucrose) was weighed and added in required quantity (3%) and allowed to dissolve. The volume was made up with double distilled water. Unless mentioned, pH of all the media was adjusted 5.8 using 0.1N NaOH or 0.1N HCl after mixing all the constituents except the gelling agent. The media were solidified by using either agar-agar (0.8%). In case of charcoal containing medium, 0.2% charcoal was added to the media before autoclaving. The media were then heated in water bath or steamer for the agar to melt. Molten medium was later dispensed into sterile culture tubes (20 ml), flasks (100 ml) or bottles (80 ml) after thorough mixing. Semisolid medium containing agar was used in most of the studies unless otherwise mentioned. All the culture media were autoclaved for 20 min. at 121°C and 15 lb/inch ^{2}. Some

heat labile substances like GA_3 and antibiotic like taxim (cefotaxime) were added aseptically to the autoclaved semisolid medium, before solidifying. In case of charcoal containing medium individual tubes were shaken after autoclaving and before setting of medium for uniform distribution of charcoal.

7.2.7 Preparation of explants and surface sterilization: The seed material of *J. curcas* was obtained from Pune region (Western Maharashtra). The seeds were germinated in the botanical garden of department of Botany of Modern College of Arts, Science and Commerce, Pune- 5. The explants used for the *in vitro* propagation of *J. curcas* were leaf disc collected from 3-4 months old plants.

The explants were washed under running tap water for 15-20 min. They were then cleaned with few drops of detergent (Labolene, Qualigens, India) and washed with distilled water. They were then treated with 10% savlon (chlohexidine gluconate solution I.P. 1.5% v/v and cetrimide I.P. 3% w/v, Johnson and Johnson, India) for 10 min followed by washings with distilled water. Explants were then treated with 1% Bavistin (carbendazim 50%WP, BASF, India) for an hour, on a gyratory shaker followed by rinsing three times with distilled water to remove Bavistin. Hereafter, the tissues were manipulated under aseptic condition in laminar airflow bench. The explants were rinsed with 70% ethanol followed by thorough washing with sterile distilled water. This was followed by 0.05-0.1% mercuric chloride treatment for specified time period depending on the nature of the explant (0.05% for 3-5 min. for leaf disc explants). Adhering mercuric chloride was removed by washing the explants repeatedly with sterile distilled water.

7.2.8 Induction of adventitious shoot buds from leaf disc explants: Very young leaves starting from one to four from nodal tips have been used for excising leaf discs. Leaf discs were prepared using a sterile cork borer approximately 3– 5 mm in diameter and placed with the abaxial side in contact with the medium.

The culture medium for induction of adventitious shoot buds consisted of MS salts with 3% sucrose (w/v) and the explants were cultured in three groups of hormone combinations with different concentrations, which were termed as induction media. The first group consisted of thidiazuron (TDZ), (2.27 and 4.55 µM) and 6-

benzylaminopurine (BA) (2.22 and 4.44 µM) in combination with IBA (0.49 and 0.98 µM). The second group consisted of TDZ (2.27 and 4.55 µM) in combination with IBA (0.49 and 2.46 µM) and the third group consisted of BA (2.22 and 4.44 µM) in combination with IBA (0.49 and 2.46 µM).

7.2.9 Shoot proliferation and elongation of induced shoot buds: Leaf-disc explants were incubated on induction medium for 4–6 weeks. For multiplication and elongation, the induced shoot buds were cultured on MS basal medium containing BA (0.44–8.88 µM), kinetin (Kn) (0.47–4.65 µM), indole-3-acetic acid (IAA) (0.29–5.71 µM) and gibberellic acid (GA_3) (0.14–1.30 µM) in combination for shoot proliferation (proliferation medium). Shoots were individually separated and the number of shoots per explant was recorded after 6 weeks. Individual shoots were further transferred on MS medium supplemented with different concentrations and combinations of plant growth regulators (PGRs), for elongation.

7.2.10 Rooting and acclimatization: Green and healthy elongated shoots with 3–4 leaves were excised and cultured on half strength MS medium supplemented with different concentrations and combinations of auxins i.e., IBA, IAA, and NAA for 8 days. These auxin treated elongated shoots were transferred to growth regulator-free half strength MS solid medium supplemented with 0.25 mg/l activated charcoal. The percentage of shoots with root induction was recorded after 6 weeks. Rooted shoots were carefully taken out of the medium and washed thoroughly in sterilized distilled water to remove medium attached to the roots. The plants were treated with 0.02% w/v carbendazim and then transferred to plastic bags containing sterilized cocopeat, sand and soil in the ratio of 1:1:1 and wetted with and covered with transparent plastic bags to maintain humidity, Partially controlled environment was provided, during hardening procedures. After 3–4 weeks, the established plants were transplanted to polybags containing garden soil and farmyard manure for further growth, for 3-4 weeks. Then plantlets were transferred to a shade net for further acclimatization. The percentage of surviving plants was recorded after 6–8 weeks.

7.2.11 Induction of embryogenic callus: The leaves were cut into small pieces and cultured with the lower surface in contact with MS (Murashige and Skoog, 1962) basal medium with 3% sucrose (w/v) (Merck, India) containing 2.3–13.9 µM Kn and

2.2–13.3 µM BA individually as well as in combination (Ref. Table 7.6) for 6 weeks. The pH of all media was adjusted to 5.8 before adding 0.8% agar (Qualigens, India) and prior to autoclaving. The medium was autoclaved at 121°C and 15 lb/ $inch^2$ for 2o min. The leaf segments placed in culture tubes (150 · 25 mm) plugged with non-absorbent cotton plugs contained 20 ml of medium each, all the cultures were incubated.

7.2.12 Induction of somatic embryos on embryogenic callus: The 4-week-old embryogenic callus cultures obtained on a concentration of 9.3 µM Kn were transferred from initiation medium [MS basal medium +3% sucrose (w/v) +9.3 µM Kn] to MS basal medium supplemented with different concentrations of 2.3–4.6 µM Kn and 0.5–4.9 µM IBA (Ref. Table 7.7). After another 6 weeks of incubation, the embryogenic calli induced globular somatic embryos in embryo tissue proliferation medium [MS basal medium +3% sucrose (w/v) + 2.3 µM Kn + 1.0 µM IBA]. The eight week-old embryogenic calli with globular somatic embryos were subcultures onto the same media for 2 subsequent weeks. Light and temperature conditions were the same as mentioned below in 7.2.14. After 10 weeks of culture, the globular somatic embryos that had developed were transferred to somatic embryo maturation medium [MS basal medium + 3% sucrose (w/v) 2.3 µM Kn + 1.0 µM IBA + 13.6 µM adenine sulphate] for 6 weeks. Different concentrations of 5.4–54.3 µM adenine sulphate were tested on somatic embryo maturation.

7.2.13 Culture conditions and data analysis: Uniform culture conditions were applied in all experiments. The pH of the medium was adjusted to 5.8 using 0.1 N NaOH or 0.1N HCl, prior to autoclaving at pressure at 15lb/$inch^2$121°C for 20 min. The cultures were maintained at 25 ± 2°C temperature under a 16-h photoperiod with light intensity 1000 to 3000 Lux (cool white fluorescent tubes).

All the experiments were set up in completely randomized design (CRD) and repeated three times with 25 replicates per treatment and one explant was cultured per test tube. Statistical difference among the means was analyzed by Duncan's multiple range test and the results were expressed as the mean ± SE of three independent experiments. Data were also subjected to analysis of variance (ANOVA).

7.2.14 Histological Techniques: Sections were prepared for histological studies following the methods described by Sharma and Sharma (1990). The tissues were cut into small pieces (approx 3 x 4 mm) and fixed in FAA (formaldehyde: glacial acetic acid: alcohol, 5:5:90, v/v) for 48 h at room temperature followed by washing and stored in 70% ethanol till further use. Tissues were dehydrated using graded concentrations of tertiary butyl alcohol and embedded in paraffin wax (MP 58-60°C). Serial sections of 10 µm were cut using a rotary microtome. Sections were double stained with 1% Haematoxylin, 1% eosin and mounted with DPX (Qualigens, India) for studies under microscope.

Preparation of Haematoxylene: 1g Haematoxylene is dissolved in 10 ml of 96% alcohol and the volume is made up to 100 ml by distilled water. The stain is then filtered and allowed to ripen for 6-8 weeks.

Preparation of Eosin: 1 g of eosin is dissolved in 100 ml of alcohol, filtered and stored.

7.2.15 Microscopy and Photography: The morphogenic response in various explants was evaluated under stereomicroscope and was photographed. Histological slides were studied under microscope and photographed. Magnifications of the photomicrographs were noted.

7.3 Result and Discussion

7.3.1 Effect of cytokinin on induction of adventitious buds: Leaf discs placed on the induction medium enlarged and exhibited varied response. The leaf discs increased in size and adventitious shoot-buds originated from cut ends exposed to the medium. Regeneration of adventitious buds was observed in leaf discs cultured on all three groups of culture media (Ref. Photo plate 7.1, a). However, the frequency of leaves showing adventitious shoot-bud induction varied with the presence of both the cytokinins. TDZ and BA in combination with IBA were best suited for maximum adventitious shoot-bud induction. TDZ at 2.27µM concentration induced adventitious shoot buds in 55% leaf disc explants whereas at 4.55µM, the shoot-bud induction was only 35% (Ref. Table 7.3).

Table 7.3 Effect of different combinations of plant growth regulators on adventitious shoot buds induction from cultured leaf explants of *Jatropha curcas* L.

Plant growth regulators (µM)			**Response of leaf disc explant (%)**		
TDZ	BA	IBA	Callus Formation	Bud induction	Nonresponsive
2.27	2.22	0.49	38±2.0	55.5±4.0	6.5
4.55	4.44	0.98	54±3.5	35±3.5	11
2.27	-	0.49	58.5±3.0	22.5±2.5	19
4.55	-	2.46	53±2.5	30.5±3.0	16.5
-	2.22	0.49	55±3.5	15±2.5	30
-	4.44	2.46	61.5±4.5	18±2.0	20.5

Data scored after 6 weeks of culture inoculation, each treatment was replicated three times and each replicate consisted of 10-15 explants, Values represent the mean ± SE

The shoot bud induction capacity was reduced in the absence of BA. TDZ and IBA in the absence of BA had less effect on shoot-bud induction. TDZ (2.27µM) in combination with IBA (0.49µM) had induced shoot buds in 22 %, whereas TDZ (4.55µM) in combination with IBA (2.46µM) induced shoot buds in 30 % of cultured leaf discs, respectively. Similarly, BA in the absence of TDZ promoted callus induction rather than shoot-bud induction. BA (2.22µM) in combination with IBA (0.49µM) induced callus in 55% and shoot buds in 15% of cultured leaf discs, whereas BA (4.44µM) in combination with IBA (2.46µM) induced callus in 61.5% and shoot buds in 18% of cultured leaf discs, respectively. Shoot-bud induction capacity was drastically reduced in the absence of TDZ. These results suggest that TDZ and BA together had a synergistic effect in adventitious shoot-bud induction in *J. curcas.* The induced shoot buds were later transferred to the multiplication medium for further growth and multiplication.

7.3.2 Effect of cytokinin on shoot proliferation and elongation of induced shoot buds: To optimize type and concentration of plant-growth regulators to achieve the highest multiplication rate, well developed shoots were transferred to the MS medium containing BA, Kn, IAA and GA_3 in combination. A wide range of concentrations of BA and Kn, used singly or in combination, did not show a considerable increase in

number of shoots. However, BA, Kn, IAA and GA_3, together recorded the highest number of shoots (11.6 shoots) after 12 weeks of culture (Ref. Table 7.4).

Table 7.4 Effect of different concentration and combination of plant growth regulators (PGRs) on multiplication of *J. curcas* L. shoots

Plant growth regulators concentration (µM)				**Number of shoots per explants**		
BAP	**Kn**	**IAA**	**GA_3**	After 4 weeks	After 8 weeks	After 12 weeks
8.88	4.65	5.71	2.89	2.25±0.6	5.20±1.2	8.60±1.6
6.66	2.33	2.86	1.45	3.10±0.8	5.60±1.0	11.20±2.8
4.44	2.33	1.43	0.72	2.85±0.4	5.55±0.9	11.60±2.6
2.22	2.33	0.69	0.35	2.45±0.5	4.75±0.8	9.40±2.1
1.11	1.16	0.29	0.14	2.20±0.4	4.10±0.7	9.00±2.3
0.44	0.47	0.29	0.14	2.00±0.2	3.70±0.9	8.10±1.8

Data scored after 6 weeks of culture inoculation, each treatment was replicated three times and each replicate consisted of 10-15 explants, Values represent the mean ± SE

The morphogenic competence for shoot regeneration was highest with 4.44µM BA, 2.33µM Kn in combination with 1.43µM IAA and 0.72µM GA_3 (Ref. Plate 7.1, b, c). Higher concentrations of BA (4.44–8.88µM), Kn (2.33–4.65µM), IAA (1.43–5.71µM) and GA_3 (0.72–2.89µM) recorded the maximum number of shoot clumps (8.6–11.6) after 12 weeks in culture with subculturing at 6 weeks interval. Proliferation and elongation of shoot buds could be achieved due to higher cytokinin/auxin ratio along with addition of GA_3 in the media.

7.3.3 Rooting and acclimatization: Percentage of rooting significantly differed depending upon the concentrations and combinations of IBA, IAA and NAA. Rooting increased with the increase in concentration of IBA and inclusion of IAA and NAA further increased the percentage of rooting (Ref. Table 7.5). Shoots of 2–3 cm were separated individually and transferred to the root-induction medium containing full strength MS basal medium. The best rooting percentage (72.9%) was observed when elongated shoots were incubated with half strength MS medium containing 15 µM

IBA, 5.7 µM IAA and 5.5 µM NAA for 8 days, followed by transfer to growth regulator-free half strength MS semi solid medium supplemented with 0.25 mg/l activated charcoal. After 6–8 weeks, more than 80% of plants survived. No morphological abnormalities were observed in regenerated plants (Ref. Plate 7.1, f).

Table 7.5 The percentage of shoot with root induction on various concentrations and combinations of auxins in half strength MS medium of *J. curcas* L.

Auxins (µM)			**Shoots with root induction (%)**
IBA	**IAA**	**NAA**	
5	-	-	30.4± 2.3
10	-	-	32.7±2.1
15	-	-	44.6±2.4
5	5.7	5.5	41.7±3.1
10	5.7	5.5	59.3±2.9
15	5.7	5.5	41.9±2.7
15	11.4	5.5	72.9±4.0
15	17.2	5.5	68.7±5.1
15	5.7	11.0	58.8±5.4
15	5.7	16.5	61.7±4.5

Data scored after 6 weeks of culture inoculation, each treatment was replicated three times and each replicate consisted of 10-15 explants, Values represent the mean ± SE

The well-developed plantlets were washed with sterile water to remove traces of agar from the roots and dipped in 0.1% (w/v) of a broad-spectrum fungicide solution (Bavistin, BASF, India) for 10 min and transferred to the potting mixture containing 1:1:1 ratio of sand:cocopeat:garden soil in 3 inch diameter pots (Hardening tray). Primary hardening took place in 3–4 weeks under high-humidity conditions created by covering pots with polythene bags. The well-acclimatized plants were further transferred to the field with more than 80% survival rate (Ref.photo plate 7.1, g).

Plant regeneration through the leaf disc is a highly practiced method in tissue cultures (Landi and Mezzetti 2006) and gene-transfer techniques (Tsugawa et al., 2004). Krikorian (1982), observed that juvenile plant tissues and organs, especially seedling parts, are highly responsive compared with mature, differentiated tissues. In case of *J.*

curcas, very young leaf explants from 2 to 4 month-old germinated seedlings exhibited great tendency for direct regeneration without the intervening callus growth.

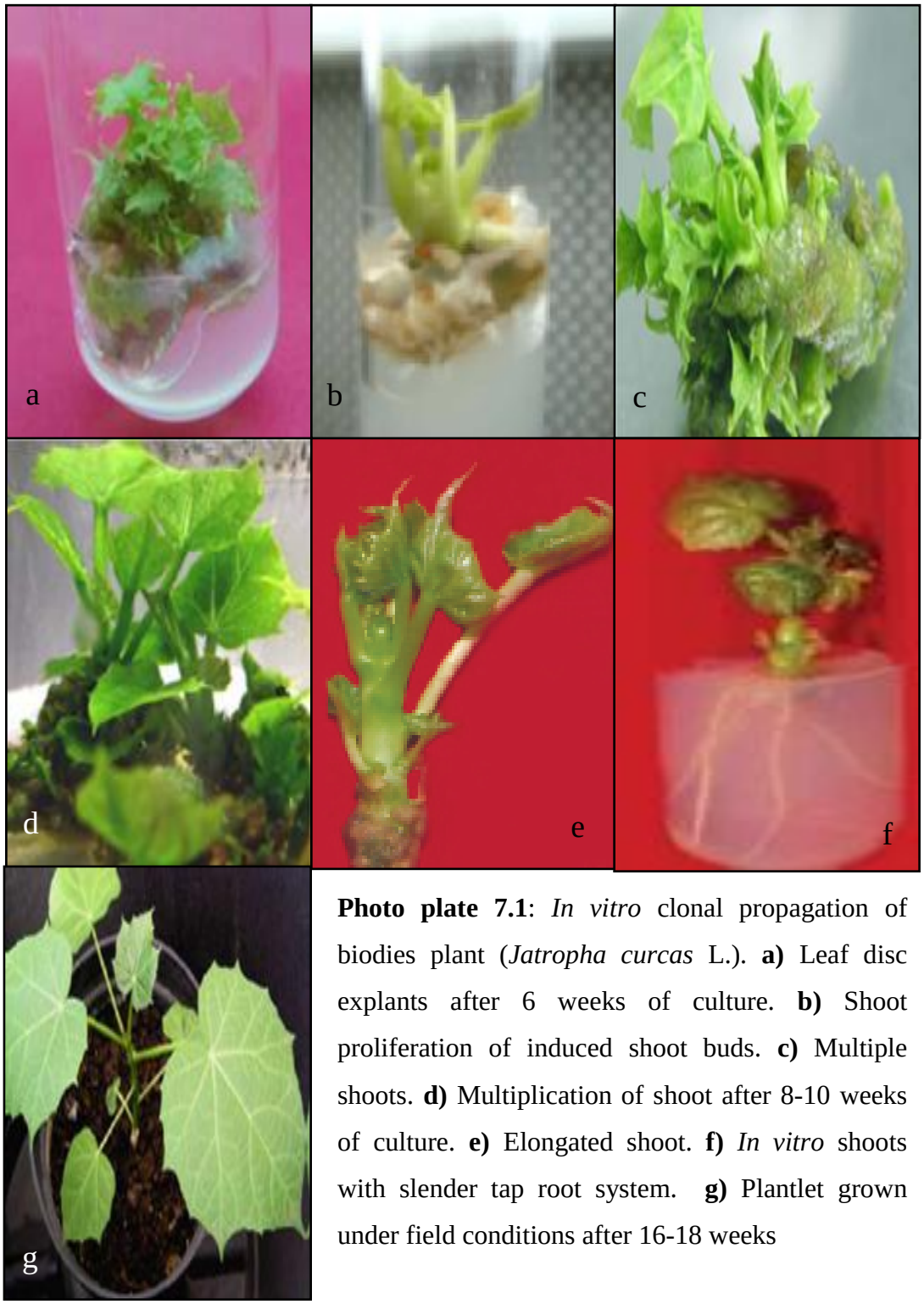


Photo plate 7.1: *In vitro* clonal propagation of biodies plant (*Jatropha curcas* L.). **a)** Leaf disc explants after 6 weeks of culture. **b)** Shoot proliferation of induced shoot buds. **c)** Multiple shoots. **d)** Multiplication of shoot after 8-10 weeks of culture. **e)** Elongated shoot. **f)** *In vitro* shoots with slender tap root system. **g)** Plantlet grown under field conditions after 16-18 weeks

Plant growth regulators such as TDZ and BA alone or in combination with auxins such as IBA (at 0.50–2.46 µM) have been tested for their ability to induce adventitious shoot buds. It was observed that these plant growth regulators exhibited greater variation in shoot bud induction. In our investigation, TDZ and BA in combination with IBA had a more pronounced effect on shoot-bud induction (Ref. Photo plate 7.1a, b).

Weida et al. (2003), reported callus-mediated regeneration of plantlets from hypocotyls, petioles and leaf explants of *J. curcas* on medium supplemented with BA and IBA. Sujatha and Mukta (1996), found that among various *Jatropha* species, *J. integerrima* was most responsive and that regeneration occurred through organogenesis in seedlings and mature explants including leaves, petioles and pedicels. However, they observed intermediary callus growth in leaf explants. TDZ is able to induce diverse morphogenic responses ranging from tissue proliferation to adventitious shoots and somatic embryo formation.

The ability of TDZ to induce high-shoot regeneration efficiency in woody plant tissues has been reported (Huetteman and Preece 1993; Meng et al., 2004). In strawberry leaf tissues, TDZ in combination with IBA induced high-frequency shoot induction (Landi and Mezzetti 2006). Our results further support the role of TDZ on high frequency shoot-bud induction from *J. curcas* leaf discs. TDZ is shown to play an important role in cultures with cytokinin-like activity. Apart from cytokinin like activity, TDZ has been suggested to be a modulator of the endogenous auxin levels. There is experimental evidence that, TDZ stimulates de novo synthesis of auxins by increasing the levels of IAA and its precursor, tryptophan (Murthy et al., 1995). Increases in endogenous auxin, cytokinin and ethylene have been seen in response to TDZ treatment (Murthy et al., 1995).

As a result, TDZ has been shown to be useful for rapid plant regeneration in several recalcitrant species through organogenesis (Malik and Saxena 1992). Similarly, in our studies, it was observed that TDZ is essential for the high frequency induction of multiple adventitious shoot buds from leaf disc cultures. The availability of highly efficient regeneration protocol using leaf discs without the intervening callus phase is highly desirable for *Agrobacterium tumefaciens*-mediated genetic transformation.

Therefore, the high frequency and efficient plant regeneration protocol standerdized in the present investigation could be useful for mass production of true-to-type plants and the production of transgenic plants through *Agrobacterium* biolistic-mediated transformation.

7.3.4 Induction of embryogeneic callus and somatic embryo: Somatic embryogenesis has been reported in some of the species of the family Euphorbiaceae as in *Hevea sp*. (Michaux-Ferriere et al., 1992) and *Cassava sp.* (Raemakers et al., 2000), but not so prominent in any of the species of *Jatropha*. The present investigation is a well-documented study of somatic embryogenesis in *Jatropha curcas*. The type and concentrations of the plant growth regulators were the strong determining factors for somatic embryogenesis in *J. curcas.* Leaf pieces were used as the primary explant. Leaf sections cultured on MS basal medium supplemented with various concentrations of cytokinin started swelling after 8-10 days. Initiation of callus was noted on the cut surfaces of the leaf sections after 2 weeks of culture. Leaf explants cultured on MS medium supplemented with 9.3 µM Kn showed significantly ($P < 0.05$) higher induction of callus (52%) in comparison to other concentrations of cytokinins studied (Ref. Table 7.6).

The initiation medium showed the development of nodular, creamish, embryogenic calli within 4 weeks of culture (Ref. Photo plate 7.2, a). Subsequent transfer of the embryogenic calli in MS medium with different lowered concentrations of Kn with IBA showed varied results (Ref. Table 7.7). The highest frequency (60%) of globular somatic embryos (48.5 ± 13.2) of callus was recorded in the combination of 2.3 µM Kn and 1.0 µM IBA after 6 weeks of culture (Ref. Photo plate 7.2, b). The role of cytokinins and auxins in the different stages of somatic embryogenesis is well-established (Fujimara and Komamine 1980; Lo Schiavo et al., 1989; Litz and Gray 1995). The important is to finding out the triggering combination and concentrations of plant growth regulators besides other factors that vary from cell to cell even within a particular type of tissue of a plant species. Earlier studies have reported that the presence of an auxin promotes the completion of the globular stage during embryogenesis (Lo Schiavo et al. 1989; Litz and Gray 1995).

Table 7.6 The influence of cytokinins on embryonic callus induction frequency from leaf explants of *Jatropha curcas* L.

Concentration of cytokinin (µM)		Callus induction (%)	Morphology of callus
Kinetin	**BA**		
2.3	0	30.2±4.6	Soft friable, light yellow
4.6	0	39.7±7.9	Soft friable, light yellow
9.3	0	52.1±9.2	Nodular, creamish, embryogenic
13.9	0	36.4±7.5	Compact, greenish brown
0	2.2	33.0±5.2	Soft friable, light green
0	4.4	38.5±8.2	Soft friable, light yellow
0	8.9	42.8±8.6	Compact, light yellow
0	13.3	35.3±6.4	Compact, light brown
2.3	2.2	20.1±3.9	Soft friable, light brown
4.6	4.4	11.2±2.4	Compact white
9.3	8.9	12.8±2.7	Soft friable, light green
13.9	13.3	10.6±2.3	Soft friable, white

Data scored after 6 weeks of culture inoculation, each treatment was replicated three times and each replicate consisted of 10-15 explants, Values represent the mean ± SE

In present study, IBA promoted the completion of the globular stage of the embryos. The combined favorable influence of auxin and cytokinins observed in the present system is in accordance with the culture response of somatic embryogenesis in *Coffea arabica* (Neuenschwander and Baumann, 1992). Clusters of globular somatic embryos were visible during the first 4-5 weeks in embryo tissue proliferation medium. Highly organized, round, creamish globular somatic embryos differentiated on the edges of the callus by the fourth week of culture. It was also found to be surrounded in the embryogenic callus tissue, while other areas of the calli remained white and translucent. Globular somatic embryos on subculture to the same embryo tissue proliferation medium were found to gradually convert into heart-shaped,

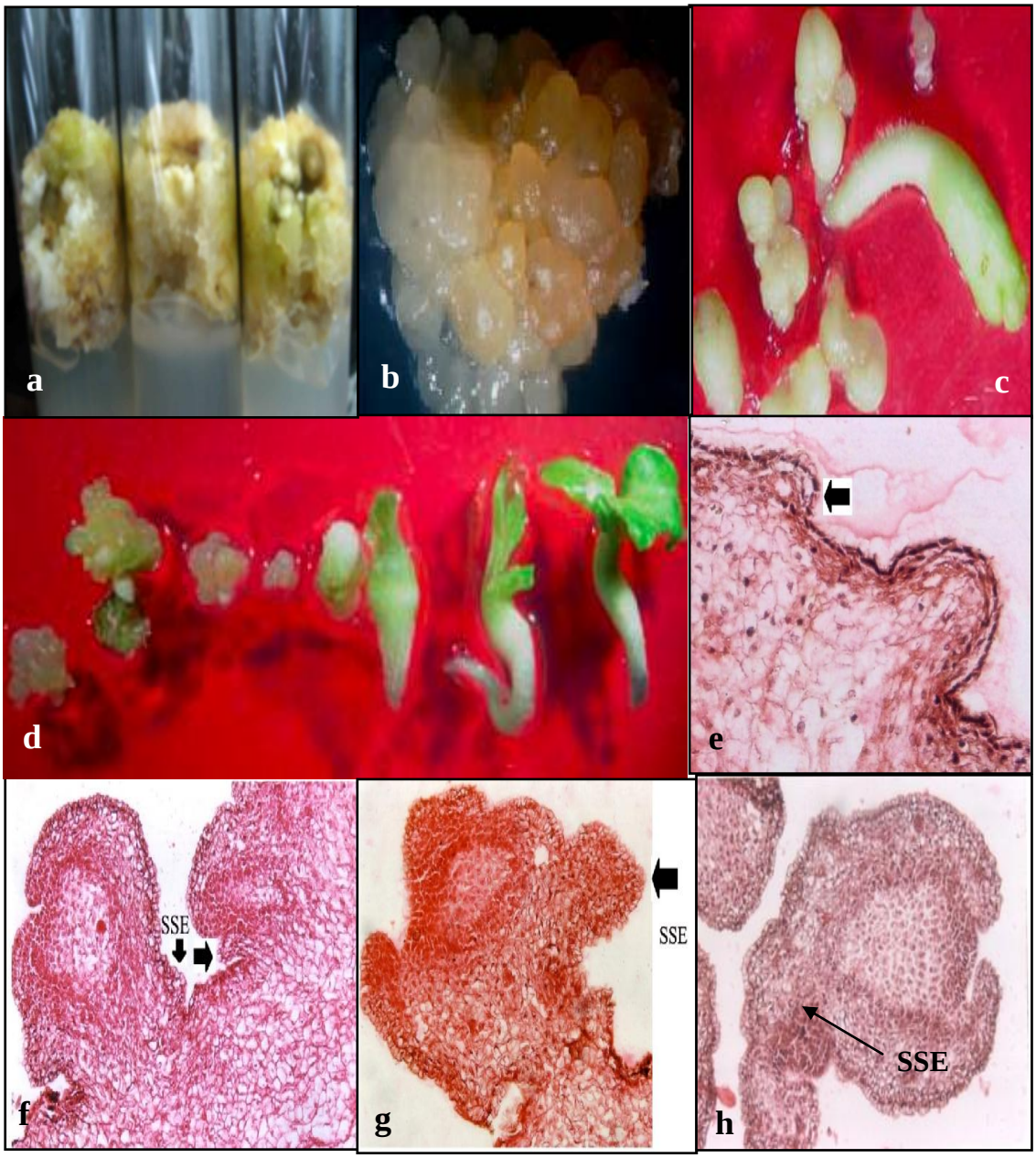
torpedo and cotyledonary stages embryos with distinct bipolarity with globular stage embryos dominating in culture (Ref. Photo plate 7.2, d).

Table 7.7 Percentage of somatic embryogenesis from embryogenic callus

Concentration of growth regulators with 13.6 µM adenine sulphate		**Percentage of embryogenesis (%)**	**No. of Somatic embryos**
Kinetin (µM)	**IBA (µM)**		
2.3	0.5	52	30.2±10.2
	1.0	60	48.4±13.2
	1.5	47	22.8±7.6
	4.9	38	27.3±8.5
3.5	0.5	45	20.9±7.1
	1.0	30	11.2±7.4
	1.5	36	10.8±6.2
	4.9	31	13.2±6.6
4.6	0.5	42	9.4±3.4
	1.0	33	2.3±1.3
	1.5	29	2.0±1.2
	4.9	21	2.1±1.2

Data scored after 6 weeks of culture inoculation, each treatment was replicated three times and each replicate consisted of 10-15 explants, Values represent the mean ± SE

Asynchronous development of different stages of somatic embryos within a culture indicates the complexity of the mechanism of somatic embryogenesis. Our study required a minimum time of 4–6 weeks for conversion of globular somatic embryos to cotylydonary stage. Addition of 13.6 µM adenine sulphate along with 2.32 µM Kn and 1.0 µM IBA led to the significantly ($P < 0.05$) highest mean number of somatic embryos (48.4 ± 13.2) after 6 weeks of culture, which served the best concentration and combination of PGRs (Ref. Table 7.7 and Photo plate 7.2, c). Adenine sulphate is known to enhance the efficiency of maturation of somatic embryos, which is a critical



(SSE = Secondary Somatic Embryo)

Photo plate 7.2 Somatic ebmryogenesis and Histology of Primary and Secondary somatic embryogenesis in *Jatropha curcas* L. **a)** Induction of callus from leaf explants. **b)** embryogenic callus after 6 week of culture. **c)** Development of cotylydonary stage of somatic embryo showing distinct bipolarity. **d)** Different developmental stages showing globular to cotyledonary stage of somatic embryogenesis. **e)** Late heart shaped primary somatic embryo with initiation of secondary somatic embryos. **f)** and **g)** Primary somatic embryos showing production of secondary somatic embryo from its epidermal layer. **h)** Secondary somatic embryo with suspensor.

step in somatic embryogenesis.Earlier reports by many authors (Das et al., 1993; Martin, 2003) also support the role of adenine sulphate in somatic embryo maturation.

To confirm the origin of embryo induction, histological studies were conducted. The initiation of direct somatic embryos was observed from the subepidermal layer of explant without an intervening callus phase (Ref. Photo plate 7.2, e). Differentiated meristematic pockets were seen at the periphery of explants that further developed into embryos. During further growth, the epidermal layer of primary somatic embryos became meristematic and produced secondary somatic embryos (Ref. Photo plate 7.2, f, g, h).

Induction of secondary somatic embryos was seen from the entire surface of primary somatic embryos. However, mostly these secondary somatic embryos were observed from the base of primary somatic embryos, especially near the suspensor (Ref. Photo plate 7.2, h). Well-differentiated cotyledonary stage primary embryos can be seen still attached to an explant at the time of secondary somatic embryo induction. These primary embryos later got detached from the mother tissue and a well developed germinating embryo with shoot-root pole along with suspensor was seen.

An interesting observation was the induction of a low frequency of secondary embryogenesis along the shoot and root poles of well-developed primary embryos grown in the medium (Ref, Photo plate 7.2, h). The synergistic combination of reduced Kn, IBA and adenine sulphate not only promoted the growth of shoot and root, but also secondary embryo formation. In present study, successful proliferation of secondary embryos was observed in some of the primary somatic embryo. Somatic embryogenic cells can act independently from neighboring cells and undergo somatic embryogenesis or they can continue to differentiate into secondary embryogenesis (Raemakers et al., 1995).

7.4 Conclusion

During present study it has been revealed that, leaf disc was the best explants for micro propagation through direct induction of adventitious shoots when cultured on Murashige and Skoog's (MS) medium supplemented with thidiazuron (TDZ) (2.27

µM), 6-benzylaminopurine (BA) (2.22 µM) and indole-3-butyric acid (IBA) (0.49 µM). The presence of TDZ in the induction medium has greater influence on the induction of adventitious shoot buds, whereas BA in the absence of TDZ promoted callus induction rather than shoot buds. Induced shoot buds were multiplied and elongated into shoots following transfer to the MS medium supplemented with BA (4.44 µM), kinetin (Kn) (2.33 µM), indole-3-acetic acid (IAA) (1.43 lµ) and gibberellic acid (GA_3) (0.72 µM). Well-developed shoots were rooted on half strength MS medium supplemented with IBA (0.5 µM) after 30 days. Regenerated plants after 2 months of acclimatization were successfully transferred to the field without visible morphological variation. This protocol might find use in mass production of true-to-type plants and in production of transgenic plants through *Agrobacterium*/ biolistic-mediated transformation.

Embryogenic callus were also obtained from leaf explants on MS basal medium. It was supplemented with only 9.3 µM Kn. Induction of globular somatic embryos 48% from of the cultures was achieved on MS medium with different concentrations of 2.3–4.6 µM Kn and 0.5–4.9 µM IBA; 2.3 µM Kn and 1.0 µM IBA proved to be the most effective combination for somatic embryo induction in *Jatropha curcas*. Addition of 13.6 µM adenine sulphate stimulated the process of development of somatic embryos. This protocol of somatic embryogenesis in *Jatropha curcas* may be used as an ideal system for future transgenic research. However, asynchronous development of somatic embryo is major limitation to use this protocol for mass multiplication, further research in this direction is required.

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8. Summary and Conclusions

Bio-diesel is fast developing alternative fuel in many developed and developing countries of the world. The bio-diesel production from vegetable oils during 2007-2008 was estimated about 2.36 billion tones globally, of these European countries accounted for about 82% and USA about 6% (Divakar et al., 2009). Global bio-diesel production is set to reach some 24 billion liters by 2017. Shortage of edible oil for human consumption in developing countries does not favor its use for bio-diesel production. Hence, over the last decade the plant species *Jataropha curcas* L. has generated the interest of many workers in the field of bioenergy. Many superior characteristics, including high yield ability, high oil content, resistant to drought and good quality of the plant oil have been attributed to this plant. However, the exact expression of these characteristics is still not well understood or researched. Preliminary findings show some of these to be true, others are exaggerated, while most will be valid only under specific conditions, which are often not indicated clearly.

The study was aimed to gain insight in the most elementary aspects of this species, before recommendation to the farmer or organizations for plantation in particular area of Maharashtra. Most elementary aspects include morphological diversity, which include agro-climatic conditions and its impact on the characteristics of this plant. Biochemical diversity comprises variation in oil content, phorbol ester (toxic compound), fatty acid composition, etc., and molecular diversity. Present study has also been focused on propagation of this plant through *in vivo* and *in vitro* system.

The present study provided most recent and useful insights on the implications for investments on *Jatropha* in Maharashtra. It also provides the most recent and realistic information to researchers, farmers and organizations who intend to set up large-scale or small scale plantation sites in Maharashtra. Much information has been collected during the research period (2007-2011) about *Jatropha*, towards learning to distinguish between ‘reality’ and ‘promise’.

Data have been presented on Morphological diversity, Biochemical diversity, Molecular diversity and Propagation systems. A reflection is also given about long term sustainability.

8.1 Morphological Diversity

A systematic collection of *Jatropha curcas* germplasm has been carried out from four distinct agro-climatic regions of Maharashtra viz, Kokan, Marathwada, Western Maharashtra and Vidarbha. The present study involved the impact of agro-climatic parameters on variability with respect to seed morphology, seed germination and seedling growth. *J. curcas* is found in Maharashtra mostly in small discrete populations which are basic resources for future improvement and breeding programmes. The results of the study will be valuable for seed zone delineations, strategies for conservation of genetic variation, prospects of improvement and assessment of the potential of locally adapted seed source.

Weight of the whole seeds and its components showed maximum weight in Marathwada sources while it was significantly low for Kokan sources. The coefficient of variance (CV) for whole seeds, seed coat and kernel were recorded 12.67, 7.05 and 10.25% respectively. Seed size, i.e. length, width and thickness, has also shown a similar trend. Maximum seed size was recorded for Marahtwada source and minimum for Western Maharashtra source. The CV of these traits was found to be 1.76, 1.00 and 2.67% respectively. Variation in seed viability, germination percentage, germination energy was found significant among different seed sources with CV of 8.49, 12.54 and 10.31 % respectively. Maximum values for these parameters were recorded for Marathwada seed sources while minimum for Kokan sources. The highest phenotypic variance was found for germination energy, while lowest for below ground biomass. The ‘genetic variance’ varied from 116.66 to 0.039 for germination energy and below ground biomass. In ‘environmental variance’, the highest value of germination was 16.52 for PCV whereas it was 16.24 for GCV.

The patterns of variation exhibited for various characters were substantially different. The presence of such difference among populations has probably produced by different intensities of natural selection acting upon these traits in their natural habitat.

Some of the variation found associated with the discrete populations from which seeds were collected. The Kokan seed sources in the present study were ranked low, in comparison to the growth producing sources i.e. Marathwada and Vidarbha. It is indicative that there is a better choice of using selected promising genotypes and population from the range of distribution. The seed sources in most of the cases were significantly different growth variables and showed a considerable amount of genetic variability within the distribution range indicating a good scope of genetic gain through selection.

The present study showed that there exists considerable amount of genetic variability in this species in Maharashtra with respect to growth performance, which offers scope to breeder for selection and breeding. It is advisable that these seed sources should be used for collection of bulk quantity of seeds to achieve better productivity. It is quite clear that Marathwada source is good in growth performance, particularly in the prevailing conditions in Maharashtra. The Western Maharashtra and Vidarbha sources also performed satisfactory in respect of growth parameters.

The work further gives a direction to effect and practice studies for genetic improvement of these species. Perhaps, this is a first attempt and report, which surveys and assesses morphological diversity of *J. curcas* population from four agro-climatic regions of Maharashtra. The results on morphological diversity and its genetic correlation of *J. curcas* obtained during present study have been duly published (Gopale and Zunjarrao, 2011)

8.3 Biochemical Diversity

The *Jatropha* oil contains Palmitic, Stearic, Oleic and Linolenic acids in good proportion. Although there is a growing demand for quality planting material of *Jatropha*, efforts are lacking for the selection of elite planting material based on their biochemical composition. In the biochemical study, twenty *Jatropha* seed samples were collected from four agro-climatic regions of Maharashtra state of India. Seed protein, oil and phorbol ester contents revealed variation with accessions from said four regions. Presence of the toxic phorbol esters is a major concern and analysis of twenty accessions resulted in identification of high and low phorbol ester contents.

The overall oil percentage in the said agro-climatic regions varied from 26 to 37%. Marathwada, Vidarbha and Western Maharashtra regions showed 34.37, 34.78 and 34.25% respectively, while Kokan region was showed 27.22%. The oil samples were further analyzed by GLC to study the variability of fatty acid composition. Significant variability of fatty acid was also observed. The Palmitic acid ranged from 14 to 36 %, Stearic acid from 4 to 15%, Oleic acid from 38 to 58% and Linolenic acid from 11 to 43%. Rich diversity among the ecotype of Maharashtra, in terms of oil percentage, Phorbol ester content and fatty acids profile, indicates the need for exploitation of germplasm in breeding programme.

There was no significant variation at inter population level of Phorbol ester in four agro-climatic regions of Maharashtra. While, at intra population level significant variations were found, which vary from 0.66 to 4.40mg/g. It indicates the synthesis of Phorbol ester was under the strong influence of genetic control. In the present study it was also observed that, there is linear relationship between protein and oil content. Significant variability in fatty acids was observed. This overall variability can be exploited for selection of elite plant of *J. curcas* and studying the genetic variability in it. It can be concluded that, out of four agro-climatic regions studied, three regions viz., Vidarbha, Western Maharashtra and Marathwada are found to be more suitable for the plantation of *J. curcas*. The results of Biochemical diversity within the four agro-climatic regions of Maharashtra has been duly published (Gopale and Zunjarrao, 2011).

8.4 Molecular Diversity

Random amplified polymorphic DNA (RAPD) markers were used to evaluate the genetic diversity in representative population of *Jatropha curcas L*. from four agro-climatic regions of Maharashtra. Each region was uniformly represented by 5 accessions. Ten selected markers have been used for amplification. A total of 125 DNA bands were obtained, of which 94 (74.62%) were polymorphic. The polymorphism was scored and used in band sharing analysis to identify genetic relationship. Cluster analysis based on Jaccard's similarity coefficient using UPGMA grouped all the 20 genotype in to two major groups at similarity coefficient of 0.54. Similarity indices from the 20 genotypes selected ranged from 0.14 to 0.98 with

average of 0.63, indicating moderate to high genetic variability among the genotypes. The highest similarity coefficient was detected from Kokan region accessions and lowest to moderate from Western Maharashtra, Marathwada and Vidarbha. The *Jatropha* populations from diverse agro-climatic regions were revealing a wide genetic base.

The results of molecular diversity study revealed that *J. curcas* germplasm within Maharashtra constitutes a broad genetic base. From the clustering pattern and genetic relationship obtained from different clusters can be employed in their future breeding programmes.

Further, observing the clustering pattern based on phenotypic traits of seed vis-à-vis the one based on RAPD markers reflected that there was similarity between the phenotypic traits and molecular diversity. Under these circumstances the selection is to be based either on the phenotypic traits or on the molecular marker separately. However, selection based on molecular marker is likely to be more effective as it is more closely linked to the genetic constitution of a genotype.

The reported variations in morphological, biochemical and molecular diversity show extremely low to high range. Some of the variations may explain differences in following factors.

a. Difference in agro-climatic factors such as soil, rainfall pattern, altitude and latitude, humidity, etc.

b. It may be due to high level of cross-pollination nature of this species and interaction of materials from different genetic sources.

8.5 *In vivo* propagation

This investigation was undertaken to establish the application of hormone, length of stem cuttings and influence of rooting media, on survival and rooting of *Jatropha curcas* stem cuttings. The experiments were performed in three seasons viz, Rainy, Winter and Spring. The first experiment evaluated the effect of different concentrations of IBA (200, 800, 1400, 2000, 4000 and 8000 mg/l.) on rooting and survival of stem cuttings. The second experiment was set up to establish the ideal

length of stem cuttings (10, 20, 30, 40, and 50cm), while third experiment was carried out to study the effect of five media substrates, viz, Sand:Soil (1:1), Sand:Soil (1:2), Sand:Soil:FYM (1:2:1), Sand:Soil:Cocopeat (1:2:1), and Sand:Soil:Vermicompost (1:2:1) on rooting and survival of stem cuttings. A cutting treated with 2000mg/l IBA showed 78% survival during Spring (summer) season while, in Monsoon and Winter it was 62% and 54%, respectively. Sand: Soil: Vermicompost (1:2:1), was found to be better substrate with 78% rooting and survival. Similarly, Sand: Soil: Cocopeat (1:2:1) and Sand: Soil: FYM (1:2:1) showed satisfactory survival percentage, which was 70% and 72%, respectively, in Spring season. Stem cuttings with 20 cm length were suitable for quicker regeneration. It showed 76% of survival and rooting in Spring season. Spring season is more suitable for quicker vegetative propagation of *Jatropha curcas*.

It could be concluded from the *In vivo* propagation study that, for large-scale production of genetically pure and improved planting material of *J. curcas,* vegetative reproduction through 20cm long stem cutting treated 2000mg/l IBA for 5min. IBA is the most effective and economic method in Spring season. A similar observation was also recorded by Dhillon R. S. (2009). Secondly, the present study revealed that, a trend of relatively better survival and rooting of stem cuttings were obtained with Sand: Soil: Vermicompost (1:2:1) as compared to other substrate with 20cm length of stem cuttings. The observed results could be due to better aeration and high water holding capacity of substrate. The further study should evaluate the field performance of such plant produce by stem cuttings.

Hence, this study implies that the stem cuttings with above mentioned growth hormone treatment, size of stem cuttings, type of substrate help to develop uniform plant stock in nursery during spring season. The results on vegetative propagation of *J. curcas* obtained during present study have been duly published (Gopale and Zunjarrao, 2010).

8.6 In vitro propagation

In vitro study revealed that leaf disc was the best explants for micropropagation through direct induction of adventitious shoots when cultured on Murashige and Skoog's (MS) medium supplemented with thidiazuron (TDZ) (2.27 µM), 6-benzylaminopurine (BA) (2.22 µM) and indole-3-butyric acid (IBA) (0.49 µM). The presence of TDZ in the induction medium has greater influence on the induction of adventitious shoot buds, whereas BA in the absence of TDZ promoted callus induction rather than shoot buds. Induced shoot buds were multiplied and elongated into shoots following transfer to the MS medium supplemented with BA (4.44 µM), kinetin (Kn) (2.33 µM), indole-3-acetic acid (IAA) (1.43 µM) and gibberellic acid (GA_3) (0.72 µM). Well-developed shoots were rooted on half strength MS medium supplemented with IBA (0.5 µM) after 30 days. Regenerated plants after two months of acclimatization were successfully transferred to the field without visible morphological variation. This protocol might find use in mass production of true-to-type plants and in production of transgenic plants through Agrobacterium/ biolistic-mediated transformation.

Embryogenic callus were also obtained from leaf explants on MS basal medium supplemented with only 9.3 µM Kn. Induction of globular somatic embryos from 48% of the cultures was achieved on MS medium with different concentrations of 2.3 to 4.6 µM Kn and 0.5 to 4.9 µM IBA; 2.3 µM Kn and 1.0 µM IBA proved to be the most effective combination for somatic embryo induction in *Jatropha curcas*. Addition of 13.6 µM adenine sulphate stimulated the process of development of somatic embryos. This protocol of somatic embryogenesis in *Jatropha curcas* may be an ideal system for future transgenic research. However, asynchronous development of somatic embryo is major limitation to use this protocol for mass multiplication of this plant, further research in this direction is required.

8.7 Recommendations

The data presented in this study recommends not engaging in large-scale plantations now. It encourages investors and developers to start on a small scale based on verified or scientific data, and learn by doing to extend the size of such plantations. In such

bio-diesel producing plants, the criteria of 'Sustainable development' are recommended to apply.

Therefore, substantial efforts should be made to streamline observations in current *Jatropha* planting sites to implement specific experiments for unraveling the impact of different production factors on crop performance. It was also found useful to exchange knowledge and information, in order to prevent unjustified investments of farmers, entrepreneur and organization.

1. As earlier report suggests that, there is strong influence of agro-climatic conditions on yield and oil content. Our study also shows similar conclusion. In Maharashtra, Marathwada, Vidarbha and Western Maharashtra agro-climatic regions are suitable for plantation of *J. curcas.* Therefore, it is recommended plantation should be promoted in these regions.

2. Morphological, Biochemical and Molecular diversity estimated moderate to high genetic diversity exist in Maharashtra, thus, it can be recommended that, there is ample scope for selection and genetic improvement of the species, through

    i. Mass selection

    ii. Mutation breeding

    iii. Inter-specific hybridization

    iv. Alien gene transfer

3. Potential of the new varieties has to be further tested for their performance through multi-location trials.

4. The tissue culture protocol serves as prelude for genetic improvement through biotechnological tools. Reported protocol in this thesis is quite efficient for production of elite *Jatropha* plantlets on large-scale with high multiplication rate.

5. Tissue culture protocol will be useful in somatic hybridization, inter-specific crosses limited by crossability barriers somatic embryogenesis not only helps

to obtain a large number of plants year round but also can act as powerful tool for genetic improvement.

6. Elite plant development may take seven to eight years. Till such time it is recommended that, the available local high yielding seeds of *Jatropha* may be used in different regions. The reported methodology for stem cuttings mentioned in this work is suitable for economical multiplication of superior accessions of *Jatropha curcas* at local nursery level.

7. Immediate action is necessary to undertake realistic assessment of type, kind, as well as availability of land suitable for *Jatropha* plantation based on agro-climatic conditions.

8. This therefore certainly calls for the dire need of joint efforts by the 'Ministry of Rural Development', Agriculture, Environment and Forest.

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